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Mira Abou-Diab

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Production éco-circulaire de peptides antibactériens, antifongiques et antioxydants déminéralisés à partir d'hémoglobine bovine par électrodialyse avec membranes bipolaires : Étude de faisabilité, mécanisme enzymatique, optimisation des paramètres, comparaison avec l'hydrolyse conventionnelle et prévention du colmatage.

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Naima Nedjar, professeur, (U. Lille - ICV) directrice de thèse en France
Laurent Bazinet, professeur (U. Laval - INAF), directeur de thèse au Canada
Pascal Dhulster, professeur (U. Lille - ICV), codirecteur de thèse en France
Ismail Fliss, professeur (U. Laval - INAF), codirecteur de thèse au Canada
Romain Kapel, professeur (U. Lorraine- BioProMo), rapporteur en France
Salwa Karboune, docteur (U. McGill), rapporteuse au Canada
Pascal Degraeve, professeur (U. Lyon), président et examinateur en France
Marie Filteau, professeur (U. Laval), examinatrice au Canada

Résumé

Le cruor bovin, la matière solide du sang, est un déchet des abattoirs produit en très grande quantité dans le monde. Ce coproduit est composé principalement d'hémoglobine, une protéine riche en peptides bioactifs après son hydrolyse enzymatique. Cependant, lors de l'hydrolyse conventionnelle par la pepsine, des agents chimiques sont nécessaires pour ajuster et réguler le pH de la solution et, les hydrolysats finaux produits contiennent des niveaux élevés en sels minéraux. Pour pallier ces inconvénients, il a été proposé d'appliquer dans cette étude, pour la première fois, une technologie verte, appelée électrodialyse avec membrane bipolaire (EDMB), comme méthode alternative à l'hydrolyse enzymatique conventionnelle de l'hémoglobine afin d'obtenir des peptides bioactifs purifiés. Les objectifs de cette thèse étaient de tester la faisabilité de ce nouveau procédé pour la production de peptides bioactifs à partir d'hémoglobine bovine, d'établir les conditions optimales, d'éviter le colmatage membranaire et d'appliquer un nouveau procédé original d'EDMB à « multiple-étapes » permettant la production de peptides bioactifs déminéralisés sans ajout de produits chimiques. Des configurations bipolaires/monopolaires (anioniques ou cationiques) utilisant les ions H^+ et OH^- générés par les membranes bipolaires pour réguler le pH ont été étudiées et comparées à un procédé conventionnel utilisant des acides et des bases chimiques. La configuration d'EDMB formée avec les membranes cationiques a permis la production d'hydrolysats contenant une faible concentration en sels minéraux mais avec la présence d'un colmatage sur la membrane cationique, alors que la configuration d'EDMB utilisant des membranes anioniques a permis la production d'hydrolysats sans colmatage mais avec une concentration en sel similaire à celle de l'hydrolyse conventionnelle (contrôle). En se basant sur ces résultats, une nouvelle configuration d'EDMB à trois compartiments a été mise en place et étudiée pour dénaturer l'hémoglobine, inactiver la réaction enzymatique et déminéraliser l'hydrolysat peptidique en simultané. Une déminéralisation de 85% a été atteinte. Cependant, un colmatage a encore été observé sur la membrane anionique en raison de la précipitation de l'hème. Pour cette raison, une étape supplémentaire de décoloration a été réalisée avant la déminéralisation pour éviter le colmatage en utilisant l'acide électro-généré. Les peptides décolorés et déminéralisés récupérés ont montré une activité antioxydante, une activité antibactérienne contre plusieurs souches bactériennes (Gram+ et

Gram-) et pour la première fois une activité antifongique contre de nombreuses souches de moisissures et de levures. Dans un contexte d'économie circulaire, cette technologie durable s'avèrerait efficace pour effectuer plusieurs opérations simultanément et présente un potentiel important au niveau industriel pour l'hydrolyse du sang, puisqu'elle produit des bio-peptides purifiés ayant une faible teneur en sels minéraux et pouvant être utilisés comme conservateurs naturels sur la viande.

Abstract

Bovine cruor, a slaughterhouse waste, is produced in large quantities all around the world. This co-product is mainly composed of hemoglobin, a protein rich in bioactive peptides after its enzymatic hydrolysis. However, during conventional hydrolysis, chemical agents are necessary to adjust or regulate the pH of the solution and the final hydrolysates produced contain high levels of mineral salts. Therefore, in this study we applied for the first time a green technology named electrodialysis with bipolar membrane (EDBM) as an alternative method to the conventional enzymatic hydrolysis of hemoglobin to obtain purified bioactive peptides. The main objectives of the present thesis were to test the feasibility of this new process to produce bioactive peptides from bovine hemoglobin, to establish the optimal conditions while avoiding membrane fouling and to apply a new original « multiple-step » EDBM process allowing the production of demineralized bioactive peptides without the addition of chemical salts. Bipolar and monopolar (anionic or cationic) configurations using the H^+ and OH^- generated by the bipolar membranes to regulate the pH were investigated and compared to a conventional process using chemical acid and base. The EDBM configuration formed with cationic membranes allowed the production of hydrolysates containing a low concentration of mineral salts but with fouling formation on the cationic membrane, while EDBM configuration formed with anionic membranes allowed the production of hydrolysates without fouling but with a salt concentration similar to the control. Based on these results, a new three-compartment EDBM configuration was carried-out to denaturate the hemoglobin, inactivate the enzymatic reaction, and demineralize the hemoglobin hydrolysate simultaneously. A demineralization level of 85% has been achieved. However, fouling was still observed on the anionic membrane due to hem precipitation. For this reason, an additional step of discoloration was tested before the demineralization to avoid fouling using the electrogenerated acid. The discolored and demineralized peptides recovered showed antioxidant activity, antibacterial activity against multiple bacterial strains (Gram+ and Gram-), and for the first time, antifungal activity against several molds and yeasts strains. Moving towards a circular economy, this sustainable technology was found to be effective in performing multiple operations simultaneously and has a great potential for industrial

hydrolysis of blood, since it produces purified biopeptides with a low mineral content that can be used as natural preservatives on meat.

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Liste des abréviations

Hb	Hemoglobin	
BH	Bovine Hemoglobin	
ED	Electrodialysis	
EDBM	Electrodialysis with Bipolar Membranes	
MEI	Membrane échangeuse d'Ion	
IEM	Ion exchange membrane	
MEA	Membrane échangeuse d'Anion	
MEC	Membrane échangeuse de Cation	
AEM	Anion exchange membrane	
MCP	Monovalent cation permselective membrane	
MPC	Membrane permsélective aux cations monovalents	
BM	Bipolar membrane	
EDBM-MCP	Electrodialysis with Bipolar and Monovalent cation perm-selective membranes	
EDBM-AEM	Electrodialysis with Bipolar and Anion exchange membranes	
CH-RH	Raw Hydrolysates (RH) produced by conventional hydrolysis (CH)	
CH-DH	Discolored Hydrolysates (DH) produced by conventional hydrolysis (CH)	
EDBM-RH	Raw Hydrolysates (RH) produced by EDBM	
EDBM-DH	Discolored Hydrolysates (DH) produced by EDBM	
NKT	Neokyotorphin	
BHT	Butylated hydroxytoluene	
LC-MS	Chromatography liquid couplée avec la spectrométrie de masse	
MS/MS	Spectrométrie de masse	
CP	Concentration de polarisation	
CEP	Champs électriques pulsé	
ABTS	2,2'-azino-bis(3-éthylbenzothiazoline-6-sulphonique)	
DPPH	2,2-diphényl 1-picrylhydrazyle	
AA	Acide Aminé	
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Acide aspartique
E	Glu	Acide glutamique
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline

Q	Gin	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophane
Y	Tyr	Tyrosine

À ma famille!

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Avant-propos

Cette thèse se compose de plusieurs sections dont l'introduction générale, six chapitres et une conclusion finale. Le premier chapitre correspond à un état de l'art sur lequel l'hypothèse et les objectifs de la recherche se sont appuyés. Le second chapitre présente ainsi la problématique, l'hypothèse de recherche et les objectifs qui en ont découlés. Les chapitres III, IV et V, constituent le corps de la thèse et correspondent aux travaux de recherche réalisés. Enfin, le chapitre VI présente une discussion générale des travaux réalisés tout au long de ce projet suivi d'une conclusion tirée des résultats obtenus et d'une série de perspectives au projet. Les trois chapitres de résultats sont présentés sous forme d'articles scientifiques publiés ou en cours de soumission :

Le chapitre III contient les résultats de l'étude de l'impact de la conductivité sur les performances d'électro-acidification et d'hydrolyse enzymatique de l'hémoglobine bovine par électrodialyse avec membranes bipolaires pour la production de peptides bioactifs. Ce chapitre a été publié sous la forme d'un article intitulé « **Impact of conductivity on the performances of electro-acidification and enzymatic hydrolysis phases of bovine hemoglobin by electrodialysis with bipolar membranes for the production of bioactive peptides** », publié dans *Separation and Purification Technology*, le 15 août 2021 (https://www.sciencedirect.com/science/article/pii/S1383586621003622?via=ihub). Les auteurs sont: Mira Abou-Diab (Candidate au Ph. D.: planification et réalisation des expériences, analyse des résultats et rédaction de l'article), Jacinthe Thibodeau (Professionnelle de recherche côté canadien : supervision de l'étudiant pour les analyses d'électrodialyse et LC-MS, correction du manuscrit), Ismail Fliss (Co-directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit), Pascal Dhulster (Co-directeur de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit), Naima Nedjar (Directrice de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit) et Laurent Bazinet (Directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit).

Le chapitre IV présente les résultats de l'optimisation du nouveau procédé vert pour la production de néokyotorphine (α137-141) et des peptides bioacifs dérivés de l'hydrolyse enzymatique de l'hémoglobine bovine utilisant deux configurations d'électrodialyse avec membranes bipolaires. Ce chapitre a été écrit sous la forme de deux articles. Le premier intitulé **« Bovine Hemoglobin Enzymatic Hydrolysis by a New Ecoefficient Process - Part I: Feasibility of Electrodialysis with Bipolar Membrane and Production of Neokyotophin (α137-141)** », publié dans *Membranes,* le 25 septembre 2020 (https://www.mdpi.com/2077-0375/10/10/257/pdf). Le second ayant pour titre : « **Bovine Hemoglobin Enzymatic Hydrolysis by a New Eco-Efficient Process - Part II: Production of Bioactive Peptides** », publié dans *Membranes,* le 29 septembre 2020 (https://www.mdpi.com/2077-0375/10/10/268). Les auteurs sont: Mira Abou-Diab (Candidate au Ph. D.: planification et réalisation des expériences, analyse des résultats et rédaction de l'article), Jacinthe Thibodeau (Professionnelle de recherche côté canadien : supervision de l'étudiant pour les analyses d'électrodialyse et LC-MS, correction du manuscrit), Barbara Deracinois (collaboratrice scientifique pour les études bioinformatiques de LC-MS), Christophe Flahaut (collaborateur scientifique pour les études bioinformatiques de LC-MS), Ismail Fliss (Co-directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit), Pascal Dhulster (Co-directeur de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit), Naima Nedjar (Directrice de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit) et Laurent Bazinet (Directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit). Ces résultats ont été présentés sous forme d'un poster lors du symposium de l'INAF (Québec, QC, Canada), du congrès Antimic (premier prix du jury) (Lille, France) et du congrès SFGP (Nantes, France) en 2019 et sous forme d'une présentation orale lors du congrès scientifique AOCS (Chicago, USA) en 2021.

Le chapitre V décrit l'étude sur la production d'hydrolysat d'hémoglobine bovine déminéralisé et décoloré actif en effectuant simultanément un processus à multiple étapes à l'aide d'un système d'électrodialyse avec membranes bipolaires. Ce chapitre fait l'objet de 2

articles. Le premier intitulé « **Eco-circular production of demineralized bioactive peptides from bovine hemoglobin by performing the necessary steps simultaneously using Bipolar Membrane Electrodialysis** », accepté dans *ACS Sustainable Chemistry & Engineering,* le 24 novembre 2021. Le second ayant pour titre : « **Production of demineralized Antibacterial, Antifungal and Antioxidant peptides from bovine hemoglobin using an optimized « multiple-steps » system: Electrodialysis with Bipolar Membrane** », soumis pour publication dans *Membranes,* le 20 août 2021. Les auteurs sont: Mira Abou-Diab (Candidate au Ph. D.: planification et réalisation des expériences, analyse des résultats et rédaction de l'article), Jacinthe Thibodeau (Professionnelle de recherche côté canadien : supervision de l'étudiant pour les analyses d'électrodialyse et LC-MS, correction du manuscrit), Ismail Fliss (Co-directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit), Pascal Dhulster (Co-directeur de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit), Laurent Bazinet (Directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit) et Naima Nedjar (Directrice de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit). Une présentation orale de ces résultats a été réalisé au congrès Green Food Tech (Québec, QC, Canada) en 2021.

Introduction

Selon un rapport publié en 2018 par la Banque mondiale, d'ici 2050, le monde devrait générer 3,40 milliards de tonnes de déchets par an, augmentant considérablement par rapport aux 2,01 milliards de tonnes d'aujourd'hui (Kaza *et al*., 2018). De plus, les procédés industriels utilisés dans les industries alimentaires participent à cette production croissante de déchets induite par une urbanisation rapide et une population grandissante. La valorisation des déchets de la filière bioalimentaire, sous-estimés et sous-utilisés, sous forme de bioproduits à haute valeur ajoutée est devenue un enjeu capital pour tendre vers une économie circulaire ou durable. L'économie circulaire est une économie fonctionnant en boucle et évitant ainsi le gaspillage. Son objectif est de produire des biens mais en même temps de limiter fortement la consommation des sources d'énergie non renouvelables et de matières premières. Comment cette économie circulaire pourrait être appliquée à l'industrie de la viande?

La population croissante augmente le besoin de se nourrir et par conséquent, la demande en produits alimentaires dont la viande. La viande comprend les bovins, la volaille, les moutons, les chèvres, les porcs et le gibier sauvage. Entre 1961 et 2013, la demande de viande a augmenté de 241,77 à 651,84 millions de tonnes au Canada, alors qu'elle a augmenté de 236,69 à 623,18 millions de tonnes en France (FAO, 2017). En 2013, la plus faible demande de viande, entre 0 et 200 millions de tonnes, est répartie dans sa majorité dans les pays d'Afrique, alors que la plus grande demande se concentre aux États-Unis, Australie, Brésil et Argentine pour des valeurs supérieures à 700 millions de tonnes comme le montre la **Figure 1**.

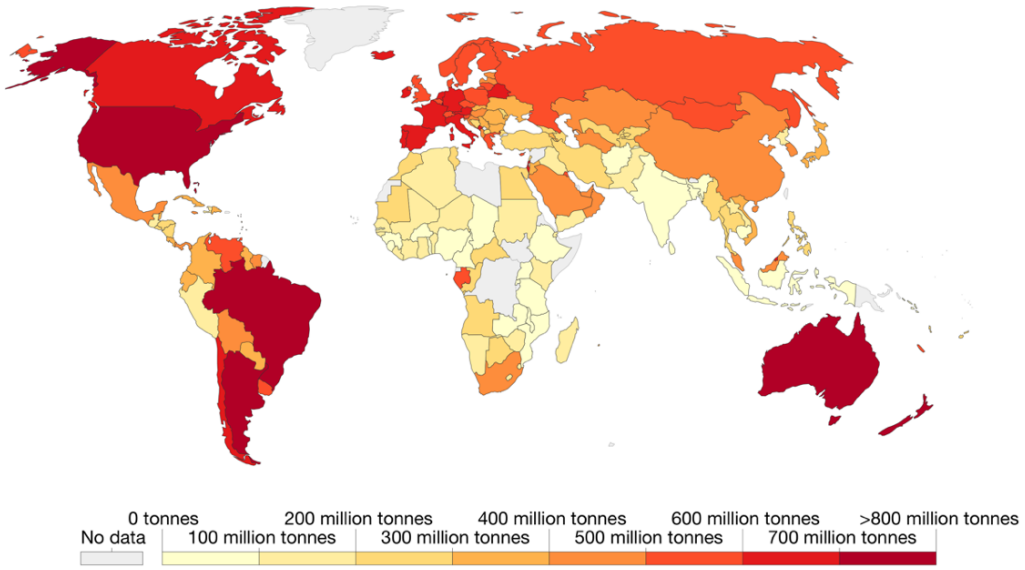


Figure 1. La répartition de la demande globale de viande dans le monde en 2013 (FAO, 2017).

En 2018, selon l'organisation pour l'alimentation et l'agriculture (FAO), 302,15 millions de bovins ont été abattus et transformés en viande dans le monde pour répondre à la demande de viande. Donc, plus de 4,53 milliards de litres de sang ont été produits en supposant une récupération de 15 L de sang par bovin (Fallows et Verner-Wheelock, 1982). Plus spécifiquement, la **Figure 2** montre qu'au Canada, 3,67 millions de bovins ont été abattus représentant 55,05 millions de litres de sang générés alors qu'en France, 4,61 millions de bovins ont été abattus équivalant à 69,15 millions de litres de sang récupérés (FAO, 2020). Ces énormes volumes de sang constituent un déchet agro-alimentaire et une ressource substantielle naturelle issue des abattoirs. Malgré l'abondance de sang récolté, il est estimé que seulement 30% sont utilisés (Gatnau *et al*., 2001). Cette grande quantité de sang animal générée est un sous-produit problématique pour l'industrie de la viande car elle a une charge polluante très élevée lorsqu'elle est rejetée dans l'environnement avec des traitements minimes (Del Hoyo *et al*., 2008; Bah *et al*., 2013).

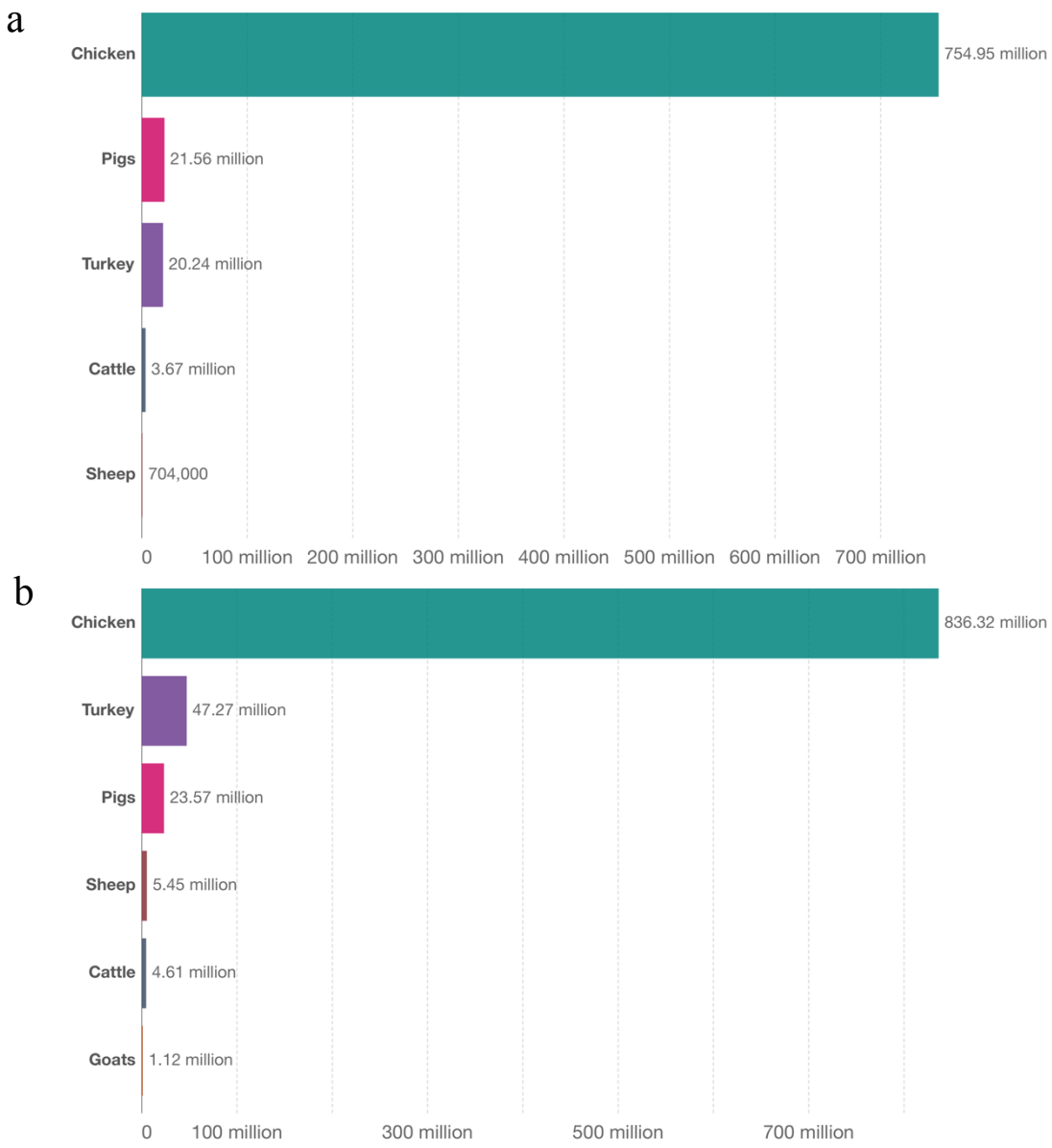


Figure 2. Nombre d'animaux abattus pour la viande au Canada (a) et en France (b) en 2018 (FAO, 2020).

Ces déchets comme de nombreux autres sont riches en protéines (Goot *et al*., 2016), qui, par l'utilisation d'enzymes adaptées, peuvent générer des peptides ayant plusieurs activités biologiques (Castro *et al*., 2015; Hartmann *et al*., 2007). L'utilisation des peptides est aujourd'hui largement répandue, que ce soit en applications thérapeutiques ou pharmaceutiques (Agyei et Danquah, 2001; Danquah et Agyei, 2012; Saadi *et al*., 2015), en usages alimentaires à visée animale (Martinez-Alvarez *et al*., 2015) ou humaine pour leur

divers effets nutraceutiques (Hartmann *et al*., 2007; Lafarga *et al*., 2014; Li-Chan, 2015). De plus, ces composés d'origine naturelle suscitent un intérêt croissant chez les consommateurs qui veulent de plus en plus manger sainement, et éviter les produits synthétiques et les additifs chimiques dont les effets à long terme sont parfois peu connus ou controversés (Agyei *et al*., 2011). Pour toutes ces raisons et dans le contexte de l'économie circulaire, l'industrie agroalimentaire s'intéresse de plus en plus aujourd'hui à la valorisation du sang. Elle cherche ainsi à développer des produits à forte valeur ajoutée tout en découvrant et appliquant de nouvelles méthodes innovatrices de production qui répondent à la demande du consommateur et contribuent à la protection de l'environnement.

Après centrifugation, le sang bovin issu des abattoirs se divise en deux parties : le plasma (60%) et le cruor (40%). Le plasma, constitue la partie incolore du sang et peut être utilisé en pharmaceutique, notamment pour sa richesse en thrombines, fibrinogènes ou albumine sérique (Bah *et al*., 2013 ; Divakaran, 1982 ; Howell *et al*., 1987 ; Pentadeo *et al*., 1979) et en alimentaire, principalement comme émulsifiant en charcuteries (Linden *et al*., 1994). Le cruor, la fraction solide du sang, est responsable de la couleur rouge et représente 70 à 75% des protéines sanguines totales. Le cruor contient plus de 90% d'hémoglobine, une protéine largement décrite comme une source riche en peptides antimicrobiens, antioxydants, antihypertenseurs, hématopoïétiques, opioïdes… après hydrolyse (Adje *et al*., 2011 ; Gomes *et al*., 2010, Nedjar-Arroume *et al*., 2008 ; Lignot *et al*., 1999 ; Zhao et Piot, 1997 ; Vercaigne-Marco *et al*., 2000 ; Daoud *et al*., 2005 ; Nedjar-Arroume *et al*., 2006 ; Przybylski *et al*., 2016). La méthode la plus couramment utilisée pour la production de ces peptides bioactifs à partir de l'hémoglobine, est l'hydrolyse enzymatique conventionnelle en lot nécessitant l'utilisation de plusieurs agents chimiques tels que l'urée, les alcools, les tampons acétate de sodium… De plus, l'utilisation de produits chimiques conduit à la production d'hydrolysats contenant des niveaux élevés de sels et présentant des niveaux de pureté faible ne répondant plus aux besoins du consommateur. Par conséquent, dans cette étude, il a été proposé d'appliquer une nouvelle technologie électromembranaire verte (Mikhaylin *et al*., 2018), appelée électrodialyse avec membranes bipolaires (EDMB), comme méthode alternative à l'hydrolyse enzymatique conventionnelle de l'hémoglobine pour obtenir des peptides bioactifs.

Les procédés électro-membranaires, reconnus comme des éco-procédés, font partie des différentes technologies de séparation par membranes qui sont de plus en plus utilisées dans les industries bioalimentaires et pharmaceutiques pour concentrer, purifier ou déminéraliser des composantes alimentaires. Contrairement aux autres techniques membranaires (microfiltration, ultrafiltration, nanofiltration et osmose inverse) dont la force motrice est liée à un gradient de pression, le déplacement des composés en cours de procédés électro-membranaires est réalisé grâce à une différence de potentiel électrique appliquée entre deux électrodes (Gardais, 1990). L'énergie électrique ainsi apportée sert principalement à assurer le transfert des espèces chargées, la nature chimique des espèces en solution restant inchangée. Récemment, le domaine des procédés électro-membranaires a connu un nouvel essor grâce au développement des membranes bipolaires. Les membranes bipolaires ont la capacité de dissocier les molécules d'eau sous l'effet d'un champ électrique pour produire des ions H^+ et OH^- générant des acides et des bases nécessaires aux applications industrielles sans ajouts de réactifs chimiques (Pourcelly, 2002). Bazinet *et al*. (1997), ont rapporté que l'avantage majeur de l'EDMB est la possibilité d'acidifier et de déminéraliser in-situ simultanément une solution, de sorte que le produit électro-acidifié a une pureté plus élevée qu'un produit chimiquement acidifié. La technologie d'EDMB a entre autres été utilisée pour la solubilisation du chitosane avant son hydrolyse enzymatique par électro-acidification (Lin Teng Shee *et al*., 2006). Récemment, l'EDMB a été appliquée pour produire un jus de canneberge désacidifié, avec une réduction drastique de la consommation d'énergie ainsi qu'une amélioration de l'éco-efficience (Faucher *et al*., 2018). Ainsi, l'utilisation de l'EDMB simplifierait l'ensemble du procédé d'hydrolyse conventionnelle actuel nécessaire à la production des peptides bioactifs à partir de l'hémoglobine bovine en une seule technologie sans ajouts de produits chimiques, et les hydrolysats produits contiendraient de faibles concentrations en sels minéraux. Cependant, l'EDMB n'a jamais été testée pour l'hydrolyse de l'hémoglobine bovine.

L'objectif général de la présente thèse sera de développer une méthode verte et innovatrice de production de peptides bioactifs de haute pureté à partir de l'hydrolyse enzymatique de l'hémoglobine bovine en utilisant EDMB. Le projet se déroulera en plusieurs phases, qui

passeront par l'évaluation de : 1) la faisabilité de l'électro-acidification et de l'hydrolyse enzymatique de l'hémoglobine bovine par la pepsine, 2) la production d'une population peptidique ayant des activités biologiques, 3) l'optimisation et la rationalisation du procédé et 4) la mise en œuvre des différentes phases d'acidification, d'hydrolyse enzymatique, d'arrêt de la réaction, de déminéralisation et de décoloration en simultané au sein d'un système unique d'EDMB. En vue de l'optimisation de ce procédé, la compréhension des phénomènes de migration, l'impact de la conductivité et du pH sur les performances du procédé et sur les paramètres de l'hydrolyse, en plus, de la compréhension de la nature et la cause du colmatage seront également au cœur du projet.

Chapitre I

REVUE DE LITTÉRATURE

I. Revue de littérature

I.1. L'hémoglobine bovine

Le cruor représente, jusqu'à 40% du volume sanguin et contient principalement de l'hémoglobine (90%) contenue au sein des globules rouges (ou hématies).

I.1.1. Structure de l'hémoglobine bovine

L'hémoglobine est le constituant spécifique de l'hématie dont la fonction est de transporter l'oxygène des poumons vers les tissus du corps et le gaz carbonique des tissus vers les poumons. L'hémoglobine bovine est une protéine formée de quatre chaînes polypeptidiques, deux chaînes α et deux chaînes β, identiques deux à deux, et ayant une masse moléculaire globale de 64 500 Da. Elle a une structure globulaire, chacune des chaînes comporte une partie protéique, la globine, et un groupement non protéique, l'hème (**Figure I-1**).

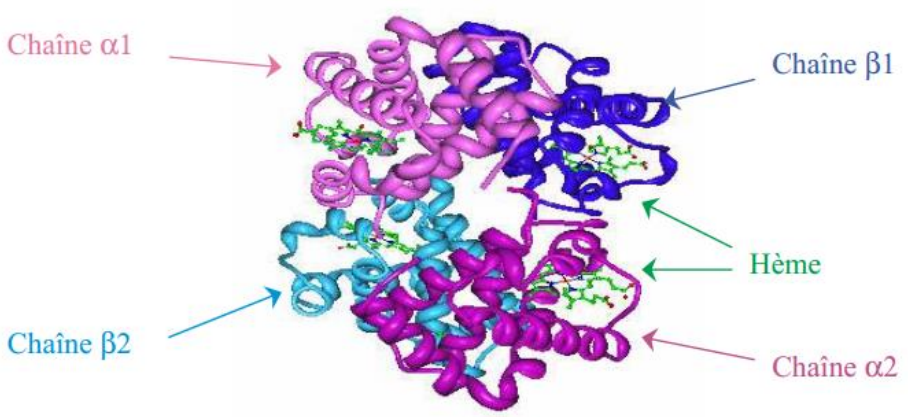


Figure I-1. Représentation spatiale de l'hémoglobine bovine (Przybylski, 2016).

I.1.1.1. La globine

La chaîne de la globine, formée selon le modèle protéique, est constituée de la succession d'acides aminés dans un ordre déterminé. Les 4 sous-unités protéiques de la globine,

semblables deux à deux (2 protomères α et 2 protomères β), sont différentes par la nature et la séquence en acides aminés.

I.1.1.1.1. La structure primaire

La structure primaire est représentée par l'enchaînement des acides aminés liés les uns aux autres par des liaisons covalentes (liaisons peptidiques).

I.1.1.1.1.1. La chaîne alpha

La chaîne α est composée de 141 acides aminés, d'une masse moléculaire globale de 15 053 Da et d'un point isoélectrique théorique de 8,19 (Sasakawa, 1961). La **Figure I-2** montre la séquence linéaire en acides aminés de la chaîne α (Satake et Sasakawa, 1962).

V[1]LSAADKGNV[10]KAAWGKVGGH[20]AAEYGAEALE[30]RMFLSFPTTK[40]TYFPHFDLS
H[50]GSAQVKGHGA[60]KVAAALTKAV[70]EHLDDLPGAL[80]SELSDLHAHK[90]LRVDPVN
FKL[100]LSHSLLVTLA[110]SHLPSDFTPA[120]VHASLDKFLA[130]NVSTVLTSKY[140]R

Figure I-2. Séquence linéaire en acides aminés de la chaîne α de l'hémoglobine bovine.

La chaîne α est constituée majoritairement d'acides aminés hydrophobes ou apolaires. Les acides aminés les plus abondants sont la leucine (L) et l'alanine (A), alors qu'il y a absence d'isoleucine (I) et de cystéine (C) (**Tableau I-1**).

Tableau I-1. Composition en acides aminés de la chaîne alpha de l'hémoglobine bovine (Adje, 2010).

Acides aminés	Quantité	Pourcentage (%)
Ala (A)	20	14,20
Arg (R)	3	2,10
Asn (N)	3	2,10
Asp (D)	8	5,70
Cys (C)	0	0
Gln (Q)	1	0,70

Glu (E)	5	3,50
Gly (G)	9	6,40
His (H)	10	7,10
Ile (I)	0	0
Leu (L)	10	14,20
Lys (K)	11	7,80
Met (M)	1	0,70
Phe (F)	7	5
Pro (P)	6	4,30
Ser (S)	13	9,20
Thr (T)	8	5,70
Trp (W)	1	0,70
Tyr (Y)	3	2,10
Val (V)	12	8,50

I.1.1.1.1.2. La chaîne bêta

La chaîne β est composée de 145 acides aminés avec un poids moléculaire total de 15 954 Da et un point isoélectrique théorique de 7,02 (Schroeder *et al.*, 1967). La **Figure I-3** montre la séquence linéaire en acides aminés de la chaîne β.

M[1]LTAEEKAAV[10]TAFWGKVKVD[20]EVGGEALGRL[30]LVVYPWTQRF[40]FESFGDLST
A[50]DAVMNNPKVK[60]AHGKKVLDSP[70]SNGMKGLDDL[80]KGTFAALSEL[90]HCDKLHV
DPE[100]NFKLLGNVLV[110]VVLARNFGKF[120]FTPVLQADFQ[130]KVVAGVANAL[140]AHRY
H

Figure I-3. Séquence linéaire en acides aminés de la chaîne β de l'hémoglobine bovine.

La chaîne β est constituée majoritairement d'acides aminés hydrophobes. Les acides aminés prépondérants sont la valine (V) et la leucine (L) (**Tableau I-2**).

Tableau I-2. Composition en acides aminés de la chaîne bêta de l'hémoglobine bovine (Adje, 2010).

Acides aminés	Quantité	Pourcentage (%)
Ala (A)	14	11,00
Arg (R)	4	2,80
Asn (N)	7	4,80
Asp (D)	9	6,20
Cys (C)	1	0,70
Gln (Q)	3	2,10
Glu (E)	8	5,50
Gly (G)	11	7,60
His (H)	6	4,10
Ile (I)	0	0
Leu (L)	17	11,70
Lys (K)	13	9,00
Met (M)	3	2,10
Phe (F)	10	7
Pro (P)	4	2,80
Ser (S)	5	3,40
Thr (T)	6	4,10
Trp (W)	2	1,40
Tyr (Y)	2	1,40
Val (V)	18	12,40

I.1.1.1.2. La structure secondaire

I.1.1.1.2.1. La chaîne alpha

La structure secondaire se rapporte aux interactions entre les acides aminés proches les uns des autres dans une séquence linéaire (Adje *et al.*, 2010). Cette structure est caractérisée par

la spiralisation de la chaîne et la formation de ponts hydrogènes. La structure de la chaîne α est formée de 8 hélices α : H1 à H8, reliées par des coudes β (**Figure I-4**).

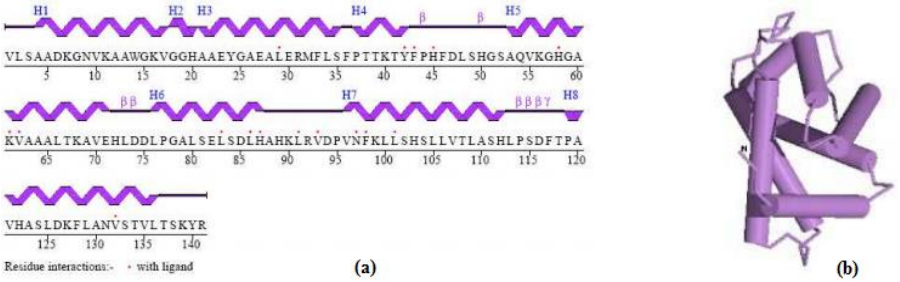


Figure I-4. Structure secondaire de la chaîne α de l'hémoglobine : représentation linéaire (a) et représentation spatiale (b) (Przybylski, 2016).

I.1.1.1.2.2. La chaîne bêta

Sa structure secondaire comporte 11 hélices α (de H1 à H11) reliées par des coudes β (Figure I-5).

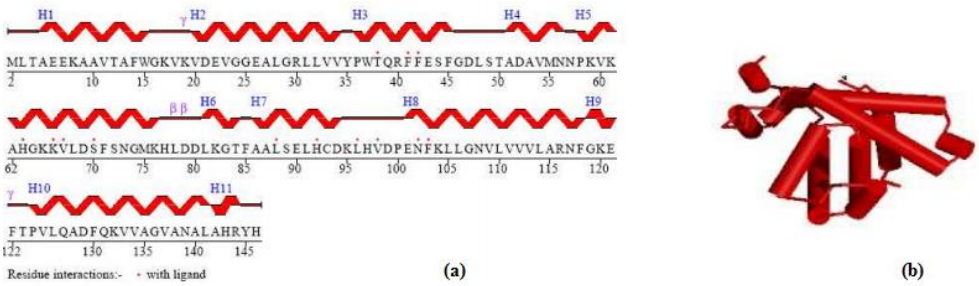


Figure I-5. Structure secondaire de la chaîne β de l'hémoglobine bovine : représentation linéaire (a) et représentation spatiale (b) (Przybylski, 2016).

I.1.1.1.3. La structure tertiaire

La structure tertiaire d'une protéine correspond au repliement de la chaîne polypeptidique dans l'espace (**Figure I-6**). La composition en acides aminés de la protéine ainsi que son environnement constituent la base de la structure tertiaire. La structure tertiaire est responsable des propriétés fonctionnelles des protéines (Adje, 2010). Des liaisons physicochimiques entraînent un pelotonnement de la molécule, l'ensemble réalise dans

l'espace une structure globulaire, ménageant une cavité où se loge l'hème. D'après les études réalisées par Kendrew dans les années 60, il a été constaté que les molécules protéiques globulaires solubles dans l'eau sont repliées grâce à l'empilement des chaînes latérales sous formes hydrophobes à l'intérieur de la molécule (Kendrew, 1958; 1959; 1962; 1963; Kendrew *et al*., 1960). La structure secondaire permet la formation des liaisons hydrogènes suite à la neutralisation des groupements NH et CO de la chaîne latérale. Plus encore, Perutz (1962), décrit la structure tertiaire de l'hémoglobine comme quatre conformations très semblables associées par des liaisons non covalentes (Adje, 2010).

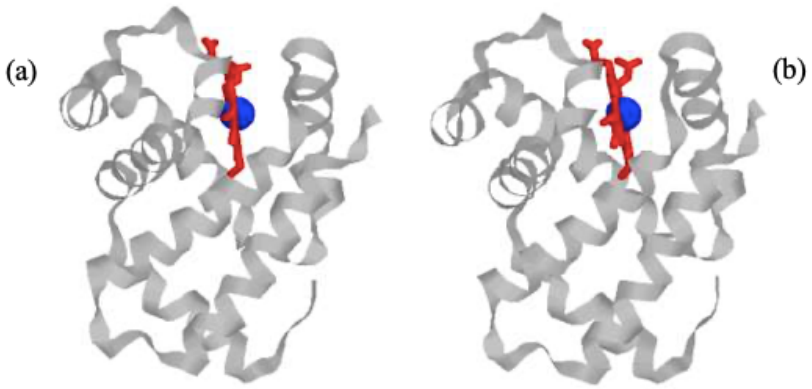


Figure I-6. Structure de la chaîne α (a) et la chaîne β (b) de l'hémoglobine (http://acces.ens-lyon.fr/biotic/gpe/dossiers/drepanocytose/html/hbstr.htm).

I.1.1.1.4. La structure quaternaire

Les différentes interactions hydrophobes, hydrogènes et non covalentes entre les chaînes polypeptidiques de la protéine et leur disposition spatiale forment la structure quaternaire (**Figure I-7**). Les chaînes de nature identique sont faiblement liées entre elles, les deux chaînes alpha d'une part et les deux chaînes bêta d'autre part. En revanche, les chaînes de nature différente ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_1\beta_2$ et $\alpha_2\beta_2$) sont fortement liées par des liaisons hydrophobes. Cette structure est modifiée suite à la fixation d'un atome d'oxygène sur le fer contenu dans l'hème (Baillet *et al.*, 2003).

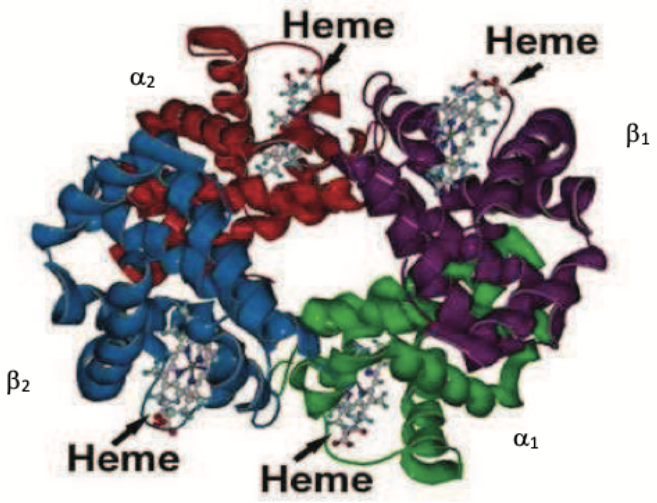
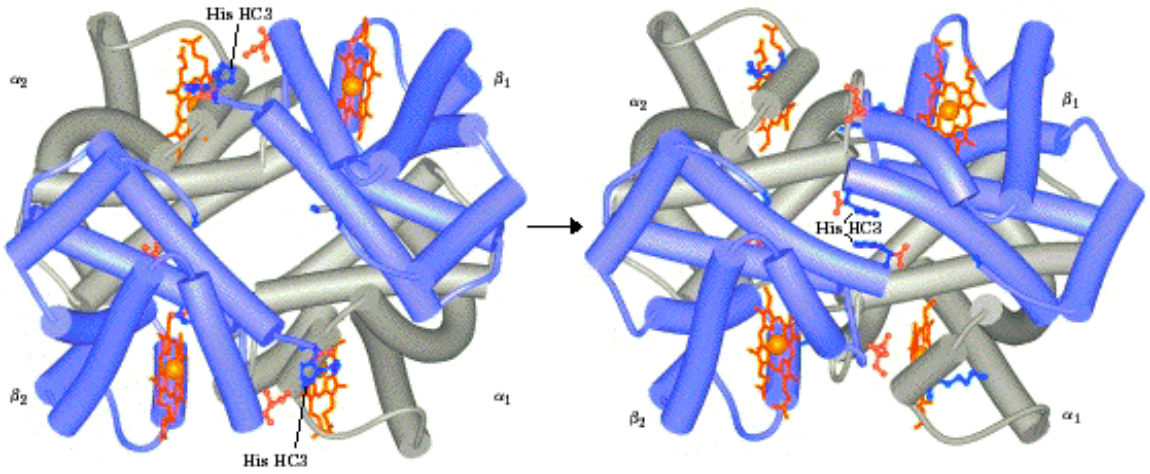


Figure I-7. Structure quaternaire de l'hémoglobine bovine (http://erasmeinfo.ulb.ac.be/globule/images/Tetra2.jpg).

Le transport de l'oxygène est assuré plus spécifiquement par les structures tertiaire et quaternaire de l'hémoglobine. Selon son affinité, la protéine existe sous deux formes : 1) la désoxyhémoglobine ayant une faible affinité pour l'oxygène, correspondant à la forme T « Tendue » et 2) l'oxyhémoglobine ayant une haute affinité pour l'oxygène, correspondant à la forme R « Relaxée » (**Figure I-8**). La présence de l'oxygène et le pH ont un impact sur ces deux formes et leur équilibre. La forme R est privilégiée à pH élevé et en présence d'O_2, par ce fait, l'hémoglobine cherche à capturer de l'oxygène. Par contre, la forme T est privilégiée à pH faible et en présence négligeable d'O_2, par la suite, la protéine relâche l'oxygène. Le passage de la forme « T » à « R », change la structure quaternaire de l'hémoglobine (Leblanc, 2013).



Tendue Relaxe

Figure I-8. Conformation quaternaire de l'hémoglobine bovine (Lehninger, 2000).

I.1.1.2.L'hème

L'hème est formé suite à plusieurs réactions qui commencent dans les mitochondries et se poursuivent dans le cytosol d'érythrocytes immatures. Cette molécule est responsable de la couleur rouge, caractéristique de l'hémoglobine et du sang. Le repliement des chaînes polypeptidiques α et β dans l'espace, leur permet d'adopter une structure globulaire et de ménager une poche où peut se nicher l'hème de l'hémoglobine dont la structure est présentée à la **Figure I-9**.

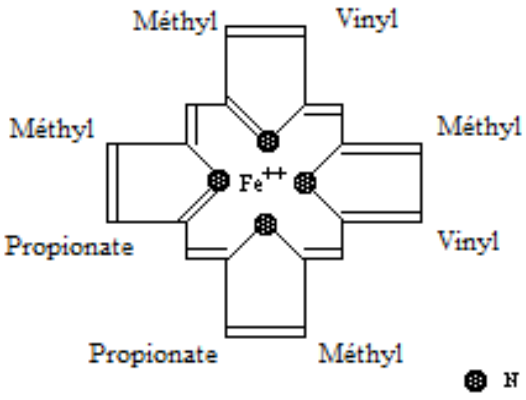


Figure I-9. Structure de l'hème
(http://acces.ens-lyon.fr/biotic/gpe/dossiers/drepanocytose/html/hbstr.htm).

L'hème est une protoporphyrine avec en son centre un atome de fer ferreux (Fe^{2+}), entouré de quatre noyaux pyrroles, reliés les uns aux autres par des ponts méthènes (-CH≡) et substitués par des groupements méthyles (4), propionates (2), et vinyls (2). Il permet d'assurer la fonction de transporteur d'oxygène (O_2) de l'hémoglobine qui se fixe au fer ferreux (Fe^{2+}) pour être distribué aux organes périphériques. L'hémoglobine passe alors de sa forme désoxygénée (ou désoxyhémoglobine) à sa forme oxygénée (ou oxyhémoglobine). Au sein de la désoxyhémoglobine, l'atome de fer possède cinq liaisons de coordination stables : quatre avec les atomes d'azote des noyaux pyrrole de l'hème et une avec l'atome d'azote d'un résidu histidine de la globine. La sixième liaison fixe l'oxygène, de façon réversible, formant ainsi l'oxyhémoglobine. Par conséquent, l'oxygène se lie à l'oxyhémoglobine dans les poumons et est ensuite transporté par le courant sanguin jusqu'à ce qu'il atteigne les tissus. Dans les tissus, l'oxygène est libéré de l'hémoglobine, et est ensuite transporté à la mitochondrie où il est utilisé pour la respiration aérobie. En échange, la désoxyhémoglobine, se lie à 2 protons et 2 molécules de CO_2 et les transporte vers les poumons, où le CO_2 est libéré par exhalation (Perutz, 1978).

I.1.2. Effet du milieu sur la structure de l'hémoglobine

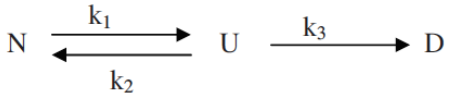
Généralement, l'environnement extérieur a un impact sur la structure des protéines qu'elles soient globulaires (hémoglobine, myoglobine, etc.) ou fibrillaires (actine, kératine, etc.). Plus spécifiquement, la modification de l'état natif et la dénaturation totale ou partielle de l'hémoglobine dépend de plusieurs facteurs. La dénaturation implique la modification des structures de l'hémoglobine suite à la rupture des liaisons qui participent sa consolidation. Par conséquent, la structure de l'hémoglobine se désorganise et la chaîne d'acides aminés prend une configuration aléatoire (Sherwood et Lockart, 2006). Des facteurs physiques ou chimiques peuvent être responsables de la variation de l'état natif des protéines et plus spécifiquement de l'hémoglobine.

I.1.2.1. Effet de quelques agents physiques

La température et le pH présentés dans cette partie sont deux éléments essentiels qui influencent la conformation des protéines et plus spécifiquement celle de l'hémoglobine et représentent des axes de réflexion qui pourraient nous aider durant notre étude pour comprendre le comportement de l'hémoglobine lors de sa dénaturation.

I.1.2.1.1. La température

L'augmentation de la température entraîne un affaiblissement des liaisons entre les différentes sous unités de l'hémoglobine et modifie les propriétés dont dépend sa conformation (pouvoir rotatoire, viscosité, absorption en UV), comme décrit par Yan *et al*. (2004). Pour toute protéine globalement, la température de dénaturation irréversible T_m peut être définie. Selon la théorie de Sanchez-Ruiz *et al.* (1988), la dénaturation irréversible des protéines en général peut être représentée par :



Dans cette représentation, *k* est la constante de cinétique de premier ordre qui varie en fonction de la température. Le passage de l'état natif (N) des protéines à l'état déplié U (« Unfolded ») est réversible. Par contre, Si $k_3 >> k_2$, les protéines atteignent l'état final irréversible D (« Denatured »), où les molécules sont transformées en agrégats (Sanchez-Ruiz *et al.*, 1988). Dans le cas de l'hémoglobine, le dépliement de la protéine a lieu autour de 42-72°C, tandis que la formation d'agrégats commence de 57-67°C, pour être complète à 87°C (Jansson et Swenson, 2008).

I.1.2.1.2. Le pH

En fonction du pH du milieu, les acides aminés des protéines ont la capacité d'être chargés positivement ou négativement, grâce aux groupements amines et carboxyles. Cette propriété

confère, à la molécule protéique, un pH isoélectrique (pH auquel la protéine a une charge neutre). La variation du pH impacte donc la structure quaternaire, tertiaire et secondaire de l'hémoglobine.

La dénaturation acide de l'hémoglobine suite à l'ajustement du pH de la solution de 6,8 à 4,4 entraîne un déséquilibre dans la structure de la protéine et une dissociation subséquente du tétramère en dimères αβ (Griffith et Kaltashov, 2003; Boys *et al.* 2007; Boys et Konerman, 2007). Pour des valeurs de pH très faibles (pH 2,1), les contacts entre les sous unités et les associations hème-protéine sont rompues et la sous-unité monomérique est fortement dépliée en solution (Dobo et Kaltashov, 2001; Konerman, 2007; Adje, 2010). Selon Boys *et al.* (2007), cette dissociation, due à l'effet du pH, peut être schématisée de la façon suivante :

$$(\alpha^h\beta^h)_2 \longrightarrow 2\alpha^h\beta^h \longrightarrow 2\alpha^h_{\text{repliée}} + 2\beta^h_{\text{repliée}} \longrightarrow 2\alpha^a_{\text{dépliée}} + 2\beta^a_{\text{dépliée}} + 4 \text{ hèmes}$$

I.1.2.2. Effet de quelques agents chimiques

Les agents présentés ci-dessous ont été démontrés comme dénaturants essentiels de l'hémoglobine. La description de leurs effets nous aidera à comprendre plusieurs notions dans notre étude.

I.1.2.2.1. L'urée

L'urée est un composé organique de formule chimique CON_2H_4. L'utilisation de l'urée a été démontré comme ayant un effet sur la dénaturation de l'hémoglobine pour des concentrations allant de 6 à 8M d'urée (Eduardo *et al*., 1983; Hedhili, 2014). Le traitement de l'hémoglobine avec de l'urée déstabilise partiellement sa structure secondaire, cependant un changement total de la disposition des groupements hydrophobes de la molécule impactant sa solubilité aura lieu.

I.1.2.2.2. Les alcools

Les alcools ont un effet sur la structure des protéines. La déformation de la structure protéique et sa précipitation peuvent être causés par des alcools tels que l'acétone et l'éthanol. La précipitation varie en fonction de la nature de l'alcool, de la longueur de la chaîne aliphatique et du nombre de groupements hydroxyles greffés sur cette chaîne. Il a été démontré que la faible constante diélectrique des alcools entraînant une diminution du pouvoir de solvatation des solutions aqueuses constituent la cause de l'agrégation protéique. De plus, les structures protéiques sont stabilisées grâce à des forces hydrophobes, cependant, les concentrations élevées de substances organiques solubles dans l'eau et ayant leurs propres interactions hydrophobes avec l'eau, interfèrent avec les interactions protéiques empêchant leur stabilisation (Kientz et Bigelow, 1996; Jacobson et Krueger, 1975; Dev *et al.*, 1984, Bhakumi, 1998). Plus spécifiquement, les alcools ont été testés à la place de l'urée lors de la dénaturation de l'hémoglobine. Il a été démontré qu'ils ont un effet sur le changement de la conformation de l'hémoglobine. Ils modifient les structures tertiaires et quaternaires de l'hémoglobine sans entraîner une modification de ses structures secondaires et favorisent une restructuration de l'hémoglobine en une conformation se trouvant entre l'état natif et l'état dénaturé (Adje, 2010).

I.1.2.2.3. Les sels

Selon Voet *et al.* (2007), certains sels comme le sulfate d'ammonium ($(NH_4)_2SO_4$), le phosphate de potassium ($K_2H_2PO_4$), le thiocyanate de potassium (KSCN) et le bromure de lithium (LiBr), stabilisent la structure protéique. Le chlorure de sodium (NaCl) et le chlorure de potassium (KCl) ont peu d'effet (Adje, 2010). L'effet « salting-out » a un effet sur l'ordre d'efficacité des sels comme défini par la série d'Hofmeister :

Anions: $SO^{2-}{}_4 > H_2PO_4^- > CH_3COO^- > Cl^- > Br^- > I^- > ClO_4^- > SCN^-$

Cations: NH_4^+ ; Cr^+ ; K^+ ; $Na^+ > Li^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$

Les ions: I^- ; ClO_4^- ; SCN^- ; Li^+ ; Mg^{2+} ; Ca^{2+} et Ba^{2+}, ont tendance à dénaturer la molécule protéique. De plus, selon Laurel *et al.* (2008), certains sels comme le thiocyanate de sodium (NaSCN), ou le perchlorate de sodium ($NaClO_4$) augmentent la solubilité de certains modèles peptidiques. L'effet « salting-in » impacte le rôle des sels sur le repliement des protéines comme décrit par Nandi et robinson (1972). Aujourd'hui, pour limiter l'utilisation d'urée, d'alcool et de tampon d'acide acétique et d'acétate de sodium, la dénaturation de l'hémoglobine se fait par la régulation du pH avec de l'HCl (Dubois *et al.*, 2005; Sion, 2019). Des concentrations élevées d'HCl peuvent provoquer la précipitation de l'hémoglobine au lieu de sa dénaturation. Cela est dû à la présence d'ions Cl^- en grande quantité, un anion chaotrope avec un β-coefficient de -0,005 (coefficients négatifs pour les ions faiblement hydratés) qui induit la déstabilisation de l'hémoglobine en perturbant sa couche d'hydratation causant par conséquent sa précipitation (Sion, 2019 ; Zhao, 2005).

I.2. Hydrolyse pepsique de l'hémoglobine bovine et peptides bioactifs

L'intérêt de l'hydrolyse enzymatique de l'hémoglobine par la pepsine réside dans l'obtention de peptides bioactifs qui ont des propriétés importantes dans le domaine de la cosmétique, pharmaceutique et alimentaire. Plusieurs études ont prouvé le potentiel de ces peptides issu de l'hydrolyse de l'hémoglobine bovine (Adje *et al.*, 2011a; Adje *et al.*, 2011b; Daoud *et al.*, 2005; Nedjar-Arroume *et al.*, 2008; Nedjar-Arroume *et al.*, 2006).

I.2.1. Généralités

Le procédé d'hydrolyse enzymatique conventionnelle se fait généralement en mode « par lot ». Pour cela, une solution d'hémoglobine ayant une concentration spécifique (p/v) dosée par la méthode de Drabkin (Crosby *et al*., 1954) doit être préparée. L'hémoglobine native est dénaturée par abaissement du pH suite à l'ajout d'une solution tampon d'acide acétique/acétate de sodium, d'urée ou d'autres produits chimiques (Adje, 2010; Nedjar *et al*., 2008; Nedjar *et al*., 2006; Lignot *et al*., 1999; Dubois *et al*., 2005; Zouari *et al*., 2020). Récemment, l'hémoglobine a été dénaturée par un abaissement du pH avec l'ajout d'acide chlorhydrique seulement (Sion, 2019). Une fois l'hémoglobine dénaturée, la pepsine est

ensuite ajoutée pour déclencher la réaction d'hydrolyse. À la fin de la réaction d'hydrolyse, l'enzyme est inactivée en élevant le pH à une valeur basique par l'ajout d'agents chimiques. La production de peptides bioactifs suite à l'hydrolyse enzymatique de l'hémoglobine bovine, consomme une grande quantité de produits chimiques, ce qui représente des volumes d'acide et de base relativement importants dans le cadre d'une production industrielle (**Figure I-10**). L'hydrolyse enzymatique de l'hémoglobine bovine peut être schématisée de la façon suivante :

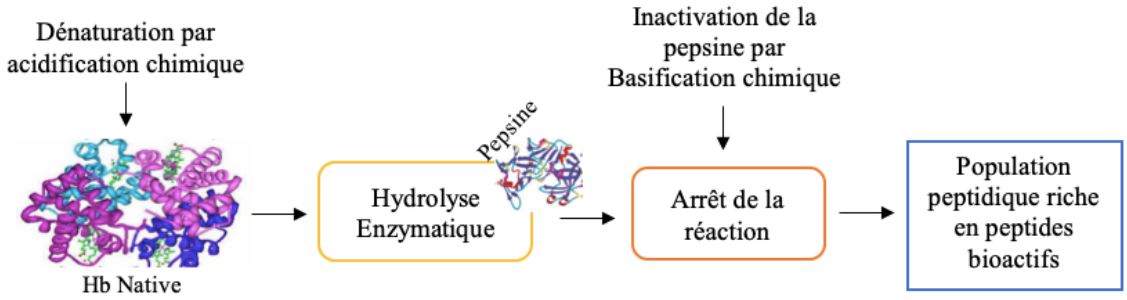


Figure I-10. Représentation schématique de l'hydrolyse enzymatique conventionnelle de l'hémoglobine bovine.

I.2.2. La pepsine porcine

La pepsine porcine est l'enzyme de choix pour l'hydrolyse de l'hémoglobine bovine. C'est une enzyme employée pour la production de peptides actifs par procédés *in vitro* (Korhonen *et al*., 2006; Danquah et Agyei, 2012; Udenigwe et Aluko, 2012) ou pour simuler *ex vivo* la digestion gastro-intestinale de protéines (Benedé *et al*., 2014; Dupont *et al*., 2010).

I.2.2.1. Structure

La pepsine porcine est une protéine enzymatique appartenant à la famille des aspartyl protéases (Dunn B.M., 2002). Elle fait partie de la même famille que la chymosine ou la cathepsine D (**Figure I-11**).

Figure I-11. Représentation spatiale de la pepsine porcine (Przybylski, 2016).

Il s'agit d'une des hydrolases prédominantes dans le suc gastrique, sécrétée dans l'estomac des vertébrés sous forme d'un précurseur inactif, le pepsinogène. Une fois qu'il est sécrété en condition acide (pH inférieur à 5) dans la lumière de l'estomac, le pepsinogène est clivé, donnant la pepsine active (Lehninger, 2000). La pepsine est une endopeptidase qui catalyse le clivage hydrolytique des liaisons peptiques au voisinage des résidus d'acides aminés hydrophobes ou aromatiques.

La chaîne peptidique de la pepsine porcine est constituée de 326 acides aminés, ayant une masse moléculaire globale de 34 600 Da. Elle est répartie en deux domaines principaux, le N-terminal (AA^{1}-AA^{176}) et le C-terminal (AA^{177}-AA^{326}) (Jin *et al.*, 2008). Elle possède essentiellement un agencement spatial en feuillets β, caractéristique des protéases à acide aspartique (Abad-Zapatero *et al.*, 1990) et trois ponts disulfures (Cys^{45}-Cys^{50}, Cys^{206}-Cys^{210} et Cys^{249}-Cys^{282}), elle possède également des hélices α et des hélices 3^{10}, mais en faible proportion.

I.2.2.2. Activité enzymatique et mode d'action de la pepsine

La réaction catalytique entre la pepsine et son substrat, l'hémoglobine bovine, est une réaction de premier ordre, où la concentration de produit formé est proportionnelle à la quantité de substrat disparu par unité de temps, ce qui est le cas de nombreuses enzymes protéolytiques (Saadi *et al*., 2015). La réaction peut donc être décrite selon le principe suivant, décrit par Michaelis *et al*. (1913), puis par Briggs *et al.* (1925) :

$$[pepsine] + [hémoglobine] \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [pepsine - hémoglobine] \xrightarrow{k_2} [pepsine] + [peptides]$$

Selon ces auteurs, la production des peptides est donc majoritairement dépendante de la constante K_2, ou K_{cat}, dont l'efficacité est soumise à la régulation ordonnée par les différentes conditions opératoires.

La production de peptides dépend du ratio enzyme/substrat, du pH et de la température de la réaction. La pepsine possède un pH optimum d'activité de 2,1 (Tanaka *et al.,* 2001) et une température optimale de 37°C (Hunkapiller *et al*., 1972). Elle perd son activité enzymatique à pH neutre. La pepsine est irréversiblement dénaturée entre pH 8,5-11 à température ambiante (Ryle, 1984).

Concernant le mode d'action de cette enzyme, il est basé sur la reconnaissance d'un motif structural dont la nomenclature a été déterminée par Schechter et Berger (1967). La nomenclature est présentée à la **Figure I-12**. Cette dernière permet de montrer que l'enzyme va hydrolyser la liaison peptidique située entre les résidus P1 et P1' des acides aminés du substrat (hémoglobine bovine) localisés au niveau des sites actifs d'interaction S1 et S1' de l'enzyme, la pepsine.

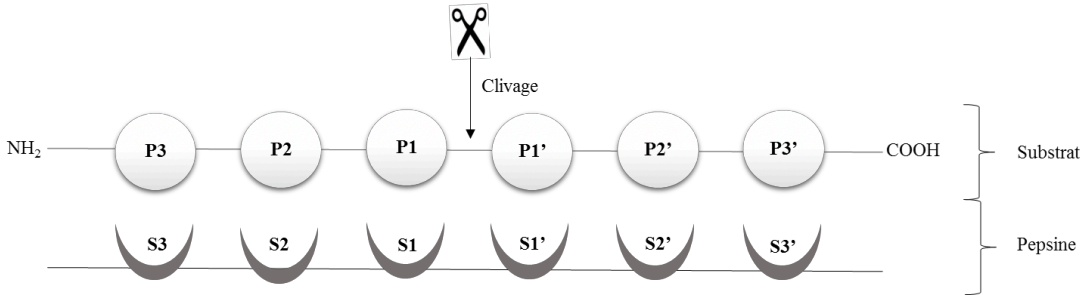


Figure I-12. Nomenclature de Schechter et Berger (1967) pour la description des sites actifs de la protéase, appliquée à la pepsine porcine.

La pepsine hydrolyse des liaisons peptidiques seulement, n'hydrolysant pas d'amide non-peptide ou de liaisons ester. De manière préférentielle, la pepsine clive les liaisons peptidiques en position C-terminale dans lesquelles un acide aminé aromatique (Tyr, Trp,

Phe) ou hydrophobe (Leu, Ala, Glu ou Gln) engage sa fonction amine en position P1 (Keil, 1992).

D'un point de vue spatial, le site actif de la pepsine comprend deux acides aspartiques proches (Asp^{32} et Asp^{215}), trois isoleucines (Ile^{30}, Ile^{120} et Ile^{213}), une sérine (Ser^{39}), deux alanines (Ala^{124} et Ala^{301}) et deux thréonines (Thr^{218} et Thr^{305}). Dunn (2002), a démontré que les deux acides aspartiques jouent simultanément les rôles de donneur et de receveur de protons. Le substrat se loge dans la crevasse formée par l'ensemble de ces acides aminés (Davies, 1990 ; Przybylski, 2016).

I.2.3. Mécanisme enzymatique

Les caractéristiques des peptides libérés par l'hydrolyse des protéines sont fortement liées au mécanisme en cours (Adje *et al.*, 2011a; b). L'hydrolyse pepsique de l'hémoglobine implique deux mécanismes principaux en fonction de sa structure : le mécanisme « zipper » et le mécanisme « one-by-one ». En effet, lorsque l'hémoglobine est à l'état dénaturé (dissociation de sa structure tétramérique), les sites de clivage à la pepsine sont plus accessibles. À ce moment, le processus d'hydrolyse conduit à la formation et au clivage successif de peptides intermédiaires plus ou moins stables selon le mécanisme « zipper » décrit pour la première fois par Linderstrom-Lang (1953). Le mécanisme « zipper » permet d'hydrolyser les sous-unités de l'hémoglobine bovine rapidement en des peptides de plus faibles masses moléculaires. Ces derniers sont appelés « peptides intermédiaires ». À leur tour, ils sont eux-mêmes digérés en des peptides plus courts, jusqu'à donner des peptides dits « finaux » qui s'accumulent tout au long de la réaction d'hydrolyse (Linderstrom-Lang, 1953). Généralement, les peptides intermédiaires sont caractérisés par une masse moléculaire supérieure à 2000 Da et présentent des caractéristiques structurelles qui leur confèrent une activité antibactérienne (Adje *et al*., 2011a). Cependant, lorsque l'hémoglobine est à l'état natif (dissociation partielle de son tétramère), son hydrolyse produit des peptides de petites tailles selon le mécanisme « one-by-one » (Linderstrom-Lang, 1953). Une mole d'enzyme dégrade alors une mole de substrat à la fois (Linderstrom-Lang, 1953). De ce fait, tout au long de la protéolyse, les concentrations en peptides augmentent de manière proportionnelle

et limitante par rapport au temps. En revanche, les peptides sont tous présents de manière qualitativement identique et cela quelle que soit la durée de l'hydrolyse. Ce mécanisme est observé pour des pH élevés et en absence de dénaturants chimiques tels que l'urée (Dubois *et al.,* 2005). Selon la littérature, il y a un intérêt principal de se placer dans des conditions permettant un mécanisme « zipper » afin de pouvoir influencer la population peptidique totale (nombre, variété et concentrations des peptides présents dans l'hydrolysat). Ce mécanisme permet d'obtenir des peptides avec des activités potentielles et valorisables. La différence entre ces deux mécanismes est présentée dans la **Figure I-13** (Dubois *et al.,* 2005).

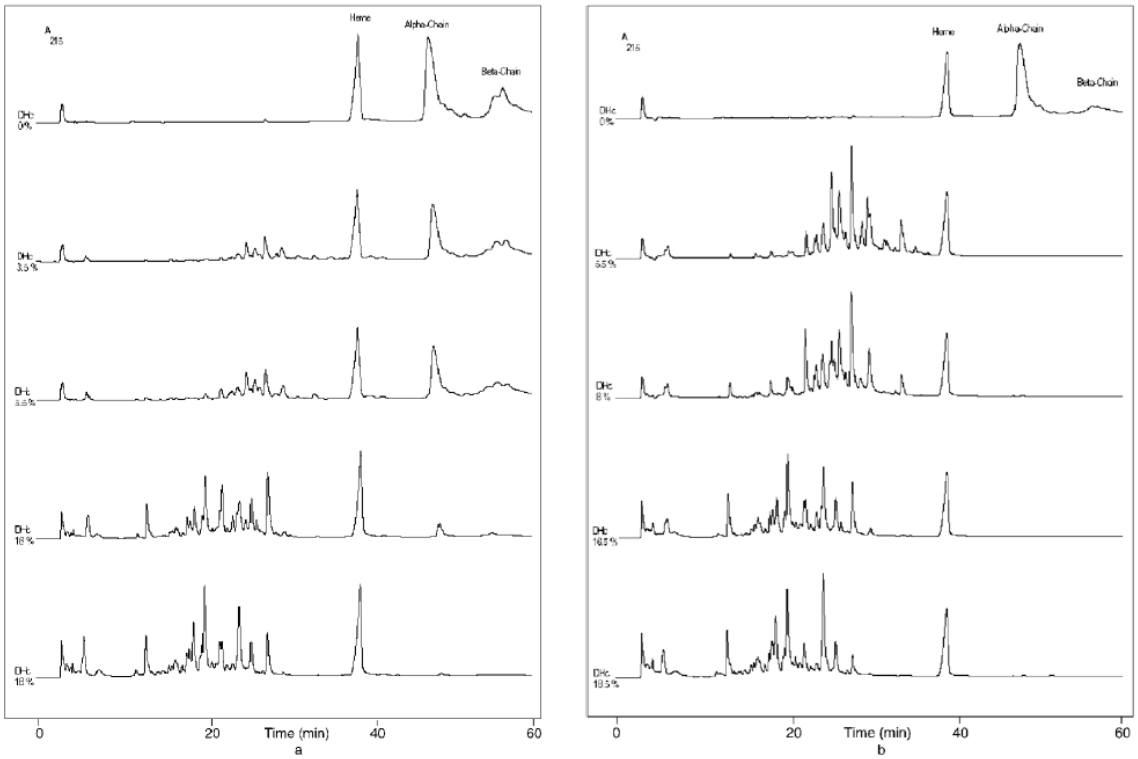


Figure I-13. Profils chromatographiques des hydrolysats d'hémoglobine bovine (a) hémoglobine native pH 4,5 sans agent dénaturant, mécanisme « one-by-one »; (b) hémoglobine dénaturée pH 3,5 sans agent dénaturant, mécanisme « zipper » (Dubois *et al.*, 2005).

I.2.4. Effet du milieu sur l'hydrolyse enzymatique de l'hémoglobine par la pepsine

I.2.4.1. Effet de quelques agents physiques

La variation de la température et du pH a un effet non seulement sur la structure de l'hémoglobine mais également sur la cinétique enzymatique par la pepsine.

I.2.4.1.1. La température

La température peut avoir un impact sur l'hydrolyse enzymatique de l'hémoglobine. Hedhili *et al*. (2013), ont étudié la cinétique de l'hydrolyse du peptide α1-32 issu de l'hémoglobine bovine par la pepsine à plusieurs températures (15, 23 et 37°C). Ils ont observé que la cinétique s'accélérait en augmentant la température. De plus, le peptide α1-32 disparaît presque complètement en environ 5 min à 15°C, 3 min à 23°C et <2 min à 37°C.

I.2.4.1.2. Le pH

L'effet du pH sur les populations peptidiques et les cinétiques d'hydrolyse de l'hémoglobine bovine par la pepsine a été étudié par Lignot (1998) et Dubois *et al*. (2005). À pH 2 et 3, l'hydrolyse pepsique de l'hémoglobine sans agent dénaturant suit le mécanisme « zipper » et aboutit à la production d'une population peptidique très complexe de tailles variables chargés positivement (Dubois *et al*., 2005). Tandis qu'à pH 4,5 et 5,5, sans agent dénaturant l'hydrolyse de l'hémoglobine suit le mécanisme « one-by-one » et aboutit à la production de peptides de tailles moins importantes et à faibles concentrations (Dubois *et al*., 2005; Su *et al*., 2007). Les auteurs ont expliqué que l'apparition de deux mécanismes différents était due à l'état de l'hémoglobine qui diffère selon le pH. À pH 4,5, l'hémoglobine est encore native, elle se déplie donc partiellement révélant ainsi certains sites inaccessibles. Plus encore, ils expliquent que l'ionisation des produits d'hydrolyse est différente selon le pH. Les peptides sont chargés positivement à pH 2 empêchant les associations par répulsions électrostatiques. Par contre, les peptides sont moins chargés à pH 4,5 favorisant les interactions. Ces interactions, vont limiter l'accessibilité de la pepsine à certains sites de coupure.

I.2.4.2. Effet de quelques agents chimiques

I.2.4.2.1. L'urée

Dans les conditions dénaturantes en présence d'urée (6M), l'hémoglobine bovine s'hydrolyse selon un mécanisme « zipper » permettant la production de plusieurs peptides bioactifs (Hedhili, 2014). La présence de l'urée provoque la dissociation complète du tétramère de l'hémoglobine favorisant l'accessibilité de la pepsine pour son substrat (l'hémoglobine) menant à la formation de peptides intermédiaires (600-7000 Da) ayant des structures variables : hélice alpha, feuillet bêta et « random coil » (Daoud *et al*., 2005). Le mécanisme « zipper » est aussi confirmé à pH 5,5 en présence d'urée 6M (Dubois *et al*., 2005).

I.2.4.2.2. Les alcools

L'effet de l'ajout d'alcools lors de l'hydrolyse enzymatique de l'hémoglobine bovine par la pepsine a été étudié. Le trifluoroéthanol (TFE) favorise la production de peptides actifs, hydrophobes et structurés puisqu'il stabilise l'activité de la pepsine à une concentration de 10% tandis qu'à des concentrations supérieures l'enzyme pourrait perdre son activité (20, 30 et 50%). Cela est lié au fait que les concentrations élevées de TFE sont à l'origine de la déstabilisation des interactions entre les molécules d'eau et l'enzyme, entraînant ainsi des modifications structurales et donc la perte de son activité (Adje, 2010). Adje (2010) a aussi démontré que le méthanol (40%), l'éthanol (30%), le propanol (20%) ou le butanol (10%), avaient des effets similaires au TFE (10%). La meilleure concentration de peptides a été obtenue lors de l'utilisation de 10% de butanol. L'hydrolyse a été effectuée à un pH 5,5 (hémoglobine native) selon un mécanisme enzymatique différent du « one-by-one » et du « zipper » permettant d'obtenir une faible population peptidique enrichie en peptides intermédiaires. Les peptides α67-106, α73-105, α99-105 et α100-105 ont été les premiers peptides antimicrobiens et antihypertenseurs obtenus par protéolyse *in vitro* d'hémoglobine bovine en présence de 30% d'éthanol. L'éthanol a permis le clivage et la stabilisation des peptides intermédiaires. Ces peptides étaient actifs contre quatre souches bactériennes : *Micrococcus luteus*, *Listeria innocua*, *Escherichia coli*, et *Staphylococcus aureus* (Adje *et*

al., 2011). Lignot *et al.* (1999a; b) ont observé une augmentation de la production de LVV-hémorphines-7, VV-hémorphines-7, néokyotorphine, VV-hémorphine-4 et du peptide potentialisateur de bradykinine à pH 4,5 suite à l'hydrolyse enzymatique de l'hémoglobine bovine en présence de 20% (v/v) d'éthanol. L'éthanol a un effet stabilisant sur la conformation de l'hémoglobine (Nedjar *et al*., 1991). La préparation des peptides actifs à pH 4,5 en présence d'éthanol pourrait être intéressant car, contrairement à pH 2, où l'hème et l'hémoglobine précipitent en partie, l'hydrolyse est réalisée dans un mélange homogène (Lignot *et al*., 1999a; b).

I.2.4.2.3. Les sels

Selon Zhao, (2005), il existe un effet des ions sur l'activité et la stabilité des enzymes suivant la série d'Hofmeister. Cependant, l'ordre des ions dans la stabilisation et la déstabilisation de diverses enzymes peut être différent selon l'application spécifique, la charge de l'enzyme, la concentration d'ions et la relation globale enzyme-milieu-substrat comme décrit par Zhao, (2005). L'effet des sels a été étudié sur la trypsine, une enzyme protéolytique, comme la pepsine, sécrétée par le système digestif. Il a été démontré par Zhao, (2005) que les sels neutres à haute concentrations (0,3-3,0 M) inhibent l'activité de la trypsine selon l'ordre d'efficacité croissante d'inhibition suivant pour les anions : CH_3COO^- < Cl^- < NO_3^- < Br^- < I^- < SCN^- < ClO_4^-, où ClO_4^- présente l'effet inhibiteur le plus élevé et suivant pour les cations : $(CH_3)_4N^+$ < Cs^+ <K^+ < Na^+ < Li^+, où Li^+ présente l'effet inhibiteur le plus élevé en raison de son plus grand coefficient β par rapport aux autres cations testés (Zhao, 2005).

I.2.5. Peptides bioactifs issus de l'hydrolyse pepsique de l'hémoglobine bovine

I.2.5.1. Peptides opioïdes

Les peptides possédant l'activité opioïde ont des propriétés analgésiques. L'activité opioïde est décrite comme simulant l'action de l'opium. Tous les peptides ayant cette activité peuvent être de potentiels substituts pharmacologiques aux traitements contre la douleur. Deux familles de peptides ayant la propriété opioïde au sein de l'hémoglobine bovine sont

représentées : Les kyotorphines, dérivées de la fin de la chaîne α et principalement du peptide néokyotorphine (α137-141), et les hémorphines, dérivées de la chaîne β, et plus précisément du fragment β31-40, également nommé LVVh-7. L'ensemble de ces peptides est résumé dans le **Tableau I-3**.

Dans la famille des kyotorphines, Takagi *et al*. (1982) et Sato *et al*. (1981) ont découverts et isolés du cerveau de bœuf deux peptides aux propriétés opioïdes et analgésiques : la kyotorphine (α140-141) et néokyotorphine (α137-141). Ces peptides agissent spécifiquement sur la met-enképhaline (neurotransmetteur et neuromodulateur possédant des propriétés analgésiques, présent dans le système nerveux central). Le tri-peptide TSK, présente une activité opioïde et antigonadotrope (Orts *et al*., 1980). Les peptides TSKY et TSKYR régulent le potentiel dépendant Ca^{++}/K^{+} au sein des fibres cardiaques chez la grenouille (Blishchenko *et al*., 1997).

La néokyotorphine présente un effet particulier comparable à celui de la Leu-enképhaline, un neuropeptide endogène produit par les cellules neuronales intervenant comme messager chimique dans les circuits de la douleur (Kiso *et al*., 1983). De plus, la néokyotorphine maintient une surexcitation neuronale en inhibant l'action de l'acide γ-aminobutyrique, ou GABA (Ueda *et al*., 1987). Ce dernier est un neurotransmetteur dit « de l'anxiété », il diminue l'activité nerveuse des neurones lors d'une situation de stress ou de peur. Plusieurs autres effets ont été décrits pour la néokyotorphine telles que des rôles dans la modulation vagale sur le rythme cardiaque (Pokrovky *et al*., 1995), dans la thermorégulation (kolaeva *et al*., 1990), dans la sortie de l'état d'hibernation chez certains animaux (Popova *et al*., 2003) ou encore dans la prolifération des adipocytes ou de certaines cellules cancéreuses (Blishchenko *et al*., 2001; Sazonova *et al*., 2003; Sazonova *et al*., 2007).

La famille des hémorphines comprend 12 peptides provenant tous du segment β31-40 de l'hémoglobine bovine. La fixation de ces peptides sur un récepteur opiacé spécifique leur permet d'exécuter leur activité opioïde. Brantl *et al*. (1986), ont montré que la chaîne β de l'hémoglobine contenait des peptides ayant une activité opiacée, les hémorphines. Il s'agit plus particulièrement de l'hémorphine-5 (β34-38) et de l'hémorphine-4 (β34-37).

Tableau I-3. Peptides opioïdes issus de l'hémoglobine bovine.

Position	Séquence	Références
α137-141	TSKYR	Takagi *et al*. (1982)
α137-140	TSKY	Blishchenko *et al.* (1997)
α137-139	TSK	Orts *et al.* (1980)
α140-141	YR	Sato *et al.* (1981)
β31-40	LVVYPWTQRF	Piot *et al*. (1992)
β32-41	VVYPWTQRFF	Garreau *et al.* (1998)
β32-40	VVYPWTQRF	Piot *et al*. (1992)
β31-39	LVVYPWTQR	Glämsta *et al*. (1991)
β32-39	VVYPWTQR	Nyberg *et al.* (1997)
β32-37	VVYPWT	Barkhudaryan *et al.* (1992)
β34-39	YPWTQR	Nyberg *et al.* (1997)
β31-38	LVVYPWTQ	Zhao *et al.* (1997)
β32-38	VVYPWTQ	Zhao *et al.* (1997)
β34-38	YPWTQ	Brantl *et al.* (1986)
β31-37	LVVYPWT	Lignot *et al.* (1999)
β32-37	VVYPWT	Lignot *et al.* (1999)
β33-36	VYPW	Lee *et al.* (2003)
β34-37	YPWT	Brantl *et al.* (1986)

I.2.5.2. Peptides hématopoïétiques

Les peptides hématopoïétiques (**Tableau I-4**) sont les peptides intervenant dans l'hématopoïèse (formation des cellules sanguines), au cours de la formation des globules rouges du sang, située principalement au sein de la moelle rouge des os.

Tableau I-4. Peptides hématopoïétiques issus de l'hémoglobine bovine.

Position	Séquence	Références
α1-8	VLSAADKG	Ivanov *et al*. (1992)
α1-13	VLSAADKGNVKAA	Ivanov *et al.* (1992)
α1-16	VLSAADKGNVKAAWGK	Ivanov *et al.* (1992)
α3-10	SAADKGNV	Ivanov *et al.* (1992)

Position	Séquence	Références
α16-28	KVGGHAAEYGAEA	Ivanov *et al.* (1992)
α17-29	VGGHAAEYGAEAL	Ivanov *et al.* (1992)
α17-28	VGGHAAEYGAEA	Ivanov *et al.* (1992)
α22-29	AEYGAEAL	Ivanov *et al.* (1992)
α25-31	GAEALER	Ivanov *et al.* (1992)
α27-30	EALE	Ivanov *et al.* (1992)
β2-12	LTAEEKAAVTA	Ivanov *et al.* (1992)
β2-11	LTAEEKAAVT	Ivanov *et al*. (1992)
β2-10	LTAEEKAAV	Ivanov *et al.* (1992)
β16-31	KVKVDE	Ivanov *et al*. (1992)
β22-31	VGGEALGRLL	Ivanov *et al.* (1992)
β115-122	RNFGKEFT	Ivanov *et al.* (1992)
α76-82	LPGALSE	Vercaigne-Marko *et al*. (2000)
β94-100	KLHVDPE	Vercaigne-Marko *et al.* (2000)

I.2.5.3. Peptides inhibiteurs de la dipeptidyl-peptidase IV

Les activités hémorphines, présentant des propriétés opioïdes, influent sur l'inhibition de la dipeptidyl-peptidase IV (DPP-IV) (**Tableau I-5**). Parmi elles, les VVh-8, VV-h4, VV-h7 et LVV-h7 sont de puissants inhibiteurs de la DPP-IV, inactivant le glucagon (GLP-1) et régulant de manière transitoire son ARNm dans des cellules humaines Caco-2 (Caron *et al*., 2016; Caron *et al*., 2017). Trois nouvelles séquences peptidiques ANVST, TKAVEH et KAAVT ont été isolées pour leurs effets stimulants sur la sécrétion de GLP-1. La séquence VAAA a été isolée pour ses propriétés inhibitrices de la DPP-IV (Caron *et al.*, 2016).

Tableau I-5. Peptides inhibiteurs de la dipeptidyl-peptidase IV.

Position	Séquence	Références
α130-134	ANVST	Caron *et al*. (2016)
α63-66	VAAA	Caron *et al*. (2016)
α68-73	TKAVEH	Caron *et al.* (2016)
β6-10	KAAVT	Caron *et al.* (2016)

I.2.5.4. Peptides antihypertenseurs, potentialisateurs de la bradykinine, hypolipidémiants, coronaro-constricteurs et stimulateurs de la croissance bactérienne

Les activités opioïde, hématopoïétique ou antimicrobienne sont largement identifiés sur les peptides générés suite à l'hydrolyse enzymatique de l'hémoglobine bovine. Toutefois, quelques activités supplémentaires sont également représentées et les peptides possédant ces activités sont référencés au **Tableau I-6.**

Les peptides potentialisateurs de la bradykinine, α129-134 et β129-134, ont été découverts par Piot *et al.* (1992) et α110-125 par Zhao et Piot, (1997). En se fixant de manière spécifique sur les récepteurs de la quinine, ces peptides possèdent des effets analgésiques.

Un peptide hypolipidémiant, le β32-35, a également été trouvé. Il inhibe l'adsorption des graisses au niveau de l'appareil digestif et/ou actionne l'activité de la lipase hépatique sur les triglycérides, par conséquent, le taux de lipides diminue au niveau hépatique et digestif (Kagawa *et al.*, 1996). Trois peptides (β32-36, β133-145 et α1-21) ont également montré une activité coronaro-constrictrice (Barkhudaryan *et al.*, 1993; Barkhudaryan *et al.*, 1992). La croissance des bactéries Gram négatif a été stimulée par l'addition du β48-52 (Zhao *et al.*, 1996a). La stimulation de ces bactéries présente un intérêt puisqu'ils ont des effets bénéfiques pour la santé comme les bactéries Lactiques (Lactobacillus casei, Streptococcus thermophilus, Lactococcus lactis…) (Zhao *et al.*, 1996a).

Enfin, récemment, Adje *et al.* (2011) ont isolé quatre peptides anti-hypertenseurs qui ont pour rôle de lutter contre l'hypertension, et donc de diminuer la tension artérielle, généralement par un mécanisme visant à inhiber l'enzyme de conversion de l'angiotensine I en angiotensine II au sein du système rénine-angiotensine.

Tableau I-6. Peptides potentialisateurs de la bradykinine, hypolipidémiants, stimulateurs de la croissance bactérienne, anti-hypertenseurs et coronaro-constricteurs issus de l'hémoglobine bovine.

	Position	Séquence	Références
Potentialisateur de la bradykinine	α110-125	ASHLPSDFTPAVHASL	Zhao et Piot (1997)
	α129-134	LANVST	Piot *et al.* (1992)
	β129-134	QKVVAG	Piot *et al.* (1992)
Hypolipidémiant	β32-35	VVYP	Kagawa *et al*. (1996)
Coronaro-constricteur	β32-36	VVYPW	Barkhudaryan *et al.* (1992)
	α1-21	VLSAADKGNVKAAWGKVGGHA	Barkhudaryan *et al.* (1993)
	β133-145	VAGVANALAHRYH	Barkhudaryan *et al.* (1993)
Stimulateur de la croissance bactérienne	β48-52	STADA	Zhao *et al.* (1996)
Anti-hypertenseur	α67-106	TKAVEHLDDLPGALSELSDL--HAHKLRVDPVNFKLLSHSLL	Adje *et al.* (2011)
	α73-105	LDDLPGALSELSDLHAHKL--RVDPVNFKLLSHSL	
	α99-105	KLLSHSL	
	α100-105	LLSHSL	

I.2.5.5. Peptides antioxydants

L'activité antioxydante montrée par les peptides est classée en deux groupes selon la base des réactions chimiques impliquées : méthodes basées sur le transfert d'atomes d'hydrogène et méthodes basées sur le transfert d'électrons (Huang *et al*., 2005).

Les tests basés sur le transfert d'atomes d'hydrogène évaluent la capacité d'un peptide à réduire les radicaux libres par le don d'hydrogène dans une réaction compétitive. Les dosages *in vitro* utilisés pour sa mesure sont le test de capacité d'absorbance des radicaux d'oxygène (ORAC), paramètre antioxydant de piégeage des radicaux totaux (TRAP) et test de blanchiment au β-carotène. Les tests basés sur le transfert d'électrons évaluent la capacité d'un antioxydant potentiel à transférer un électron pour réduire un oxydant. Les tests

d’activité de piégeage des radicaux 2,2'-azino-bis(3-éthylbenzothiazoline-6-sulphonique) (ABTS), test de pouvoir antioxydant à réduction ferrique (FRAP) et activité de piégeage des radicaux 2,2-diphényl 1-picrylhydrazyle (DPPH) sont utilisées pour sa mesure (Huang *et al.*, 2005).

Selon Liu *et al.* (2016), la plupart des peptides antioxydants ont entre 4 et 16 acides aminés, avec une masse moléculaire d'environ 400-2000 Da. Le poids moléculaire affecte l’atteinte des sites cibles et pourrait augmenter la capacité antioxydante *in vivo* (Li *et al*. 2004). Le type d'acide aminé joue un rôle important dans la détermination de l'activité antioxydante des peptides. À cet égard, les acides aminés aromatique tels que tyrosine (Tyr), histidine (His), tryptophane (Trp) et phénylalanine (Phe) peuvent donner des protons contribuant aux propriétés anti-radicalaires (Rajapakse *et al*., 2005). D'autre part, les acides aminés hydrophobes ont été décrits comme étant capables d'augmenter la présence de peptides à l'interface eau-lipide leur permettant d’accéder plus facilement au piégeage des radicaux libres de la phase lipidique (Ranathunga *et al*. 2006). Enfin, les acides aminés acides utilisent le carbonyle et les groupes aminés dans la chaîne latérale qui fonctionnent comme des chélateurs d'ions métalliques (Suetsuna *et al*. 2000).

Il a été démontré que les hydrolysats d’hémoglobine et de cruors bovin et porcin suite à leur hydrolyse conventionnelle par la pepsine sont une source de peptides bioactifs ayant une activité antioxydante élevée (Sion *et al.*, 2019; Zouari *et al.*, 2020). Przybylski *et al.* (2016), ont démontré le rôle de la néokyotorphine comme un conservateur naturel pour protéger la viande pendant son stockage ou sa distribution. Par son application sur la viande, le peptide α137-141 a montré une réduction de 60% de l’oxydation des lipides et a inhibé la croissance microbienne jusqu’à 14 jours au réfrigérateur avec des effets proches du butylhydroxytoluène (BHT), couramment utilisé comme conservateur de viande.

I.2.5.6. Peptides antimicrobiens

I.2.5.6.1. Généralités

Le peptide α1-23, actif contre *Micrococcus luteus* A270, a été le premier peptide antimicrobien isolé par Froidevaux *et al.* (2001) suite à l'hydrolyse pepsique de l'hémoglobine bovine. À l'issue de l'hydrolyse pepsique de l'hémoglobine, 30 peptides présentant une activité antimicrobienne ont été découverts après fractionnement par HPLC : 24 proviennent de la chaîne α de l'hémoglobine et 6 sont des dérivés de la chaîne β de l'hémoglobine (Nedjar *et al*., 2008). Ils présentent une activité antibactérienne contre certaines souches comme : *Micrococcus luteus, Listeria innocua, Escherichia coli* et *Salmonella enteritidis.* De nouveaux peptides ont été identifiés à partir de l'hydrolyse pepsique de l'hémoglobine bovine purifiée en présence de différents solvants par Adje *et al*. (2011). L'ensemble des peptides antimicrobiens issus de la chaîne alpha et bêta de l'hémoglobine bovine sont présentés dans le **Tableau I-7.**

Tableau I-7. Peptides antimicrobiens issus de l'hydrolyse pepsique de l'hémoglobine bovine (d'après a : Nedjar-Arroume *et al*., 2008) et b : Adje *et al*., 2011).

Position	Séquence	Références
	Milieu aqueux	
α1-32	LSAADKGNVKAAWGKVGGHAAEYGAEALERM	a
α1-29	VLSAADKGNVKAAWGKVGGHAAEYGAEAL	a
α1-28	VLSAADKGNVKAAWGKVGGHAAEYGAEA	a
α1-27	VLSAADKGNVKAAWGKVGGHAAEYGAE	a
α1-23	VLSAADKGNVKAAWGKVGGHAAE	a
α33-98	FLSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAALTKAVEHLDD LPGALSELSDLHAHKLRVDPVNF	a
α33-97	FLSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAALTKAVEHLDD LPGALSELSDLHAHKLRVDPVN	a
α34-98	LSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAALTKAVEHLDDL PGALSELSDLHAHKLRVDPVNF	a
α36-97	FPTTKTYFPHFDLSHGSAQVKGHGAKVAAALTKAVEHLDDLP GALSELSDLHAHKLRVDPVN	a

α37-98	PTTKTYFPHFDLSHGSAQVKGHGAKVAAALTKAVEHLDDLPG ALSELSDLHAHKLRVDPVNF	a
α33-83	FLSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAALTKAVEHLDD LPGALSEL	a
α34-83	LSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAALTKAVEHLDDL PGALSEL	a
α33-66	FLSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAAL	a
α34-66	LSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAAL	a
α33-46	FLSFPTTKTYFPHF	a
α33-45	FLSFPTTKTYFPH	a
α34-46	LSFPTTKTYFPHF	a
α36-45	FPTTKTYFPH	a
α37-46	PTTKTYFPHF	a
α107-141	VTLASHLPSDFTPAVHASLDKFLANVSTVLTSKYR	a
α107-136	VTLASHLPSDFTPAVHASLDKFLANVSTVL	a
α107-133	VTLASHLPSDFTPAVHASLDKFLANVS	a
α133-141	STVLTSKYR	a
α137-141	TSKYR	a
β1-30	MLTAEEKAAVTAFWGKVKVDEVGGEALGRL	a
β1-13	MLTAEEKAAVTAF	a
β114-145	ARNFGKEFTPVLQADFQKVVAGVANALAHRYH	a
β121-145	FTPVLQADFQKVVAGVANALAHRYH	a
β126-145	QADFQKVVAGVANALAHRYH	a
β140-145	LAHRYH	a
	Milieu hydroalcoolique	
α1-32	VLSAADKGNVKAAWGKVGGHAAEYGAEALERM	b
α1-33	VLSAADKGNVKAAWGKVGGHAAEYGAEALERMF	b
α1-46	VLSAADKGNVKAAWGKVGGHAAEYGAEALERMFLSFPTTKT YFPHF	b
α100-105	LLSHSL	b
α106-141	LVTLASHLPSDFTPAVHASLDKFLANVSTVLTSKYR	b
α107-128	VTLASHLPSDFTPAVHASLDKF	b
α107-133	VTLASHLPSDFTPAVHASLDKFLANVS	b
α107-136	VTLASHLPSDFTPAVHASLDKFLANVSTVL	b
α107-141	VTLASHLPSDFTPAVHASLDKFLANVSTVLTSKYR	b
α110-141	ASHLPSDFTPAVHASLDKFLANVSTVLTSKYR	b

α137-141	TSKYR	b
α3-36	SAADKGNVKAAWGKVGGHAAEYGAEALERMFLSF	b
α34-65	LSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAA	b
α62-91	VAAALTKAVEHLDDLPGALSELSDLHAHKL	b
α62-106	VAAALTKAVEHLDDLPGALSELSDLHAHKLRVDPVNFKLLSHS LL	b
α73-105	LDDLPGALSELSDLHAHKLRVDPVNFKLLSHSL	b
α86-109	LHAHKLRVDPVNFKLLSHSLLVTL	b
α96-106	VNFKLLSHSLL	b
α99-105	KLLSHSL	b
α99-106	KLLSHSLL	b
β1-30	MLTAEEKAAVTAFWGKVKVDEVGGEALGRL	b
β77-125	LDDLKGTFAALSELHCDKLHVDPENFKLLGNVLVVVLARNFG KEFTPVL	b
β79-104	DLKGTFAALSELHCDKLHVDPENFKL	b
β85-105	AALSELHCDKLHVDPENFKLL	b
β114-124	ARNFGKEFTPV	b
β114-145	ARNFGKEFTPVLQADFQKVVAGVANALAHRYH	b
β126-145	QADFQKVVAGVANALAHRYH	b

I.2.5.6.2. Caractéristiques des peptides antimicrobiens

La classification des peptides antimicrobiens est extrêmement difficile à cause de leur grande diversité. En règle générale, pour interagir avec les membranes biologiques, un peptide antimicrobien doit posséder plusieurs caractéristiques. La charge, la conformation, l'hydrophobicité et le caractère amphiphile de la séquence peptidique sont autant d'éléments influençant son action (Yeaman et Yount, 2003). Pour cela, il doit d'abord posséder une charge globale positive, permettant l'interaction avec la membrane. Une conformation particulière est aussi importante formant dans la plupart des cas, des pores essentiels à l'insertion membranaire. Ensuite, un caractère amphiphile semble souvent nécessaire afin qu'il puisse s'insérer dans la membrane, puis un caractère hydrophobe afin de la perméabiliser (Powers *et al*., 2003; Yeaman et Yount, 2003).

I.2.5.6.2.1. Charge

La charge du peptide antimicrobien est un élément primordial de son activité (Yeaman et Yount, 2003; Van Der Weerden *et al*., 2013; Bahar *et al*., 2013; Kumar *et al*., 2018). Des domaines cationiques sont indispensables à l'établissement des interactions électrostatiques entre la membrane phospholipidique chargée négativement, et les peptides chargés positivement. Plus la charge du peptide est élevée, plus le potentiel antimicrobien du peptide est important. Cependant, au-delà d'une charge de +5, l'activité antimicrobienne diminue (Dathe *et al*., 2001). Pour des charges positives élevées, l'interaction du peptide avec les têtes polaires des membranes bactériennes devient trop importante, empêchant la translocation de la séquence à l'intérieur de la membrane (Akkam, 2016).

I.2.5.6.2.2. Conformation

L'organisation des peptides en structures secondaires, a une grande importance lors de l'interaction de la séquence peptidique avec la membrane microbienne. Les peptides antimicrobiens peuvent être répartis en quatre grands groupes : les peptides en hélice α amphiphile, en feuillet β stabilisé par un ou plusieurs ponts disulfures, les peptides linéaires riches en acides aminés particuliers et, enfin, les petits peptides à structure dite « random coil ».

I.2.5.6.2.2.1. Peptides en hélice α amphiphiles

Les peptides en hélice α amphiphiles sont présents chez de nombreux organismes comme l'homme, les insectes ou même les champignons (Powers *et al*., 2003). Ils ont été les plus étudiés et apparaissent comme possédant une structure et un mode d'action simple. Ils sont cationiques possédant 40 à 60% de résidus hydrophobes, leur taille est inférieure à 40 acides aminés et ne possèdent pas de résidu cystéine. Ces peptides linéaires se structurent au contact de la membrane bactérienne en adoptant une conformation hélicoïdale en hélices α amphiphiles (Bechinger *et al*., 1993) (**Figure I-14**). Ces peptides forment des pores au sein

de la membrane, induisant une perméabilisation membranaire et facilitant leur insertion après interaction avec les phospholipides membranaires.

Figure I-14. Structure secondaire d'un peptide antimicrobien en hélice α amphiphile (Powers *et al*., 2003).

I.2.5.6.2.2.2. Peptides en feuillets β

La famille des peptides en feuillets β contient des cystéines leur permettant de stabiliser leur structure au moyen de ponts disulfures (**Figure I-15**). Généralement, ils vont être caractérisés par une série de feuillets β anti-parallèles. De plus, la présence de portions organisées en hélices α permet de maintenir un caractère amphiphile et d'adopter des conformations diverses, aboutissant le plus souvent à la formation de pores nécessaire à l'interaction avec la membrane biologique. Ces peptides ne sont pas linéaires.

Figure I-15. Structure secondaire des peptides antimicrobiens organisés en feuillets β (Powers *et al*., 2003).

I.2.5.6.2.2.3. Peptides non structurés riches en acides aminés particuliers

Les peptides antimicrobiens riches en acides aminés rigides et particuliers comme l'histidine, la proline, l'arginine, la phénylalanine et le tryptophane (Epand *et al*., 1999; Powers *et al*., 2003) n'adoptent pas de structure secondaire organisée en hélice α ou en feuillet β. Ils

possèdent un mécanisme d'action différent induisant la modification de leur structure quaternaire (Yeaman et Yount, 2003) et agissent au niveau de la synthèse de protéines contrairement aux peptides organisés en hélice α qui induisent une perméabilisation de la membrane bactérienne (Andreu *et al*., 1998). Ils sont appelés « extended peptide » (Powers *et al*., 2003), comme illustré en **Figure I-16**.

Figure I-16. Structure secondaire d'un peptide non structuré (Powers et al., 2003).

I.2.5.6.2.2.4. Peptides à structure « random coil »

Les peptides à structure « random coil » sont caractérisés par une séquence courte, souvent inférieure à dix acides aminés, par une charge globale positive et présentant peu de résidus hydrophobes, voire même aucun (Catiau *et al*., 2011; Nedjar-Arroume *et al*., 2008). Nedjar-Arroume *et al.* (2008), ont décrit que les peptides en structure « random coil » s'organisent au contact de la membrane et possèdent des mécanismes d'action particuliers, faisant intervenir un déterminant antimicrobien tel que le Lys-Tyr-Arg (KYR) issus de la chaîne alpha ou le Arg-Tyr-His (RYH) issus de la chaine béta. La néokyotorphine qui représente les cinq derniers acides aminés en C-terminal de la chaîne α de l'hémoglobine, à savoir : Thr^{137}-Ser^{138}-Lys^{139}-$Tyr^{140}Arg^{141}$ (TSKYR), ayant une masse molaire de 653 Da, un point isoélectrique de 10,5 et une charge +2 à un pH de 7 (Catiau *et al*., 2011) fait partie de cette catégorie. Son mécanisme d'action n'est pas classique, parce qu'il ne possède pas de résidus hydrophobes ni de structure secondaire. Pour interagir avec la membrane biologique, il fait intervenir un déterminant antimicrobien le Lys-Tyr-Arg (ou KYR), constitué des trois derniers acides aminés en C-terminal du peptide (Catiau *et al*., 2011). De plus, sans intervention d'une structure secondaire, les charges positives de la lysine et de l'arginine de la néokyotorphine permettent l'interaction du peptide chargé positivement avec les

phospholipides des membranes biologiques chargés négativement (Powers *et al*., 2003). La **Figure I-17**, montre que suite à l'interaction avec la membrane, un changement conformationnel de la tyrosine (Y), un acide aminé contenu dans la séquence de l'α137-141, a lieu. Le changement se traduit par une modification de l'orientation de son noyau phénolique par rapport à la membrane lipidique, devenant parallèle aux phospholipides (Lopes *et al*., 2005). Suite à cette nouvelle orientation, le peptide applique son effet antimicrobien particulier puisqu'il est capable de pénétrer plus profondément dans la membrane, déstabilisant par la suite totalement la structure (Catiau *et al*. 2011; Lopes *et al.* 2005; Przybylski, 2016).

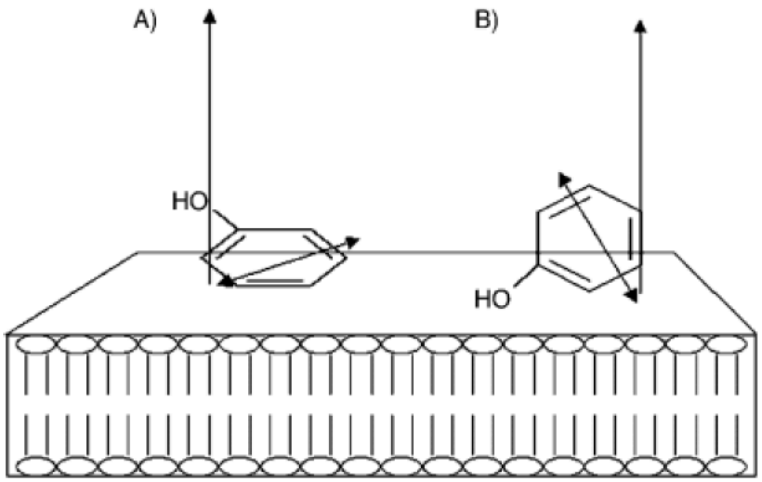


Figure I-17. Changement d'orientation du noyau aromatique de la tyrosine de la néokyotorphine après interaction avec une membrane biologique (Lopes *et al.*, 2005). A : noyau phénolique perpendiculaire à la membrane biologique induisant une pénétration réduite du peptide au sein de la structure; B : noyau phénolique parallèle à la membrane biologique favorable à une pénétration importante du peptide au sein de la structure.

I.2.5.6.2.3. Hydrophobicité

L'hydrophobicité d'un peptide est définie comme étant le pourcentage de résidus hydrophobes au sein de sa séquence en acides aminés. Cette caractéristique influe sur la capacité intrinsèque du peptide à se mouvoir depuis une phase aqueuse vers une phase hydrophobe (Dathe *et al*., 1999). L'hydrophobicité est une exigence essentielle pour l'interaction avec les membranes peptidiques ainsi que la perméabilisation membranaire. La

plupart des peptides antimicrobiens possèdent entre 40 et 60% de résidus hydrophobes, alternés avec des domaines cationiques. Ainsi, chaque caractéristique a son rôle, la partie cationique permet de fixer le peptide aux têtes polaires des phospholipides des membranes, alors que la partie hydrophobe permet d'insérer le peptide au sein de la membrane, la déstabilisant et engendrant la lyse de l'agent microbien par entrée d'eau (Hancock *et al*., 2000; Akkam, 2016). L'augmentation de l'hydrophobicité des peptides pourrait signifier une affinité plus importante pour les lipides, augmentant ainsi l'activité antimicrobienne, mais elle a aussi été liée à une augmentation de l'activité hémolytique (Jiang *et al*., 2008; Yeaman et Yount, 2003).

I.2.5.6.2.4. Caractère amphiphile

L'ensemble des peptides antimicrobiens possède des groupements hydrophiles et hydrophobes, formant ainsi une structure amphiphile. La plupart des peptides antimicrobiens forment des structures amphipathiques lors de l'interaction avec les membranes cibles. L'augmentation du nombre d'hélices α peut induire une augmentation dans le caractère amphiphile, entraînant l'augmentation de l'activité antimicrobienne (Yeaman et Yount, 2003).

I.2.5.6.3. Mécanisme d'action des peptides antimicrobiens

De par leurs structures et caractéristiques physico-chimiques variées, les peptides antimicrobiens possèdent des mécanismes d'action divers qui diffèrent en fonction de la cible microbienne. Globalement, les peptides antimicrobiens agissent soit directement au niveau de la membrane cellulaire, entraînant sa perturbation puis la mort de la cellule, soit par la mise en jeu de phénomènes osmotiques rapides ou soit sur des mécanismes intracellulaires, par exemple sur la synthèse de protéines (Jenssen *et al.*, 2006).

I.2.5.6.3.1. Mécanisme d'action des peptides perméabilisant les membranes

Globalement, le mécanisme d'action des peptides antimicrobiens se fait en plusieurs étapes: la fixation à la membrane (dépendante de la concentration du peptide et de sa capacité à s'auto-structurer), l'intégration dans la membrane (dépendante de la conformation et de la capacité d'auto-assemblage des peptides) et l'action antimicrobienne (généralement la perméabilisation membranaire).

I.2.5.6.3.1.1. Fixation du peptide antimicrobien à la membrane bactérienne

La fixation du peptide antimicrobien à la membrane bactérienne représente l'étape initiale de l'action antimicrobienne et implique d'une part des interactions hydrophobes entre les chaînes acyles lipidiques et les chaînes hydrophobes des peptides, et d'autre part, les interactions électrostatiques entre les chaînes hydrophiles des peptides et les têtes polaires des phospholipides. Selon le type de bactérie-cible, l'établissement de l'interaction entre les charges positives des peptides et les charges négatives des phospholipides de la membrane bactérienne s'opère différemment (**Figure I-18**). Cette fixation se fait grâce aux :

- Lipopolysaccharide (ou LPS) des bactéries à Gram-négatif, par le biais de ses groupements phosphate,
- Peptidoglycane des bactéries à Gram-positif, par le biais d'acides lipoteichoïques présents à la surface (Jenssen *et al*., 2006).

Une fois fixés à la membrane, il a été décrit que les peptides antimicrobiens possédaient deux états de fixation. Le premier est un état dit « de surface » (ou S). Le second, ou état I, est un état de formation de pores, où le peptide est prêt à intégrer la membrane biologique (Huang, 2000).

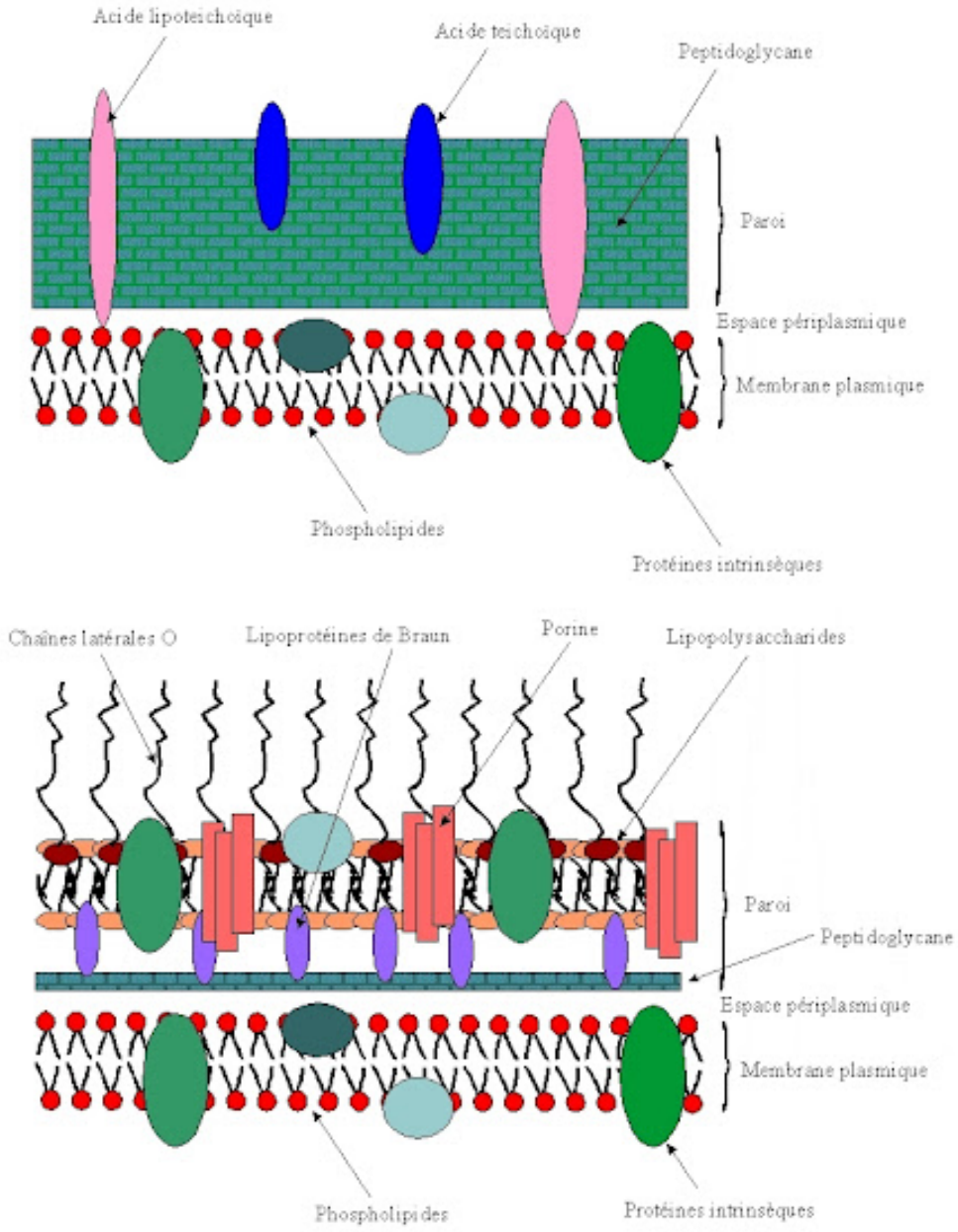


Figure I-18. Description de la paroi des bactéries à Gram-positif et Gram-négatif (Michel, 2010).

I.2.5.6.3.1.2. Intégration du peptide antimicrobien au sein de la membrane bactérienne

Dans un premier temps, où le ratio peptides/lipides est faible, les peptides sont sous état inactif, ou état S, adsorbés sur la membrane. Pour cet état, les peptides sont orientés de manière parallèle à la membrane. Ensuite, avec l'augmentation de peptides fixés, le ratio

peptides/lipides augmente lui aussi, permettant aux peptides de s'orienter de manière perpendiculaire à la membrane. Ils peuvent alors s'y insérer et former des pores transmembranaires : les peptides sont en état I (Huang, 2000).

I.2.5.6.3.1.3. Actions antimicrobiennes

Une fois la membrane lipidique passée, les peptides peuvent agir avec la membrane cytoplasmique et exercer leur activité antimicrobienne. Cela peut s'effectuer par le biais de la lyse membranaire aboutissant à une fuite du contenu cytoplasmique. Suite à la formation des pores par les peptides, la perméabilité membranaire est ainsi modifiée. Différents modèles ont été décrits : le modèle en douves de tonneau, le modèle en pores toroïdaux et le modèle en tapis. Exposés ci-après successivement, ces modèles historiques ont ensuite fait l'objet du modèle de Shai, Matsuzaki et Yang (Matsuzaki, 1999 ; Shai, 1999 ; Yang et al., 2000).

Le modèle en douves de tonneau est principalement décrit pour des peptides adoptant une structure en hélice α lors de leur interaction avec la membrane phospholipidique, comme décrit par Breukink *et al.* (1999) et Yeaman et Yount, (2003). Pour ce modèle, les résidus hydrophobes des peptides sont en contact avec les lipides de la membrane. Les peptides adaptent une orientation perpendiculaire comme on l'observe dans la **Figure I-19**. Lors de l'internalisation dans les membranes, les peptides forment des canaux en forme de tonneau suite à l'exposition de leurs régions peptidique hydrophiles vers l'intérieur de la membrane. À travers ces canaux, une fuite du contenu cytoplasmique s'effectue induisant un déséquilibre osmotique ainsi que la mort de la bactérie.

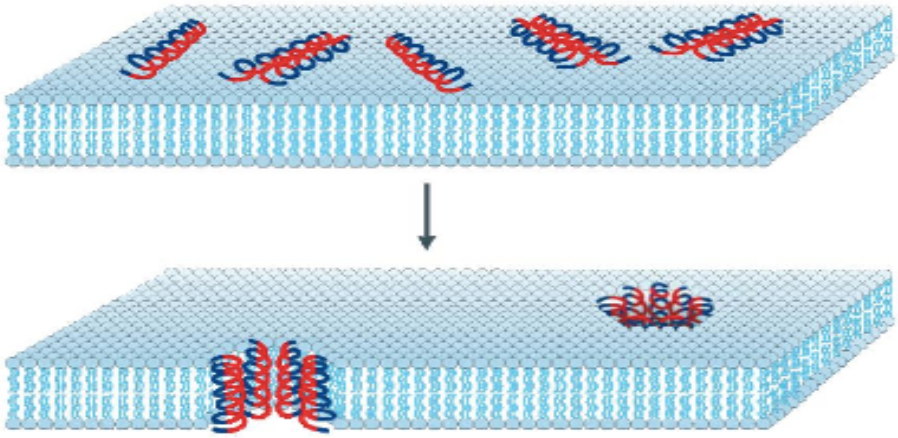


Figure I-19. Représentation du modèle en douves de tonneau (Brogden, 2005).

Le modèle en pores toroïdaux est similaire au précédent du fait de la formation de pores transmembranaires par les peptides qui sont organisés en hélice α. Ces derniers forment des structures amphiphiles, en contact avec la membrane et exposent leurs régions hydrophobes. Suite à l'exposition de ces régions, la **Figure I-20** montre une courbure de la membrane, induite par un déplacement des têtes polaires des phospholipides (Hirsh *et al*., 1996). Les pores sont formés suite à l'orientation perpendiculaire des peptides à la membrane dont les parois sont constituées par les domaines hydrophiles des peptides et les phospholipides. Ceci contraste avec le modèle en douves de tonneau où les peptides s'insèrent en forant la membrane, sans la déformation de la double couche lipidique et où l'intérieur du pore n'est formé que de la partie hydrophile des peptides antimicrobiens (Matsuzaki *et al*., 1996 ; Yang *et al*., 2001 ; Yeaman et Yount, 2003).

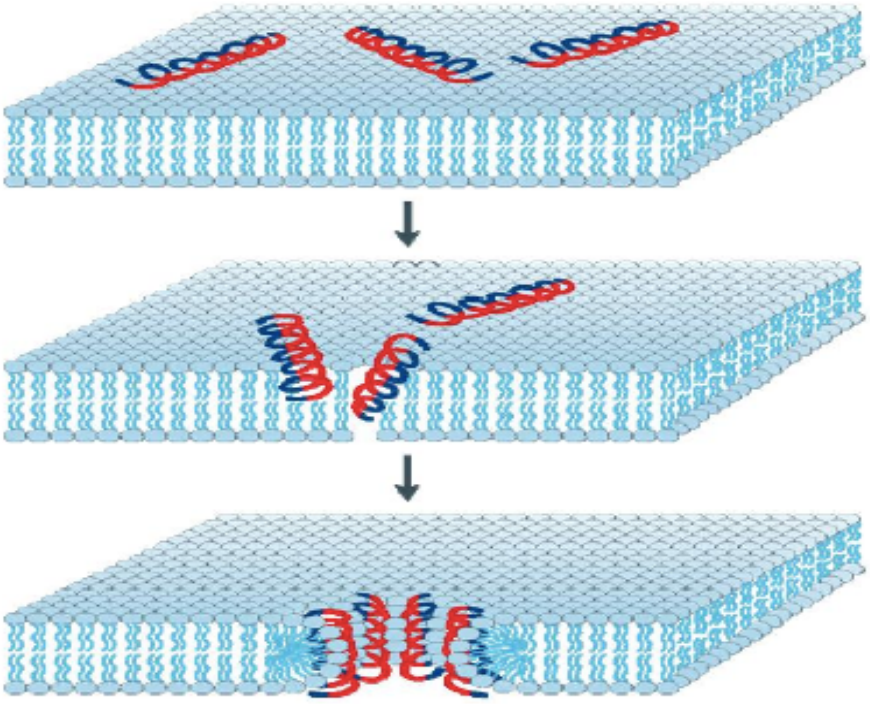


Figure I-20. Représentation du modèle en pores toroïdaux (Brogden, 2005).

Dans le modèle en tapis, contrairement aux deux modèles précédents, l'orientation des peptides s'effectue uniquement de manière parallèle à la membrane et par des interactions électrostatiques (Pouny *et al.*, 1992). Cela se traduit par le fait que durant l'ensemble du processus de perméabilisation de la membrane, les peptides restent en contact avec les têtes polaires des lipides sans pour autant s'insérer au niveau de son cœur hydrophobe (**Figure I-21**). L'accumulation des peptides à la surface de la membrane forment un véritable tapis aboutissant à la perturbation de la fluidité membranaire et à sa rupture. Lorsque le ratio peptides/lipides est élevé, les peptides présents engendrent la formation de micelles et de pores, entraînant une perforation de la membrane et la libération du contenu cytoplasmique (Ehrenstein et Lecar, 1977 ; Jenssen *et al.*, 2006). Contrairement aux modèles en douves de tonneau et pores toroïdaux, les peptides ne s'insèrent pas dans la membrane lipidique et il n'est pas nécessaire que les peptides adoptent une structure en hélice α dans ce mode d'action.

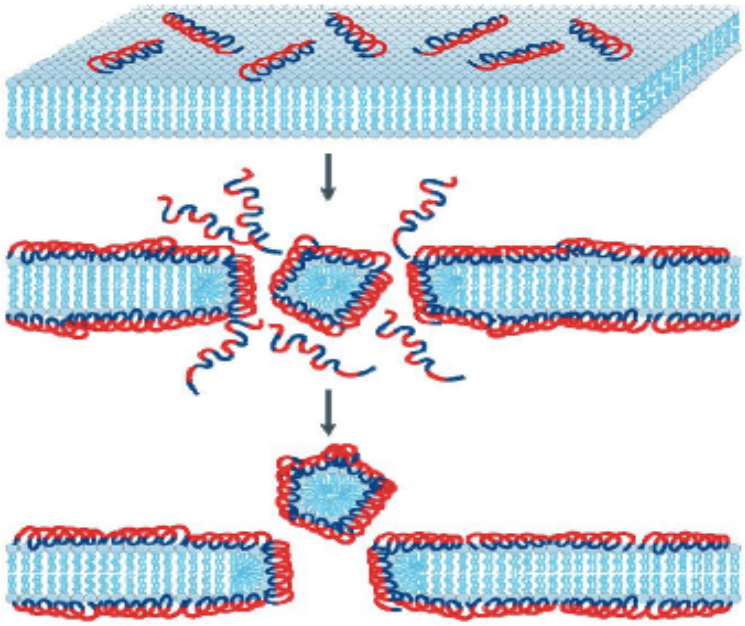


Figure I-21. Représentation du modèle en Tapis (Brogden, 2005).

L'ensemble des trois modèles précédents (en douves de tonneau, en pores toroïdaux et en tapis) a abouti à un modèle décrit par trois auteurs : Shai, Matsuzaki et Yang. Ils ont émis l'hypothèse que ces modèles n'en étaient en réalité qu'un seul et unique modèle où les différents états survenaient de manière chronologique (Matsuzaki, 1999 ; Shai, 1999 ; Yang *et al*., 2000). Zasloff, (2002) décrit ce modèle comme étant la succession de quatre étapes menant à deux finalités (**Figure I-22**). Dans un premier temps, les peptides s'associent sous la forme d'un tapis à la surface de la membrane bactérienne, suite à des interactions électrostatiques entre les peptides chargés positivement, et les phospholipides chargés négativement. Ces derniers, s'ils ne sont pas suffisants pour rompre la membrane, conduisent à la pénétration membranaire de peptides organisés en hélice α et, enfin, à la formation de pores. La formation de pores entraîne la fuite de composants intracellulaires. Ces désordres osmotiques engendrent soit la mort de la cellule, soit la diffusion de fragments peptidiques à cible intracellulaire.

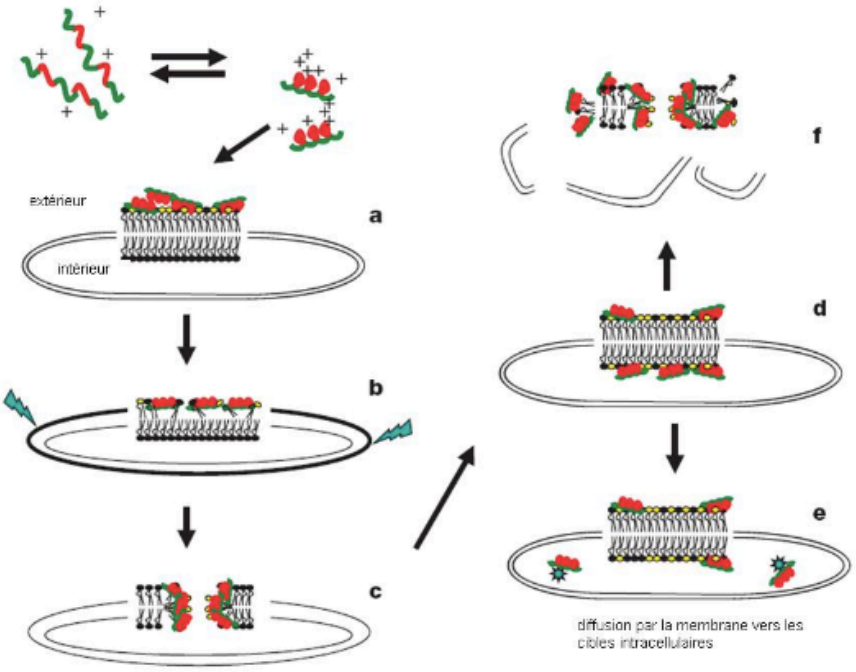


Figure I-22. Représentation du modèle de Shai, Matsuzaki et Yang (Zasloff, 2002).

I.2.5.6.3.2. Mécanismes d'action des peptides antimicrobiens à action intracellulaire

Un autre mécanisme d'action des peptides antimicrobiens fonctionnant comme un couple ligand-récepteur a été décrit, le mécanisme à action intracellulaire. Le principe de ce mécanisme d'action se situe au niveau des interactions avec des protéines, de l'ADN ou de l'ARN, afin d'inhiber des voies métaboliques menant à la mort cellulaire (Brogden, 2005 ; Jenssen *et al.*, 2006). Ce mécanisme s'applique aux peptides dont la composition en acides aminés leur permet d'être facilement transportés au sein du milieu intracellulaire, que ce soit pour une bactérie à Gram-positif ou à Gram-négatif comme par exemple, les peptides riches en un acide aminé particulier, comme les peptides riches en arginine (Powers *et al.*, 2003).

Les peptides bioactifs issus de l'hydrolyse de l'hémoglobine bovine par la pepsine porcine sont nombreux et variés. De ce fait, la valorisation du co-produit de l'industrie agroalimentaire, l'hémoglobine bovine, est importante pour la production de peptides bioactifs ayant des intérêts particuliers et majeurs.

La récupération des peptides bioactifs à haute valeur biologique dépend des conditions d'hydrolyse et des mécanismes enzymatiques. Néanmoins, toutes les conditions d'hydrolyse classique nécessitent l'utilisation de produits chimiques de haute concentration pour dénaturer l'hémoglobine et pour arrêter la réaction. Ainsi, le développement d'autres techniques propres et innovantes est important.

I.3. L'électrodialyse avec membranes bipolaires

L'électrodialyse avec membranes bipolaires fait intervenir un système d'électrodialyse (ED) intégrant des membranes dites « bipolaires ». Dans cette partie de la revue de littérature, nous introduirons la technologie d'électrodialyse conventionnelle en décrivant ses principes et ses applications, puis nous développerons plus spécifiquement l'EDMB.

I.3.1. L'électrodialyse conventionnelle

I.3.1.1. Principes et généralités

L'électrodialyse conventionnelle a vu le jour à la fin du XIX^{ième} siècle et est utilisée dans le cadre de la production d'eau potable depuis un peu plus de 50 ans (Bazinet, 2005; Strathmann, 2010). L'électrodialyse (ED) est un procédé de séparation électrochimique par lequel des espèces ioniques (chargées électriquement) sont transportées, d'une solution à l'autre, en traversant une ou plusieurs membranes permsélectives, sous l'influence d'un courant électrique continu (Bazinet, 2005). Aujourd'hui, l'électrodialyse (ED) trouve une utilisation croissante dans les industries agroalimentaires pour concentrer, purifier ou modifier les aliments. La faible consommation d'énergie, la conception modulaire, l'efficacité et la facilité d'utilisation, ainsi que la sensibilité à la chaleur de nombreux produits alimentaires figurent parmi les raisons de sa croissance rapide (Bazinet, 2019). Contrairement aux autres techniques membranaires (ultrafiltration, nanofiltration, osmose inverse) dont la force motrice est due à un gradient de pression, le déplacement des composés de l'ED est généré par une différence de potentiel appliquée entre deux électrodes (Bazinet, 2019).

En 1940, Meyer et Straus, ont été les premiers à proposer les systèmes d'électrodialyse dont le concept se base sur la formation d'une cellule par une alternance de membranes cationiques et anioniques pour former des compartiments de solutions situées entre une paire d'électrodes.

La configuration d'ED utilisée pour des applications de dilution-concentration conventionnelle est la configuration la plus simple et la plus répandue (**Figure I-23**). Une alternance de membranes anioniques et cationiques constitue la configuration de cet électrodialyseur, délimitant ainsi des compartiments de dilution et de concentration. Les compartiments de dilution s'appauvrissent en sels ou autres composés tels que des minéraux, acides organiques, lactose, etc., tandis que les compartiments de concentration s'enrichissent en sels. Sous l'influence d'un champ électrique, les cations migrent vers la cathode ; ils quittent le compartiment de dilution en traversant la membrane échangeuse de cations (MEC) et sont alors retenus dans le compartiment de concentration à cause de la membrane échangeuse d'anions (MEA), qui est imperméable à leur passage. Simultanément, les anions migrent vers l'anode ; ils quittent le compartiment de dilution et traversent la MEA, puis sont retenus dans le compartiment de concentration, à cause de la présence de la MEC, qui bloque leur passage (Gavach, 1998).

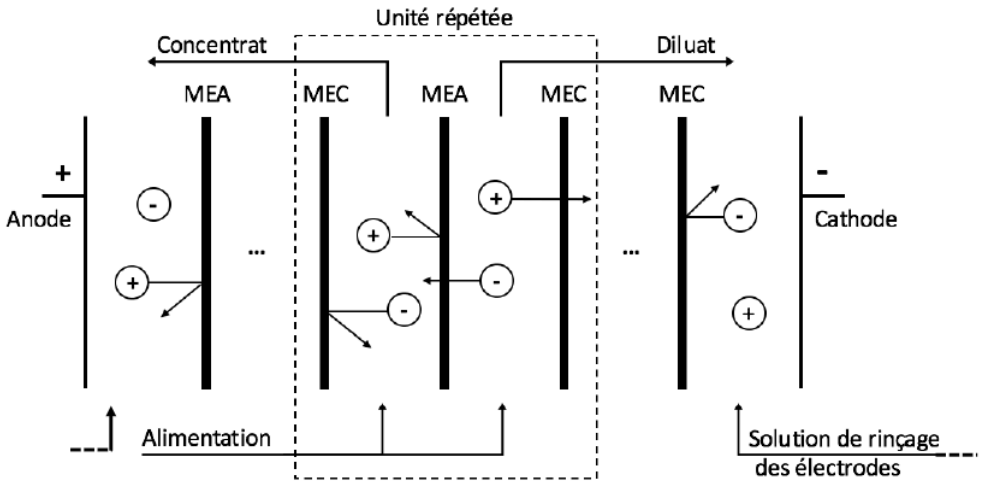


Figure I-23. Schéma d'une cellule d'ED. Principe de migration des ions au travers d'un empilement de membranes échangeuses d'ions lors de l'application d'un champ électrique (Dufton, 2019).

Les membranes sont séparées par des cadres séparateurs ou espaceurs qui sont équipés de grilles servant à la fois au support des membranes, mais aussi à la promotion de la turbulence. La cellule élémentaire formée de deux compartiments, soit le diluat et le concentrat est définie par une unité de base d'un électrodiayseur comprenant une membrane anionique, un espaceur, une membrane cationique, et un autre espaceur. Aux extrémités de chaque module, dans un circuit indépendant où circule l'électrolyte, sont placés l'anode et la cathode (les électrodes). Chaque compartiment est relié à son propre réservoir dont le contenu est mis en circulation par des pompes (Bazinet, 2019).

I.3.1.2. Les membranes échangeuses d'ions

Les membranes homopolaires ou monopolaires ne contiennent qu'un seul type d'échangeur d'ions. Ce sont des conducteurs ioniques et ils ont un effet barrière semi-perméable pour empêcher le mélange des solutions de part et d'autre. Il s'agit soit des MEC ou des MEA. Un transport ionique sélectif au travers des membranes peut être établi sous l'effet d'une force motrice, généralement un champ électrique ou un gradient de concentration. Les MEC imperméables aux anions, sont dotées de groupement chargés négativement tels que les sulfonates SO_3^-, COO^-, PO_3^{2-}, HPO_2^-; tandis que les MEA imperméables aux cations, présentent des sites chargés positivement tels qu'ammonium tertiaire NH_3^+ ou quaternaire NH_4^+ (Bazinet, 2019). Dans ce réseau, les groupes ionisables sont à l'origine de la sélectivité membranaire. Dans le cas d'une membrane échangeuse de cations, les cations sont des contre-ions qui se déplacent sous l'influence d'un champ électrique d'un site à un autre dans le réseau des groupements fonctionnels anioniques fixés sur la matrice et traversent la membrane, tandis que les anions sont des co-ions qui sont expulsés de la membrane par répulsion électrostatique, car ils sont chargés négativement comme les groupes fonctionnels. Le principe de fonctionnement est inversé pour les membranes échangeuses d'anions qui laissent passer les anions et rejettent les cations. Cette exclusion, qui en résulte, est appelée exclusion de Donnan, du nom F.G. Donnan qui a signalé le phénomène pour la première fois en 1911 (Pourcelly et Bazinet, 2005). Une structure schématique d'une MEC est illustrée dans la **Figure I-24** lors de l'application d'un champ électrique.

Les propriétés recherchées pour les MEI sont les suivantes (Nagarale *et al*., 2006) :

- Une haute permsélectivité : une MEI doit être très perméable aux contre-ions, mais doit rester imperméable aux co-ions.
- Une faible résistance électrique : une MEI doit présenter une faible résistance électrique pour permettre une bonne conduction du courant électrique dans la cellule d'électrodialyse.
- Une bonne stabilité mécanique : une MEI doit être mécaniquement forte et avoir un faible degré de gonflement et de rétrécissement en solution saline.
- Une bonne stabilité chimique : une MEI doit être stable sur un grand spectre de pH allant de 0 à 14 et en présence d'agents oxydants.

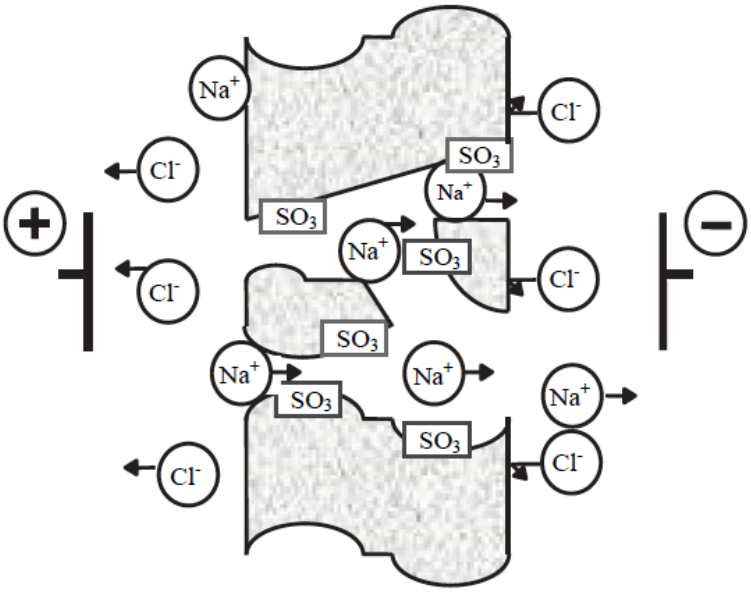


Figure I-24. Principe de fonctionnement d'une membrane échangeuse de cations (Bazinet, 2019).

Les membranes permsélectives aux ions monovalents sont une autre catégorie de membranes échangeuses d'ions permettant la migration des ions monovalents de charge opposée tout en conservant les ions polyvalents. Les membranes permsélectives aux anions monovalents (MPA) sont constituées de résine contenant des charges positives dont la surface est recouverte d'une très mince couche négativement chargée. Inversement, les membranes permsélectives aux cations monovalents (MPC) sont constituées de résine contenant de charges négatives recouverte d'une fine couche de charge positive (**Figure I-25**). De telles

couches sur l'interface membranaire sont capables de séparer les ions de même charge, mais de valences différentes. Les MPA ne sont hautement perméables qu'aux anions monovalents comme les ions chlorure, alors que les MPC sont hautement perméables uniquement aux cations monovalents comme les ions sodium. Avec ces couches particulières à leurs interfaces, les MPA et MPC présentent des résistances au colmatage peptidique (Persico et Bazinet, 2018).

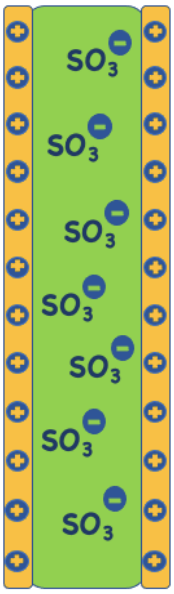


Figure I-25. Représentation schématique d'une membrane permsélective aux cations monovalents (MPC).

I.3.1.3. Phénomènes de transport

I.3.1.3.1. Modes de transport

Lors du procédé ED, différents modes de transport vont intervenir au sein de la MEI et sont donnés par l'équation de Nernst-Plank :

$$Ji = Di\left(\frac{dCi}{dx} + \frac{ZiFCi}{RT}\frac{d\varphi}{dx}\right) + CiV$$

Où Ji est la densité du flux ionique, Di le coefficient de diffusion, Ci la concentration ionique, Zi le nombre de charge, φ le potentiel électrique, V la vitesse du fluide, F la constante de Faraday, R la constante des gaz parfaits et T la température. Les différents modes de transport sont :

- La convection a lieu quand la molécule est entraînée par une force extérieure. Dans les procédés de séparations membranaires cette force extérieure est un gradient de pression. Elle est négligeable dans la MEI.
- La migration des ions d'un compartiment à un autre, qui est due à l'application d'un courant électrique. Les charges vont être attirées par l'électrode de charge opposée. Ainsi, les cations vont traverser le compartiment en direction de l'anode chargée positivement. Des compartiments vont donc être enrichis en espèces ioniques (les concentrats), alors que d'autres vont être appauvris (les diluats) (Strathmann *et al*., 2010).
- La diffusion, est le mouvement d'espèces ioniques via un gradient de concentration. Elle se fera ici au niveau des interfaces et couche limite des MEI. Celle-ci se compose d'un réseau de macromolécules sur lequel sont fixés des groupements ionisants.

Le transport des ions est régi par la loi de l'électro-neutralité. Ainsi en électrodialyse, les ions vont migrer vers les électrodes de charge opposée, l'important est que le nombre de charges positives soit égal au nombre de charges négatives, selon l'équation (Bazinet, 2019) :

$$\Sigma_{ZiCi} = 0 \text{ ou } \Sigma_{(NBt^{+} + NBt^{-})} = 1$$

Où

z_i : valence de l'espèce i

C_i : concentration de l'espèce i (en mol/m^3)

Nbt^+ : nombre de transport des cations

Nbt^- : nombre de transport des anions.

I.3.1.3.2. Concentration de polarisation et courant limite

De ces différents modes de transports des ions vont découler deux principes majeurs de l'ED : la concentration de polarisation (CP) et la densité de courant limite. La CP est due à la variation de concentration du contre-ion entre la membrane et la solution. Lors de l'application d'un champ électrique, il s'établit un gradient de concentration lié à la différence de mobilité des contre-ions en solution et dans la membrane. La mobilité des contre-ions dans la membrane sera supérieure à celle des contre-ions en solution. Cela se traduit par la création de gradients de concentration à l'interface membrane/solution dans une fine couche nommée couche limite de polarisation (δ) (Baker, 2004). Afin de maintenir un courant constant, la diffusion ionique va se faire rapidement, et donc la concentration en contre-ions sera plus faible au sein de cette couche limite (**Figure I-26**). Lorsque le courant est augmenté de façon intense, la concentration d'ions à l'interface membrane/solution dans le compartiment de dilution sera plus faible (valeur proche de 0), que celle au sein du compartiment de concentration à cause de la migration des ions du diluat vers le concentrat. Cela détermine la densité du courant limite (Bazinet, 2005; Strathmann, 2010).

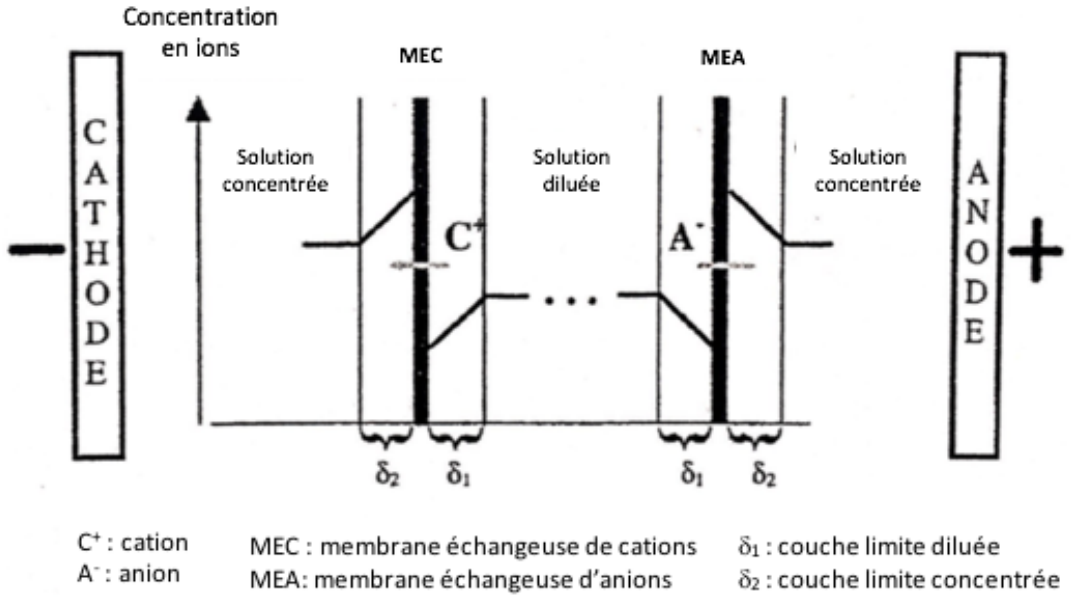


Figure I-26. Formation de gradients de concentration à l'interface membrane/solution (Bazinet, 2005).

Suite à l'atteinte et au dépassement du courant limite, la concentration en ions au sein de la couche limite n'est plus suffisante pour maintenir le passage du courant. À ce moment, l'eau à la surface de la membrane va se dissocier en ions H^+ et OH^- pour fournir les ions nécessaires au transfert du courant (Bazinet, 2019; Mavrov *et al*., 1993; Ibanez *et al*., 2004). Suite à cette dissociation, le pH à l'interface des membranes peut varier causant dans certain cas la précipitation des composés de la solution traitée et peut aboutir au colmatage membranaire. Afin d'éviter le colmatage et les dépenses d'énergie associés à la dissociation des molécules d'eau, l'intensité utilisée en électrodialyse doit être contrôlée et inférieure à l'intensité du courant limite. L'intensité de courant limite peut être déterminée en traçant une courbe courant-tension, et fait apparaître trois régions comme montré dans la **Figure I-27** (Bazinet, 2019 ; Ibanez *et al*., 2004) :

(1) Une zone appelée **région ohmique** (I), où la densité de courant augmente linéairement en fonction de la tension. Dans cette région, le système suit la loi d'Ohm donnant la mesure de la résistance de la solution et de la membrane entre les électrodes de référence.
(2) Une région « plateau », nommée **région limite** (II), où la densité de courant limite est atteinte. Cette région est causée par l'épuisement des ions dans la couche limite hydrodynamique. La densité de courant reste constante lorsque la tension augmente. Cette zone correspond à la valeur maximale de courant à partir de laquelle commence la dissociation des molécules d'eau qui vont permettre le transfert du courant.
(3) Une troisième région, appelée **région surlimite** (III), où la densité de courant croît avec l'augmentation de la tension. C'est la zone de dépassement du courant limite pendant laquelle l'énergie électrique sert à dissocier les molécules d'eau. Le transfert de masse est augmenté (Bazinet, 2019).

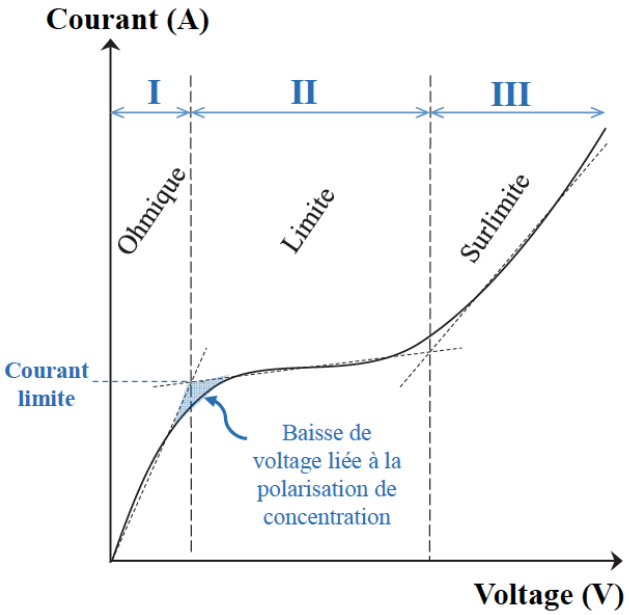


Figure I-27. Courbe courant-voltage aux bornes d'un système d'électrodialyse (Bazinet, 2019).

Lorsque la valeur de la densité du courant limite est dépassée, plusieurs effets défavorables se produisent sur la solution et la membrane. Il peut y avoir surconsommation néfaste d'énergie, des variations du pH non contrôlées et des changements pouvant agir sur, entre autres, les peptides en modifiant leurs charges et ainsi diminuer leurs migrations à travers la membrane (Suwal *et al*., 2005).

I.3.1.4. Colmatage membranaire

I.3.1.4.1. Définition et impacts du colmatage

Le colmatage membranaire est le phénomène d'attachement indésirable de certaines espèces (organismes vivants ou substances non vivantes) à la surface ou à l'intérieur des membranes. Il représente le premier facteur limitant dans l'utilisation du procédé d'ED. Le colmatage peut avoir lieu à la surface des membranes et/ou à l'intérieur. Différentes conséquences peuvent avoir lieu suite à ce colmatage :

- Une diminution des performances membranaires.
- Une réduction d'environ 10 à 40% des taux de déminéralisation (Cifuentes-Araya *et al*., 2011; Zhao *et al*., 2018).
- Une augmentation de la résistance électrique du système au cours du procédé de 5 à 40% dépendamment du type de colmatage et des membranes (Balster *et al*., 2007; Bleha *et al*., 1992).
- Une diminution de la valeur du courant limite qui doit être déterminée normalement au préalable de toute étude en électrodialyse.
- Une augmentation de 20 à 30% de la consommation énergétique (Lee *et al*., 2009; Lee *et al*., 2002).
- Une diminution de la permsélectivité membranaire (Thompson et Tremblay, 1983**;** Bleha *et al*., 1992).
- Une dégradation des membranes qui peut être irréversible nécessitant leur remplacement et entrainant des conséquences économiques majeures pour les industriels (Grossman et Sonin, 1973).
- Une augmentation de la fréquence et de l'intensité de nettoyage du système représentent un coût majeur et élevé pour les industries (Grebenyuk *et al*., 1998). Dans le cas d'un colmatage réversible, un simple traitement acide pourrait résoudre le problème, néanmoins cette opération nécessite d'arrêter le traitement d'électrodialyse et de remplacer les effluents soumis à l'électrodialyse par des acides ou des bases qui devront ensuite être traités.

Pour l'ensemble de ces raisons, il est important de comprendre la nature et la cause de la formation du colmatage afin de proposer des solutions pour le prévenir ou du moins en réduire la sévérité.

I.3.1.4.2. Nature du colmatage

Différentes natures de colmatages ont été décrites dans la littérature. Les membranes anioniques et cationiques présentent un comportement très différent vis-à-vis du type de colmatage. Étant donné que les membranes échangeuses d'anions (MEA) présentent des sites

chargés positivement, elles seront plus sensibles à un colmatage par des espèces chargées négativement. Par contre, les membranes échangeuses de cations (MEC) sont dotées de groupement chargés négativement, elles seront alors plus sensibles à un colmatage par des espèces chargées positivement.

I.3.1.4.2.1. Le colmatage colloïdal

Les colloïdes sont des solides en suspension non dissous, qui sont présents dans les eaux naturelles et les eaux traitées ainsi que dans de nombreux effluents sous forme de minéraux argileux, de silice colloïdale, d'oxyde de fer, d'oxyde d'aluminium, d'oxyde de manganèse, de colloïdes organiques, etc. (Lee *et al*., 2003; Brunelle, 1980). Ces particules ont fait l'objet de plusieurs études puisqu'ils ont été révélées responsables du colmatage membranaire (Lindstrand *et al.*, 2000). Dans les processus électrodialytiques, il y a une attention particulière sur les dépôts colloïdaux de la MEApuisque la plupart des colloïdes traités par ED sont chargés négativement. Ce colmatage résulte d'interactions entre les colloïdes et les groupements d'ions chargés positivement de la MEA. Par exemple, Lee *et al*. (2008), ont étudié la formation d'un colmatage colloïdal particulier tel qu'un colmatage par un agent tensioactif (Sodium Dodécyl-Benzène-Sulfonate; SDBS). Le schéma présenté sur la **Figure I-28** résume le mécanisme de formation du colmatage développé par ces auteurs où les micelles de SDBS ont formé une couche sur la surface de la membrane en raison de la taille moléculaire accrue et des charges très négatives. Un simple lavage ne sera pas possible pour rompre le colmatage par des tensioactifs à cause des fortes interactions qui peuvent être irréversibles. Lindstrand *et al.* (2000), ont observé une augmentation significative de la résistance électrique de la membrane suite au colmatage par adsorption de tensioactifs. Les facteurs affectant le colmatage colloïdal sont la concentration de particules colmatées ainsi que la concentration en sel dissous, le pH, la température, les propriétés de la membrane, le mode de fonctionnement et les conditions hydrodynamiques (Mikhaylin et Bazinet, 2016).

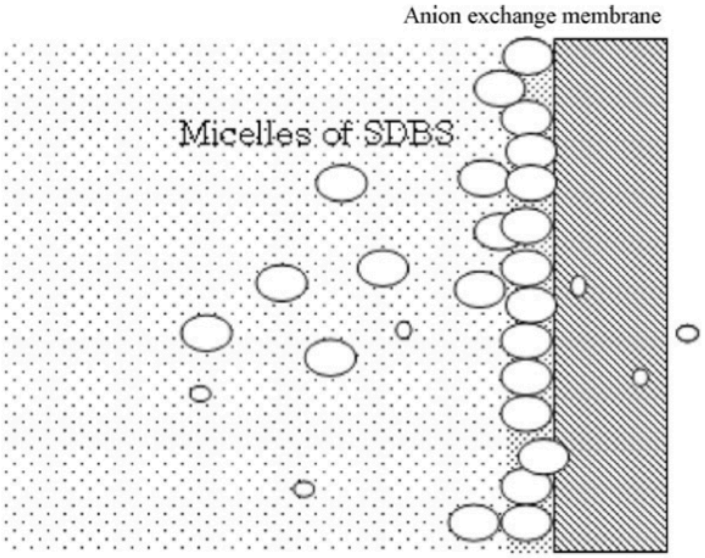


Figure I-28. Mécanismes de colmatage liés à la formation de micelles SDBS (Lee *et al.*, 2008).

I.3.1.4.2.2. Le colmatage minéral

Les membranes échangeuses d'ions sont particulièrement sensibles à un colmatage minéral. Le colmatage minéral est causé lorsque la solubilité des ions diminue en dessous d'un seuil d'équilibre ionique. Plusieurs facteurs peuvent provoquer la précipitation minérale tels que :

- Le changement de la température, du pH et de la concentration des ions (Casademont *et al*., 2008 ; Chandrapala *et al*., 2015).
- Le type, la structure et les propriétés de surface des membranes utilisés (Balster *et al*., 2005 ; Mikhaylin *et al*., 2016).
- Le gradient de concentration des ions à proximité des membranes dépendamment du régime d'ED appliqué.
- L'atteinte du courant limite qui provoque la dissociation des molécules d'eau en protons et ions hydroxyle influençant la précipitation des ions divalents (Mikhaylin et al., 2016).

Bazinet *et al.* (2000), ont rapporté que le colmatage minéral peut être réversible ou irréversible en fonction de la perm-sélectivité membranaire. Lorsque le courant limite est dépassé, une grande quantité d'ions hydroxyles va être produite suite à la dissociation des

molécules d'eau. La présence de ces ions hydroxyles vont avoir un impact sur le pH. Par conséquent, le changement du pH va causer les réactions de précipitation. De plus, la solubilité des ions dans les compartiments d'ED peut être causée par le phénomène de compétition des ions pour la migration à travers les membranes entraînant l'effet de concentration de certains ions. Les travaux réalisés par Casademont *et al*. (2010) et Cifuentes-Araya *et al*. (2012), ont de même démontré que la formation de différents types de précipités était établie suite à l'établissement de colmatages successifs à l'intérieur et à la surface des membranes modifiant les conditions chimiques et favorisant ainsi la formation du colmatage minéral. Comme exemple d'ions reconnus comme étant particulièrement prédisposés à la précipitation durant les procédés d'ED, les ions calcium, magnésium et phosphate qui sont présents dans le lactosérum acide (Atamanenko *et al*., 2004 ; Ayala-Bribiesca *et al*., 2007 ; Casademont *et al*., 2008 ; Cifuentes-Araya *et al*., 2014).

I.3.1.4.2.3. Le colmatage organique

Le colmatage organique se produit lorsqu'une solution traitée contient des substances organiques telles que de l'huile, des glucides, des protéines, des substances aromatiques, de l'acide humique et des agents anti-moussants (Lindstrand et al., 2000; Banasiak et Schäfer, 2009; Bleha *et al*., 1992). Ces substances organiques se collent à la surface de la membrane et/ou se logent à l'intérieur de la membrane. Ce type de colmatage a une réelle importance en raison du grand nombre de produits traités avec des matrices différentes contenant de telles matières organiques (**Figure I-29**). Plusieurs études rapportent des informations sur les mécanismes de formation du colmatage organique. Le colmatage organique membranaire est formé par le biais d'interactions électrostatiques, mais aussi hydrophobes ou encore par affinité structurelle et géométrique (Banasiak et Schäfer, 2010; Guo *et al*., 2014; Lee *et al*., 2009; Lindstrand *et al*., 2000; Park *et al*., 2005; Wang *et al*., 2017; Zhao *et al*., 2018). L'influence de certains paramètres sera particulièrement importante dans l'établissement de colmatage organique telles que la concentration, la taille et la structure des molécules organiques ainsi que la composition et la structure de la membrane (Persico et Bazinet, 2018; Wang *et al*., 2017) et les régimes de traitement électromembranaire (mode et intensité de courant électrique) (Bukhovets *et al*., 2010; Persico *et al*., 2017). Il a été constaté qu'à des

densités de courant proches des valeurs limites, où la dissociation de l'eau a lieu, les MEI se colmatent par la phénylalanine. Langevin et Bazinet, (2011) ont étudié l'effet de l'acidification et de la basification de la surface des MEI sur le colmatage par les peptides. Ces conditions sont proches de celles qui se produisent lorsque le courant atteint une valeur limite et qu'il y a une dissociation des molécules d'eau. Il a été proposé que les liaisons principales entre les peptides et les MEI étaient dues aux interactions électrostatiques (Langevin et Bazinet, 2011). Persico *et al*. (2016; 2017) ont étudié le colmatage peptidique des membranes échangeuses d'anions et de cations dépendamment du pH sur un hydrolysat protéique bien caractérisé. Pour les MEA, Persico *et al.* (2016) ont démontré par l'étude de la structure tridimensionnelle des peptides présents dans l'hydrolysat que le colmatage peptidique était sévère à pH 10, deux fois inférieur à pH 6 et absent à pH 2. Il était dû aux interactions électrostatiques produites entre les charges peptidiques négatives (COO^-) à pH 6 et 10 et les groupes chargés positivement des MEA ($N^+(CH_3)_3$). De plus, travailler à un pH acide a empêché complètement le colmatage des MEA en raison de répulsion entre les peptides et les groupes MEA (Persico *et al*., 2016). Tandis que pour les MEC, le colmatage peptidique était important à pH 6, deux fois plus faible à pH 2 et négligeable à pH 10. Ce colmatage était aussi dû aux interactions électrostatiques produites entre les charges peptidiques positives (NH_3^+) et les groupes chargés négativement des MEC (SO_3^-) et aux interactions hydrophobes peptides/peptides créant une deuxième couche de colmatage à pH 6. À pH 2, les peptides interagissent uniquement électro-statiquement avec la membrane. À pH 10, aucun colmatage n'a été observée en raison de l'absence de charges peptidiques positives (Persico *et al*., 2017).

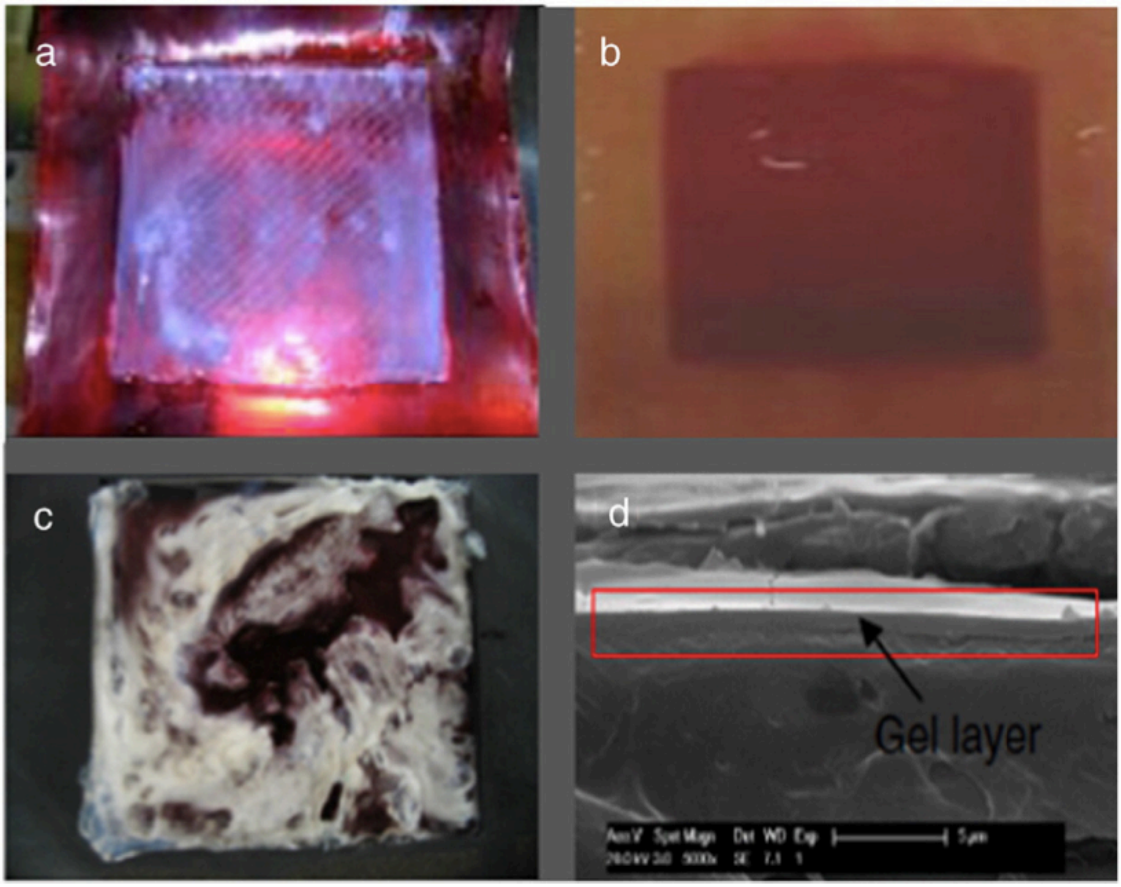


Figure I-29. Exemples de colmatage organique (Mikhaylin et Bazinet, 2016) : a) colmatage de chitosane (Lin Teng Shee *et al*., 2007), b) colmatage d'anthocyanine (Husson *et al*., 2013), c) colmatage protéique (Ruiz et al., 2007), d) colmatage de polyacrylamide (Guo *et al*., 2014).

I.3.1.4.2.4. Colmatage biologique

Le colmatage biologique est le type de colmatage le moins étudié. La plupart des études sur ce colmatage sont dédiées à la micro-, ultra-, nanofiltration et à l'osmose inverse (Baker et Dudley, 1998; Huertas *et al*., 2008). Cependant, le développement rapide des biotechnologies avec l'application des MEI fait du colmatage biologique un problème réel. Par exemple, l'encrassement biologique est un problème majeur dans les cellules de dessalement microbien. La caractéristique principale de ces cellules microbiennes est l'implication de bactéries spéciales sur la surface de l'anode. Ces bactéries peuvent oxyder les substrats biodégradables et produire des électrons et des protons. La croissance continue entraîne le développement de micro-colonies. Au fur et à mesure que les micro-colonies continuent

d'augmenter en taille, les cellules à l'intérieur des micro-colonies connaîtront un surpeuplement, une diminution de la disponibilité des nutriments et une augmentation des concentrations de déchets, de toxines et de métabolites excrétés, y compris les molécules de signalisation des cellules. Cela peut avoir des effets négatifs sur les membranes (Flemming *et al*., 2011; Mikhaylin et Bazinet, 2016).

I.3.1.4.3. Influence des paramètres opératoires sur la présence de colmatage

Dans cette partie seront présentés les paramètres et les conditions opératoires pouvant avoir des répercussions sur la présence ou l'absence de colmatage. Fondamentalement, le colmatage est affecté par différents paramètres liés à la morphologie de la membrane et aux propriétés de surface (type de matériau, charge de surface, taille des pores, rugosité, hydrophobicité), les caractéristiques de la solution (pH, force ionique et concentration) et la force motrice (champ électrique), le débit de la solution (Nichka *et al*., 2020; 2021) et la présence ou non de turbulence (Huisman et Wislon, 2000). Dans cette partie, il ne s'agit pas de donner une liste exhaustive, mais d'une sélection de paramètres sur lesquels notre travail pourrait nous amener à réfléchir.

I.3.1.4.3.1. La morphologie de la membrane et ses propriétés de surface

Le choix et la nature des membranes revêtent une importance significative sur la présence ou non de colmatage. Plusieurs études ont ainsi été réalisées sur la porosité du film, la micro-hétérogénéité ou encore la perm-sélectivité membranaire.

I.3.1.4.3.1.1. Porosité

La taille du réseau du film échangeur d'ions affecte la mobilité des espèces ioniques et leur migration à travers la membrane. Une faible mobilité induite par un réseau plus petit ou de même taille que celle des ions peut provoquer un colmatage. Par conséquent, de nouvelles membranes ont été proposées par Neosepta, telles que les membranes AMX, ayant un réseau plus adapté et une perméabilité plus élevée aux anions (Ho et Sirkar, 2001).

I.3.1.4.3.1.2. Hétérogénéité

Le colmatage membranaire peut être causé par le phénomène de micro-hétérogénéité à la surface de la membrane. Oda et Yawataya, (1967) et Korngold *et al*. (1970), ont décrit ce phénomène comme étant des zones plus conductrices du courant que d'autres. Ces zones vont créer de fortes densités locales du courant et vont être responsables de la concentration de polarisation et générer ainsi la dissociation des molécules d'eau. Les membranes hétérogènes sont plus sensibles à ce type de phénomènes que les membranes homogènes (Volodina *et al*., 2005).

I.3.1.4.3.1.3. Modification de surface

Des travaux ont été réalisés par Persico et Bazinet, (2018), pour étudier l'impact de la modification de la surface membranaire sur le colmatage peptidique. Le choix des membranes s'est porté sur les membranes permsélectives aux anions monovalents (MPA) et les membranes permsélectives aux cations monovalents (MPC) et ont été comparées aux membranes conventionnelles anionique et cationique (AMX-SB et CMX-SB). L'étude a été réalisée dans le cadre de la déminéralisation des hydrolysats de protéines de lactosérum par électrodialyse. Concernant les membranes anioniques, il a été constaté que plus le pH est élevé et plus le colmatage peptidique est élevé pour les deux membranes permsélective (MPA) et conventionnelle (AMX-SB). Cependant, le colmatage peptidique a été drastiquement diminué sur les membranes permsélectives aux ions monovalents par rapport aux membranes conventionnelles, puisque l'abondance relative totale de la MPA était extrêmement faible (environ 97 à 100% inférieur) par rapport à l'AMX-SB par UPLC-MS. Encore une fois, le colmatage peptidique était plus faible sur la MPC avec une abondance relative totale des peptides de 95 à 100% de moins par rapport au CMX-SB. Cette diminution du colmatage sur les membranes MPA et MPC permsélectives aux ions monovalents est due à la présence d'une couche mince chargée de manière opposée à leur matrice. Les auteurs ont trempé ensuite les membranes dans une solution de Rhodamine B pour détecter par fluorescence la distribution des charges sur les membranes. La **Figure I-30** montre une capture de fluorescence utilisant la rhodamine B chargée positivement sur les surfaces de

MPC et CMX-SB. Il est apparu que la CMX-SB était totalement recouverte par la rhodamine B en jaune qui montre que ses charges étaient réparties de manière homogène. Cependant, la majeure partie de la surface de MPC n'a pas interagi avec la rhodamine. La couche mince sur la matrice de MPC n'était pas homogène, ne couvrait pas complètement sa matrice et a modifié l'équilibre entre les peptides et la surface membranaire. Par conséquent, un petit nombre de peptides a pu s'adsorber dans les cavités où les charges étaient accessibles. Ainsi, selon Persico et Bazinet, (2018), lorsque l'on travaille avec des solutions peptidiques, les membranes permsélectives peuvent être utilisées pour éviter le colmatage dans les processus d'ED.

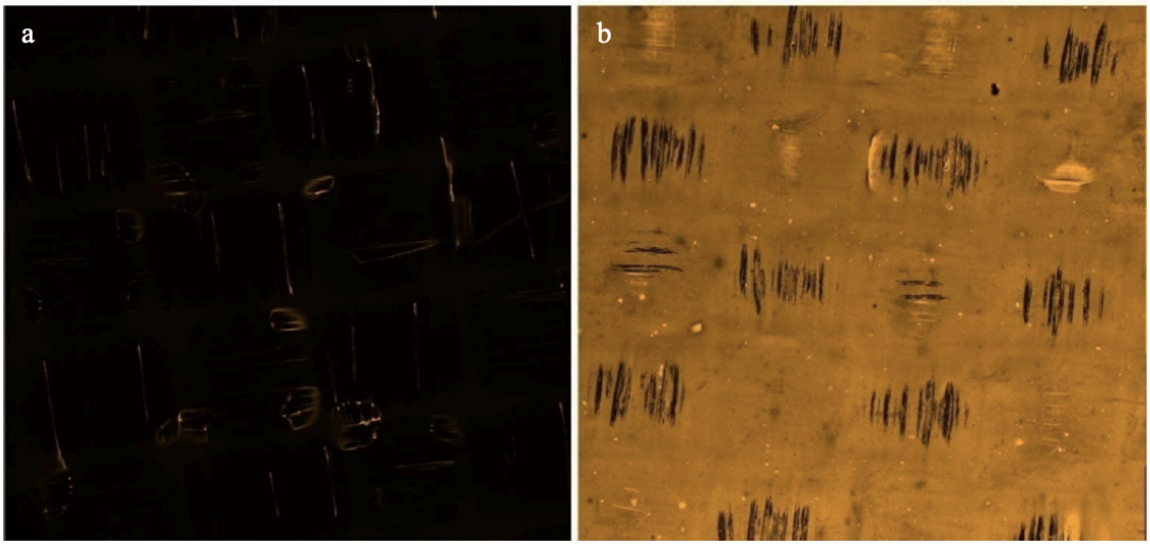


Figure I-30. Surfaces fluorescentes de la MPC (a) et de la CMX (b) en utilisant la rhodamine B (Persico et Bazinet, 2018).

I.3.1.4.3.2. Caractéristiques de la solution

I.3.1.4.3.2.1. Le pH

Le pH joue un rôle dans la migration des espèces chargées sous l'influence d'un champ électrique dans l'ED ainsi que lors de la formation du colmatage. Dans le cadre de l'étude sur l'acidification du lait bovin en électrodialyse avec membranes bipolaires réalisée par Bazinet *et al.* (2001), ils ont observé un dépôt d'origine minérale du côté de la membrane cationique

en contact avec la base électro-générée par la membrane bipolaire. Ce type de colmatage peut être prévenu en neutralisant les OH^- par une alimentation acide en continu dans le compartiment où ils étaient électro-générés comme décrit par Davis (1990). Par la suite, Bazinet *et al*. (2003), ont appliqué ce concept lors de l'acidification par électrodialyse avec membranes bipolaires pour éviter le colmatage minéral et aucun dépôt ne fut observé. Concernant les composés organiques, Persico *et al*. (2016 ; 2017) ont étudié le colmatage peptidique des membranes échangeuses d'anions et de cations à différents pH. Pour les MEA, il est apparu que le colmatage peptidique était sévère à pH 10, deux fois inférieur à pH 6 et absent à pH 2. Les trois principaux peptides responsables du colmatage portaient des charges nettes négatives sur leur extrémité C-terminale à pH 6 et 10. Par conséquent, les interactions de nature électrostatique étaient principalement produites entre les charges peptidiques négatives (COO^-) et les charges positives de la MEA ($N^+(CH_3)_3$). De plus, le pH acide empêcherait complètement le colmatage des MEA en raison de la répulsion entre les peptides et les groupes chargés de la MEA (Persico *et al*., 2016). Concernant la MEC, les résultats ont démontré que le colmatage peptidique était important à pH 6, deux fois plus faible à pH 2 et négligeable à pH 10. Ils ont observé qu'à pH 6, les séquences peptiques qui ont colmaté ont d'abord établi des interactions électrostatiques avec les groupes chargés négativement des MEC (SO_3^-) grâce à leurs résidus K et R positifs (NH_3^+) créant une première nano-couche. Deuxièmement, des interactions peptide/peptide se sont produites à travers leurs résidus hydrophobes créant une seconde nano-couche. À pH 2, ils ont observé que les séquences des peptides qui ont colmaté n'interagissaient que de manière électrostatique et cela dans une moindre proportion puisqu'à pH acide, la plupart des charges des MEC seraient protonnées et non chargées (HSO_3) et limitaient alors le potentiel électrostatique d'interactions. Aucun colmatage n'a été observé à pH 10 en raison de l'absence de charges peptidiques positives (Persico *et al*., 2017).

I.3.1.4.3.2.2. La force ionique et concentration

La force ionique a un effet sur l'interaction des peptides avec les membranes puisque les interactions électrostatiques sont les principaux liens formés lors du colmatage des membranes par les peptides (Lindstrand *et al*., 2000). Langevin et Bazinet, (2011) ont

comparé l'effet de deux concentrations de solution salines, NaCl de 2 et 5%, sur l'élimination des peptides précipités sur les membranes. Ils ont observé que l'ajout d'une étape de nettoyage avec une solution saline de 5% pouvait améliorer la procédure de nettoyage et éliminer les peptides de poids moléculaire plus élevé ayant colmaté notamment sur la MEC. La concentration la plus élevée de NaCl ayant la force ionique supérieure était plus efficace pour désorber les peptides avec des poids moléculaires élevés qui sont susceptibles de présenter plus de charges sur leur surface et donc plus efficace pour rompre les interactions électrostatiques. Récemment, le colmatage a été éliminé en trempant les membranes échangeuses d'ions dans une solution de NaCl (2% (v/v)), la désorption des peptides était liée à l'augmentation de la conductivité et au fait que les sels vont interagir de façon privilégiée avec les sites de la membrane (Persico *et al*., 2017).

I.3.1.4.4. Prévention ou réduction du colmatage

Il existe un certain nombre de stratégies différentes permettant la prévention et le contrôle du colmatage dans les processus d'ED. Cependant, il faut toujours tenir compte de la nature du colmatage, car une stratégie peut être appropriée contre un type de colmatage et inefficace contre un autre. Cette section fournira des informations générales sur les stratégies de prévention et de contrôle du colmatage des membranes.

I.3.1.4.4.1. Régimes électriques

L'application de certains régimes électriques s'est montrée particulièrement efficace dans le cadre de la réduction du colmatage membranaire. Parmi les moyens utilisés pour réduire ou prévenir le colmatage : l'inversion de polarité, le courant sur-limite et les champs électriques pulsés (CEP).

I.3.1.4.4.1.1. Inversion de polarité

Le principe de l'inversion de polarité est représenté en **Figure I-31** et consiste à inverser périodiquement les polarités des électrodes (c'est-à-dire à des intervalles de temps variant de

quelques minutes à plusieurs heures) ainsi qu'une inversion des compartiments de concentrat et de diluat grâce à une installation particulière (Katz, 1979; Fuboa, 1985; Valerdi-Pérez *et al*., 1996; Chao et Liang, 2008; Turek *et al*., 2007; Valero et Arbos 2009; Strathmann, 2004). Lorsque la polarité des électrodes est inversée, les composés chargés s'étant fixés à la surface de la membrane vont se détacher pour migrer dans la direction opposée. En augmentant la fréquence d'inversion, le risque de colmatage est considérablement diminué. Cependant, il y a un inconvénient lié au temps. En effet, après chaque inversion, une perte de temps de quelques minutes est nécessaire afin de retrouver un flux de solution ainsi qu'une efficacité de procédé optimale (Fubao, 1985; Katz, 1981). Récemment, Shyam *et al*. (2016) ont démontré l'effet de l'inversion de la polarité sur la conservation des propriétés physico-chimiques et la diminution du colmatage spécifiquement de la MEA. Toutefois c'est une méthode qui présente un certain nombre de contraintes pratiques et ne peut pas être employée pour toutes les applications d'électrodialyse. Notamment pour les membranes bipolaires qui sont polarisées, une inversion de la polarité détruirait l'intégrité physique (Mikhaylin et Bazinet, 2016).

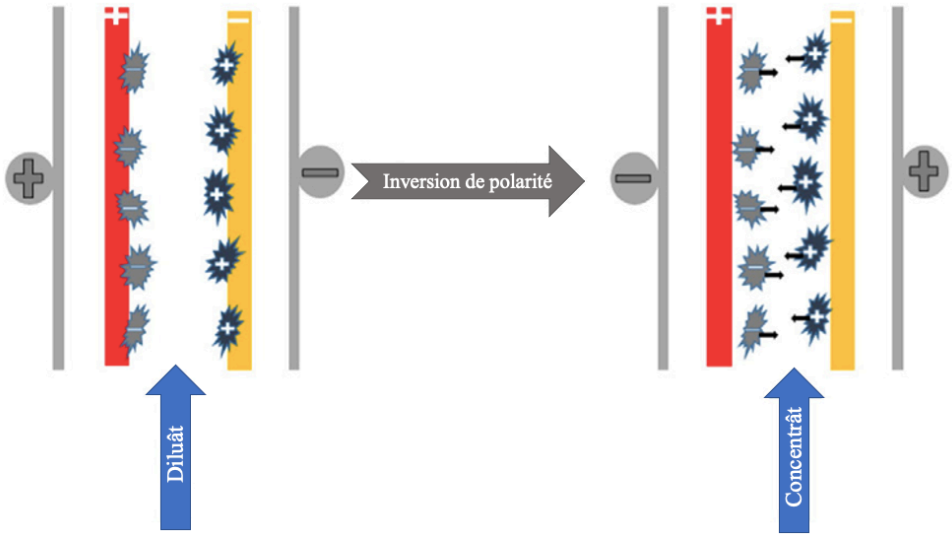


Figure I-31. Représentation de l'ED avec une inversion de polarité et en présence du colmatage (Mikhaylin et Bazinet, 2016).

I.3.1.4.4.1.2. Champ électrique pulsé (CEP)

Un nouveau régime de courant a permis de réduire considérablement l'effet de la concentration de polarisation. Ce régime, appelé champ électrique pulsé (CEP), développé par Karlin et Kropotov en 1995, repose sur le principe de l'application d'un courant ou d'un voltage alterné (pulse) durant un temps déterminé (t_{on} ou temps durant lequel le courant est appliqué) suivi d'un temps de relaxation (t_{off} ou temps durant lequel le courant n'est pas appliqué) et est représenté en **Figure I-32** (Bazinet et Geoffroy, 2020).

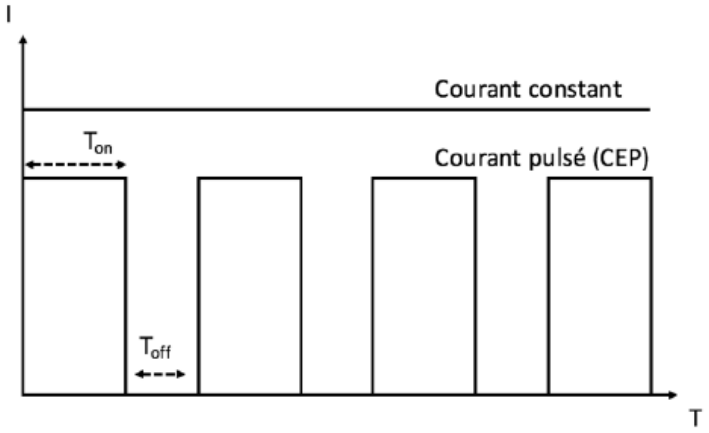


Figure I-32. Représentation de l'intensité de courant en fonction du temps dans le cas d'un courant continu constant et d'un champ électrique pulsé (Dufton, 2019).

Grâce à ce régime, la concentration de polarisation diminue jusqu'à disparaître durant chaque temps de pause. Ce temps, nécessaire à la disparition de la couche limite, est appelé temps de transition et permet de revenir à un état de concentration initial similaire à celui de la solution homogène. Il dépend principalement de la diffusion des ions à travers les membranes et dans la couche limite, de la concentration en ions ainsi que du voltage appliqué au système (Malek *et al*., 2013; Mishchuk *et al*., 2001). Le CEP présente un certain nombre d'avantages tels que :

- Un accroissement de la mobilité des ions dans la couche limite.
- Une diminution de la résistance électrique et une augmentation de l'efficacité du courant et donc une diminution de l'énergie consommée (Malek *et al*., 2013).

- Un retard dans l'apparition de la polarisation de concentration.
- Une diminution du colmatage (Andreeva *et al*., 2018).
- Une simplicité dans les équipements nécessaires, ce qui rend l'intégration d'une telle approche à l'échelle industrielle facile et peu coûteuse.

L'application des CEP sur des solutions alimentaires est encore récente et tire principalement parti des avantages en termes de consommation énergétique et d'amélioration des transferts (Lemay *et al*., 2019; Pelletier *et al*., 2017). Cette nouvelle approche en électrodialyse a été appliquée dans la lutte contre le colmatage par Lee *et al*. (2002; 2005) sur des matières organiques et par Ruiz *et al*. (2007) sur des gels de caséines. De plus, Dufton *et al.* (2020), ont montré que l'utilisation de l'ED avec application du champ électrique pulsé était efficace pour produire du lactosérum acide déminéralisé avec une réduction du colmatage minéral et une diminution de la consommation d'énergie. L'efficacité du champ électrique était étroitement liée au choix des paramètres et des différences significatives ont été observées en fonction des combinaisons de temps de pulsation et de temps de pause choisis (Bazinet et Geoffroy, 2020).

I.3.1.4.4.1.3. Courant sur-limite

Il est connu que l'atteinte et le dépassement du courant limite induisent un colmatage membranaire (Bazinet et Geoffroy, 2020). Cependant, des études ont montré qu'une certaine maîtrise des conditions appliquées en densité de courant sur-limite permettait d'améliorer les performances des procédés d'ED et de réduire les colmatages membranaires (Belova *et al*., 2006; Zabolotskii *et al*., 2012). Les courants sur-limite conduisent à la formation d'une convection induite par le courant, en particulier l'électro-convection, dont l'apparition de vortex aux abords des membranes sont les principaux éléments responsables de ce phénomène. Les vortex électro-convectifs réduisent les couches limites de diffusion d'une manière considérable, facilitant ainsi le transport des ions vers la surface des MEI et permettant le contrôle du phénomène de dissociation des molécules d'eau ce qui peut entraîner par la suite une diminution du coût de l'ED (Mikhaylin, 2015; Nikonenko *et al*., 2014). Bukhovets *et al*. (2011) ont proposé l'effet « washing out » de l'électro-convection

sur le colmatage organique. L'effet positif de l'électro-convection sur la diminution du colmatage minéral sur les MEC a été rapporté récemment par Mikhaylin *et al*. (2015) et Bazinet et Geoffroy (2020).

I.3.1.4.4.2. Contrôle des conditions hydrodynamiques

Dans certains cas, le contrôle des conditions hydrodynamiques du traitement d'ED peut être suffisant pour éviter la formation de colmatage. Grossman *et al*. (1972; 1973), ont rapporté qu'une augmentation du débit et l'introduction d'espaceurs favorisant la turbulence pourront être avantageux pour diminuer le colmatage. La conception des espaceurs est importante en termes d'augmentation de l'efficacité du courant (Mikhaylin et Bazinet, 2016). De plus, Nichka *et al*. (2021), ont étudié l'effet combiné de trois différents débits relativement élevés d'une solution d'alimentation (correspondant aux nombres de Reynolds (Re) de 187, 374 et 560) et divers rapports impulsion-pause du régime CEP (10s/10s, 10s/20s, 10s/33s et 10s/50s) sur la cinétique de colmatage des protéines au cours de EDMB d'une solution modèle de caséinate. Ils ont constaté que l'augmentation de la durée de pause et le débit de la solution de caséinate a eu un impact positif sur la minimisation du colmatage des protéines se produisant sur l'interface cationique de la membrane bipolaire (MB) pendant l'EDMB. À la fois une longue pause et un débit élevé, contribuent à une diminution plus efficace de la concentration de protons et d'anions caséinates à la surface de la MB : une très bonne performance membranaire a été obtenue avec 50s de durée de pause de CEP et un débit correspondant à Re = 374 (Nichka *et al*., 2021).

I.3.1.4.4.3. Modification des membranes

La modification de la matrice membranaire (Gudernatsch *et al*., 1990) ou le greffage de groupements en surface sont des méthodes qui permettent de résoudre un certain nombre de problèmes liés au colmatage (Nagarale *et al*., 2004; Qiu *et al*., 2007). Généralement, la modification des MEI vise à changer les propriétés de la surface de la membrane telles que la quantité et la distribution de leurs charges de surface, leur degré et l'équilibre d'hydrophobicité/hydrophilicité ou bien la rugosité (Balster *et al*., 2005; Gil *et al*., 2018; Jin

et al., 2018; Mulyati *et al*., 2012; Zhao *et al*., 2016). Grebenyuk *et al*. (1998) ont démontré que la modification de la MEA par des tensioactifs de haute masse moléculaire était efficace contre le colmatage organique ce qui a conduit par conséquent à une réduction de 1,7 fois de la consommation d'énergie électrique. Kusumoto *et al*. (1974), ont constaté que le traitement oxydant de la MEA peut générer une couche mince et neutre sur la surface de la membrane rendant la membrane résistante au colmatage organique. La modification de la surface de la MEA par des agents sulfonants améliore considérablement le potentiel anti-colmatage (Mulyati *et al*., 2012; Kusumoto et Mizutani, 1975). Cette amélioration se produit en raison de la diminution de l'hydrophobicité de la surface de la membrane et de l'augmentation de sa densité de charge négative de surface. Les auteurs ont également souligné que l'augmentation de la charge négative de surface et la diminution de l'hydrophobicité de la surface ont une influence positive sur le potentiel anti-colmatage, tandis que l'augmentation de la rugosité de la surface diminue le potentiel anti-colmatage (Mikhaylin et Bazinet, 2016).

I.3.1.4.4.4. Traitement de la solution

Une autre possibilité pour éviter le colmatage est d'éliminer les éléments susceptibles de colmater en appliquant un traitement préalable à la solution soumise à l'ED. En 2003, Bazinet *et al*. ont appliqué le principe de la neutralisation des ions hydroxyles (OH^-) électro-générés par une membrane bipolaire lors du traitement par électro-acidification du lait bovin écrémé, et l'ont considéré comme une approche consistant à traiter en continu les solutions pour éviter la précipitation et le dépôt de composés colmatant indésirables.

I.3.1.4.4.5. Nettoyage en place (Cleaning in place)

L'application de produits nettoyants (acide chlorhydrique, EDTA (sel de l'acide éthylène-diamine-tétra-acétique), solutions anti-colmatante, etc.) est utilisée parfois comme moyen pour éliminer le colmatage déjà formé ou pour limiter sa formation (He *et al*., 2009; Wang *et al*., 2011). Les agents de nettoyage sont choisis en fonction de la nature du colmatage. Cependant, le nettoyage par des agents peut avoir des impacts négatifs. Par exemple, il présente l'inconvénient de devoir arrêter le traitement et de vider le système pour y faire

circuler ces agents nettoyants. En appliquant cette méthode, il y a nécessité de répéter l'opération à plusieurs reprises ce qui peut provoquer un surcoût et des conséquences néfastes d'un point de vue environnemental. De plus, cette méthode peut avoir une influence négative sur les performances des membranes. Par exemple, le nettoyage des MEI avec des solutions alcalines ou des oxydants peut conduire à la dégradation des groupes échangeurs d'ions (Bauer *et al*., 1990; Garcia-Vasquez *et al.,* 2014). De plus, cette méthode présente un risque de contamination dans le cas de procédés de l'industrie alimentaire (Garcia-Vasquez *et al*., 2016). Toutefois, Ayala-Bribiesca *et al*. (2005) ont démontré que lors de la déminéralisation de lactosérum, qui représente l'une des principales applications industrielles de l'ED, la procédure de nettoyage en place et son coût pourraient être justifiés par la haute valeur ajoutée des produits (Mikhaylin et Bazinet, 2016).

I.3.1.5. Applications de l'électrodialyse conventionnelle

Un grand nombre d'applications de l'ED a été identifié depuis très longtemps. Il y a eu des évolutions au niveau des connaissances et du matériel. Les principales applications en industrie alimentaire sont présentées dans le **Tableau I-8**.

Tableau I-8. Les applications de l'ED en industrie alimentaire.

Applications	Références
Déminéralisation du sucre de mélasse	Maigrot et Sabates (1890); Kokubu *et al.* (1983)
Déminéralisation du lactosérum	Delanay (1979); Bazinet (2005); Strathmann (2010)
Dessalement des eaux saumâtres	Katz (1982)
Stabilisation tartrique du vin	Escudier *et al.* (1995)
Dessalement de sauce soja	Mok *et al.* (2001)
Séparation et purification des acides aminés	Zhang *et al.* (2011); Yuan *et al*. (2016); Kattan Readi *et al.* (2013); Wang *et al*. (2016)
Élimination des résidus acides de sucre	Wang *et al*. (2015)

Cette partie de la synthèse bibliographique a permi de montrer que l'électrodialyse est un procédé électro-membranaire ayant une importance particulière. La présentation qui fait suite concerne l'électrodialyse avec membranes bipolaires, ses caractéristiques et son mode d'action.

I.3.2. L'électrodialyse avec membranes bipolaires (EDMB)

I.3.2.1. Principe de l'EDMB

Nous allons nous intéresser maintenant à un type particulier d'électrodialyse qui met en œuvre des membranes bipolaires. Cette technologie est connue sous le nom d'électrodialyse avec membranes bipolaires (EDMB).

I.3.2.1.1. Les membranes bipolaires

Les membranes bipolaires sont en général préparées en structure composite multicouches et formées de trois parties : une MEC, une MEA et une couche intermédiaire hydrophile au centre dépourvu de toute charge. Elles ont la propriété de dissocier les molécules d'eau en présence d'un champ électrique. Ainsi, elles produisent des protons H^+ à partir de l'interface cationique provoquant une acidification dans le compartiment proche de la cathode et des ions hydroxyles OH^- du côté de l'interface anionique provoquant une basification dans le compartiment proche de l'anode (Bazinet, 2019). Le principe de ce phénomène est illustré à la **Figure I-33**.

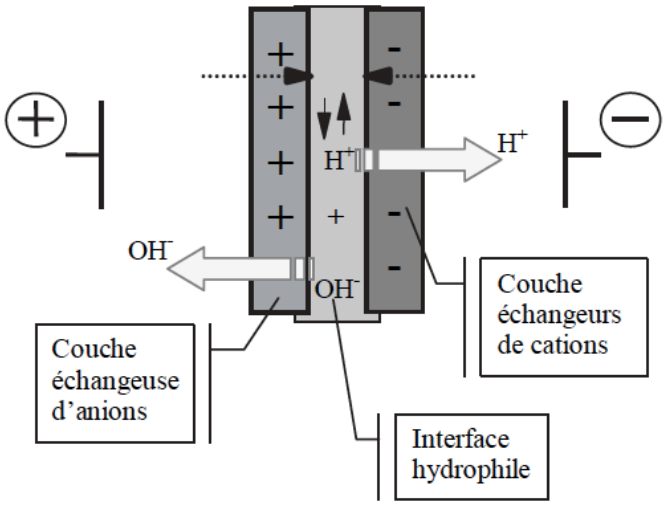


Figure I-33. Principe de fonctionnement de la membrane bipolaire (Bazinet, 2019).

I.3.2.1.2. Génération d'H^+ et d'OH^-

I.3.2.1.2.1. Généralités

Le procédé conventionnel de génération d'ions H^+ et OH^- à partir de l'eau est l'électrolyse. L'électrolyse génère également de l'O_2 et de l'H_2, et la surtension de cette génération consomme environ la moitié de l'énergie électrique du processus. L'EDMB est une méthode alternative à l'électrolyse pour la génération d'ions H^+ et OH^-, qui peut être utilisée pour générer de l'acide et de la base à partir de sels, sans production d'oxygène et d'hydrogène (Mani, 1991). La molarité des solutions générées est de l'ordre de 4 M à 5 M, une molarité très élevée de 30 M, par exemple, ne sera jamais atteinte. De plus, il a été décrit que l'application des MB réduit les coûts énergétiques associés à la polarisation des électrodes dans l'approche électrolytique plus conventionnelle (Pourcelly et Bazinet, 2005).

Le mécanisme de dissociation de l'eau dans les membranes ioniques et bipolaires a fait l'objet de nombreuses études et discussions. Deux explications principales ont pu être suggérées : le second effet de Wien et le phénomène de protonation-déprotonation.

I.3.2.1.2.2. Le second effet de Wien

Le second effet de Wien représente la première explication avancée pour pouvoir comprendre ce mécanisme. Un fort champ électrique a pour effet d'induire et d'augmenter la dissociation des molécules d'eau. Cependant, la recombinaison des ions H^+ et OH^- n'est pas affectée par ce champ électrique, permettant aux protons et ions hydroxyles de rester sous forme dissociée (Strathmann *et al*., 1993). Suite aux calculs effectués selon ce modèle, il a été remarqué qu'ils ne correspondent pas tout à fait à la quantité réelle de protons et d'ions hydroxyles générés. Par conséquent, le second effet de Wien ne suffit pas à lui seul pour expliquer la dissociation de l'eau au sein des membranes bipolaires (Pourcelly et Bazinet, 2005).

I.3.2.1.2.3. Le phénomène de protonation-déprotonation

Des considérations théoriques et expérimentales ont conduites à l'hypothèse que l'accélération de la dissociation de l'eau est aussi provoquée par un transfert réversible de protons entre l'eau et les groupements ioniques fixes de la membrane (Simons, 1985). Les résultats expérimentaux montrent que la dissociation de l'eau se produit principalement à la surface des membranes et que le fractionnement de l'eau n'a pas lieu dans la solution mais dans la phase membranaire. Simons a suggéré qu'avec les MEA, la dissociation de l'eau est provoquée par la protonation réversible de groupes faiblement basiques (amines tertiaires).

Pour les MB, les réactions de transfert de protons se font par les deux groupes échangeurs de cations et d'anions du polymère membranaire adjacent qui réagissent avec les molécules d'eau à la surface de la membrane par les mécanismes suivants de dissociation de l'eau :

$$B + H_2O \leftrightarrow BH^+ + OH^-$$

$$BH^+ + H_2O \leftrightarrow B + H_3O^+$$

Et

$$A^- + H_2O \leftrightarrow AH + OH^-$$

$$AH + H_2O \leftrightarrow A^- + H_3O^+$$

Où B est une base neutre, BH^+ est un centre actif catalytique (généralement le groupe chargé fixe de la MEA), A^- est le groupe chargé fixe de la MEC et AH est un acide neutre (Xu, 2005). Les groups BH^+ ou A^- sont considérés comme des centres catalytiques favorisant la réaction de dissociation de l'eau dans la membrane (Pourcelly et Bazinet, 2005).

I.3.2.1.2.4. Modèle global

Ce modèle de dissociation de l'eau développé par Strathmann *et al*. (1997) est décrit comme étant une combinaison du second effet de Wien, des phénomènes de protonation-déprotonation des groupes fonctionnels dans la membrane et de l'épaisseur de la zone de réaction. Dans les MB, la dissociation de l'eau a lieu dans une très petite région de la couche échangeuse d'ions mono-polaire (Simons and Khanarian, 1978). La protonation et la déprotonation peuvent avoir lieu au niveau des groupes basiques et acides. Selon le modèle global, les auteurs ont démontré que pour les groupes fortement basiques, la protonation est très rapide mais la déprotonation est très lente, donc les groupes fixés dans la membrane restent sous la forme protonés. Pour les groupes fortement acides, tous les groupes restent sous les formes dissociées et déprotonées. Par conséquent, les réactions de transfert de protons sont capables d'expliquer la dissociation accrue de l'eau. Les protons se déplaceront d'abord plus rapidement que les ions hydroxyles, en raison de leur mobilité plus élevée. Cela perturbe l'électroneutralité dans la couche de transition et conduit à un champ électrique supplémentaire, qui augmente la migration des ions hydroxyles et ralentit les protons. Il est important de mentionner que l'épaisseur de la zone de transition entre les couches échangeuses d'ions, qui comprend la zone de réaction, est de quelques nanomètres. En raison de la rugosité des surfaces polymères, dans certains cas, il peut y avoir de fines régions neutres (couche d'eau) entre les couches échangeuses d'ions. La présence de ces régions dépend de la production de la MB (Pourcelly et Bazinet, 2005).

I.3.2.1.3. Catalyseurs pour améliorer la dissociation de l'eau

Le potentiel théorique pour atteindre la capacité de séparation de l'eau est de 0,83 V à 25°C. En raison de l'ajout de catalyseurs, le potentiel réel à travers la membrane bipolaire est assez

proche de celle-ci étant de l'ordre 0,9 à 1,1 V pour des densités de courant comprises entre 50 et 150 mA/cm^2. Donc, pour améliorer la dissociation des molécules d'eau dans la MB, des catalyseurs sont nécessaires pour réduire son potentiel électrique à une valeur proche de 0,83 V. Cet effet est dû en principe à la réduction de l'énergie d'activation de la réaction globale de dissociation (Mafé *et al*., 1998). La dissociation de l'eau est une réaction de dismutation ou de transfert de protons catalysée par des acides et des bases faibles (Kemperman, 2000).

Il est supposé que le meilleur effet catalytique soit obtenu si la valeur pK_a ou pK_b du matériau en interphase est proche de 7 (Rapp, 1994). Le matériel catalytique doit être stable et efficace pendant une longue période. Il doit également rester dans l'interphase, où il est le plus actif, pendant la durée de vie prévue de la membrane. Certains hydroxydes de métaux, tels que l'hydroxyde de chrome (III) et de fer (III), sont adaptés pour être déposés en tant que catalyseur dans l'interphase entre les couches échangeuses de cations et d'anions de la MB. D'autres composants catalytiques utilisés dans la préparation de MB, tels que les amines tertiaires ou les acides phosphoriques, sont généralement liés directement à la matrice polymère de la membrane (Kemperman, 2000; Strathmann, 2004). Les catalyseurs utilisés dans l'industrie alimentaire sont de grade alimentaire. Les composants catalytiques les plus couramment utilisés dans les MB sont énumérés dans le **Tableau I-9** (Pourcelly et Bazinet, 2005).

Tableau I-9. Principaux catalyseurs utilisés dans les membranes bipolaires (Pourcelly et Bazinet, 2005).

Matériaux	Remarques	Valeur de pK_a	Références
$Cr(OH)_3$	Après traitement en OH, et sous forme de sel dans la couche d'échange de cations ou l'interphase		Simons, 1993; Hurwitz and El Moussaoui, 1996
$Fe(OH)_3$	Appliqué en tant que sel dans la couche		Hanada *et al*., 1991.

	d'échange de cations ou l'interphase		
$-NR_2/-NH^+R_2$ (amines tertiaires)	Les amines tertiaires se lient à la matrice de la couche échangeuse d'anions	≈ 9-10	Gnusin *et al*., 1980 ; Bauer *et al*., 1988
Sn ou Ru ion			Hanada *et al*., 1991; Simons, 1989.
$R-PO_2H^-/-COO^-$	Liaison à la matrice de la couche échangeuse de cations	≈ 7	Shel'deshov et al., 1986
$R-COOH/-COO^-$		4.8	Bauer, 1992
Pyridine		5.2	Bauer, 1992

I.3.2.2. Les configurations d'EDMB

Bien que le mécanisme de dissociation de l'eau dans les membranes bipolaires reste aujourd'hui sujet à de nombreuses discussions, les applications de l'électrodialyse avec membranes bipolaires à l'échelle laboratoire et industrielle existent depuis une trentaine d'années. Selon le type d'application recherchée, les procédés d'EDMB peuvent être réalisés avec différentes configurations de cellule : cellule à 2 compartiments ou cellule à 3 compartiments (Pourcelly et Bazinet, 2005).

I.3.2.2.1. Une cellule à 2 compartiments

Il existe des applications où l'obtention d'une haute pureté de l'acide et de la base n'est pas possible, ou même, elle peut générer des problèmes pendant le processus. Par exemple, dans la séparation d'un sel à partir d'un acide faible (acétate de sodium), l'acide pur est faiblement

dissocié, sa conductivité est donc faible. Il ne serait donc pas pratique de réaliser le fractionnement de l'acétate de sodium dans une cellule à trois compartiments. De ce fait, une cellule à deux compartiments plaçant la solution d'acétate entre une MB et une MEC est recommandée (Pourcelly, 2002) (**Figure I-34a**). Lorsque la conversion du courant de sel atteint 95 %, la conductivité devient très faible. Il n'est donc pas possible de continuer le processus d'EDBM. L'EDBM est alors arrêté et les ions Na^+ résiduels sont éliminés à l'aide de résines échangeuses de cations. De manière similaire, un sel d'ammonium ou d'amine peut être traité dans une cellule à deux compartiments avec une MEA au lieu d'une MEC (**Figure I-34**(**b**)) (Pourcelly et Bazinet, 2005). De plus, les performances de la cellule à deux compartiments peuvent être améliorées par l'introduction d'une troisième chambre (Mani, 1991). Lors de l'opération, la solution saline est d'abord introduite dans la chambre entre les deux MEC, puis passe dans le compartiment acide. Cela donne un flux sel/acide ayant une concentration d'acide plus élevée que la cellule standard à deux compartiments. La cellule anionique multichambre est utilisée d'une manière similaire à celle de la cellule cationique. Il est clair que cet arrangement multicellulaire entraîne un coût d'énergie électrique plus élevé en raison de la tension de cellule plus élevée que celle nécessaire dans la cellule à deux compartiments (Pourcelly et Bazinet, 2005).

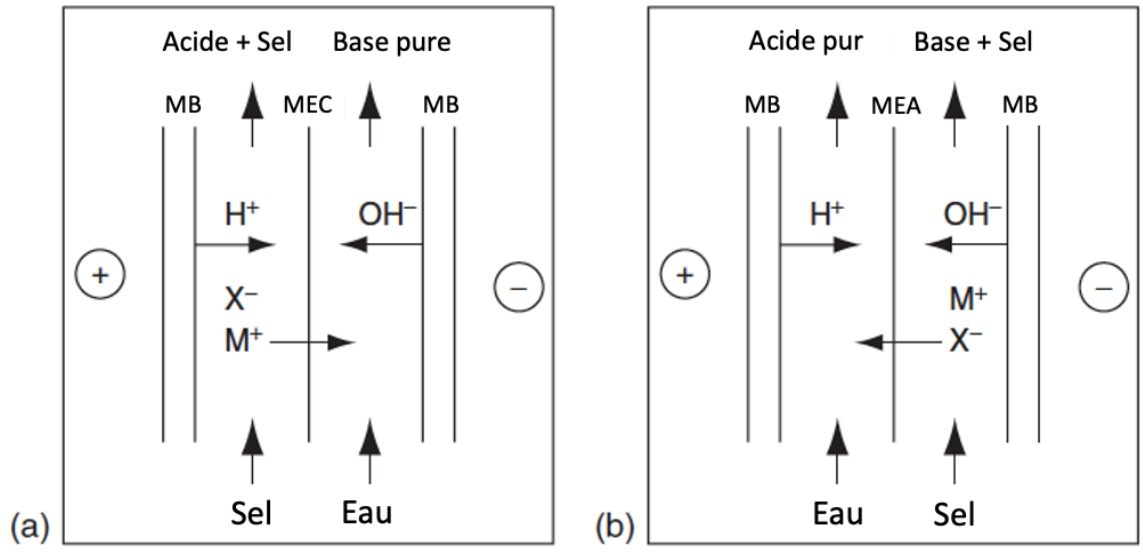


Figure I-34. Cellule d'électrodialyse avec membranes bipolaires à 2 compartiments produisant : (a) une base pure et (b) un acide pur : MEC, membrane échangeuse de cations ; MB, membrane bipolaire ; MEA, membrane échangeuse d'anions (Pourcelly et Bazinet, 2005).

I.3.2.2.2. Une cellule à 3 compartiments

Une cellule à 3 compartiments est généralement utilisée comme cellule élémentaire pour la conversion d'une solution concentrée de sels comme le Na_2SO_4 en acides et bases tels que les H_2SO_4 et NaOH (**Figure I-35**). Le système est constitué d'une MEA, une MEC et deux MB. Cette cellule élémentaire est répétée et placée entre deux électrodes. La solution Na_2SO_4 circule entre la MEA et la MEC. Lorsqu'un courant électrique est appliqué, l'eau se dissocie dans les MB pour former des quantités équivalentes d'ions H^+ et OH^-. Dans ce type de configuration, les ions H^+ générés du côté cationique de la membrane bipolaire réagissent avec les ions sulfate (SO_4^{2-}) fournis par le compartiment adjacent pour former l'acide H_2SO_4. En parallèle, les ions OH^- générés du côté anionique de la membrane bipolaire forment la base NaOH à partir des cations sodium (Na^+) de la solution de sel ayant traversé la membrane cationique. Le résultat final est la production de NaOH et de H_2SO_4 à partir de Na_2SO_4 à un coût nettement inférieur à celui d'autres méthodes (Pourcelly et Bazinet, 2005).

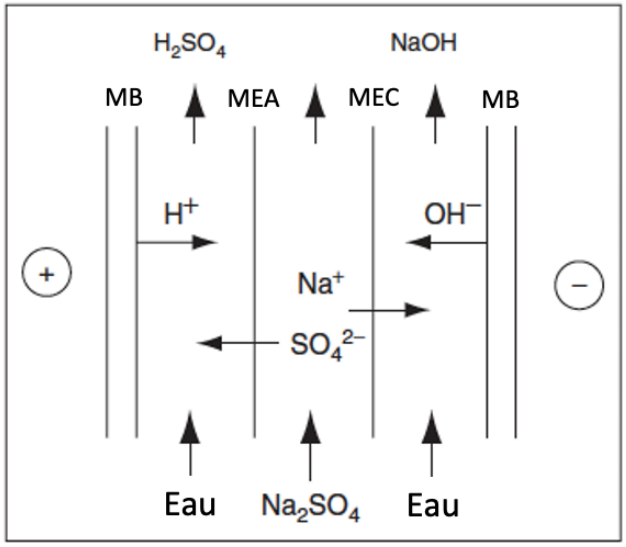


Figure I-35. Cellule d'électrodialyse avec membranes bipolaires à trois compartiments produisant un acide et une base à partir d'un sel : MEC, membrane échangeuse de cations ; MB, membrane bipolaire ; MEA, membrane échangeuse d'anions (Pourcelly et Bazinet, 2005).

I.3.2.3. Les caractéristiques de l'EDMB et ses limites

Les principales caractéristiques de l'EDMB sont (Pourcelly et Bazinet, 2005) :

- Un taux élevé de dissociation de l'eau en ions H^+ et OH^-.
- Une faible résistance électrique à haute densité de courant permettant une bonne conduction du courant électrique.
- Un taux de transport des co-ions faible.
- Une forte sélectivité aux ions (95-99%).
- Il n'y a pas génération de gaz lors du fractionnement de l'eau.
- Il n'y a pas génération d'effets indésirables puisque dans son mécanisme d'action, il n'y a pas de réaction électrochimique (réaction d'oxydation ou de réduction).
- Une bonne tenue mécanique et une stabilité thermique.
- Une bonne stabilité chimique aux acides et bases fortes (stable sur un grand spectre de pH entre 2 et 11,5).
- Elle possède des propriétés uniformes sur une large surface.
- La MB peut être utilisée en association avec la MEA et la MEC pour produire des acides et des bases à partir de sels par électrodialyse.

Cependant, et comme pour tous les processus, l'EDMB présente des limites (Mani, 1991). Des processus indésirables qui peuvent être externes ou internes peuvent réduire l'efficacité de génération d'acide et de base à partir de sel et d'eau. Il peut y avoir perte de la perm-sélectivité de chaque couche homopolaire de la MB. Ce facteur agit directement sur la pureté de l'acide et de la base produits. Il peut de même y avoir une perte de la perm-sélectivité des membranes homopolaires associées (MEA ou MEC). Enfin, il peut y avoir des pertes par diffusion dues aux gradients de concentration, ce qui se produit de manière significative pour les petites molécules faiblement ionisées (Pourcelly et Bazinet, 2005).

I.3.2.4. Principales applications de l'EDMB en transformation alimentaire

Un grand nombre d'applications de l'EDMB a été identifié depuis les années 80. Ce procédé a un grand intérêt dans la résolution de problèmes de pollution, de récupération des ressources

et de traitement chimique. Il a aussi un grand rôle dans les industries alimentaires. Dans cette section, les principales applications de cette technologie sur les produits alimentaires seront présentées, puis nous focaliserons sur une application particulière proche du sujet de la présente thèse.

I.3.2.4.1. Principales applications

En 1990, Hatzidimitriu a breveté un procédé permettant d'ajuster le pH des fluides aqueux. Ce procédé peut être utilisé pour ajuster l'acidité d'un fluide comestible (notamment des sirops de sucre, des jus de fruits et de légumes, des vins, des sauces et de la pâte de tomates) par électrodialyse dans une cellule contenant des paires de membranes comprenant une membrane bipolaire et une membrane sélective pour les ions. Plus spécifiquement, Dykalo *et al*. (1996) ont utilisé une membrane bipolaire en ED pour contrôler l'acidité de produits laitiers contenant une phase aqueuse. Boyaval *et al*. (1993), Siebold *et al*. (1995) et Alvarez *et al*. (1997) ont utilisé respectivement cette technologie pour la production à l'échelle laboratoire d'acide propionique, lactique et salicylique. Parmi les applications potentielles de l'EDMB on peut aussi citer : la production d'isolats de protéines de soja (Bazinet *et al*., 1997), l'inhibition du brunissement du jus de pomme (Tronc *et al*., 1997 ; Lam Quoc *et al*., 2000), la production d'isolats de caséine de lait bovin (Bazinet *et al*., 1999), la désacidification de jus de fruits de la passion (Vera *et al*., 2003), la délipidation du lactosérum et de concentré protéique de lactosérum (Lin Teng Shee *et al*., 2005 et 2007), la production et la déminéralisation des oligomères de chitosane (Lin Teng Shee et al., 2006, 2007 et 2008), la désacidification de jus de canneberge d'une manière eco-efficiente (Serre *et al*., 2016 ; Faucher *et al*., 2018) et la production de protéine de lait en tenant compte des préoccupations environnementales et en favorisant le développement d'une société humaine saine (Mikhaylin *et al*., 2018).

Nous allons présenter maintenant les études réalisées par Lin Teng Shee sur l'électroacidification et l'électrobasification du chitosane avec les membranes bipolaires, l'application la plus proche du sujet de la présente thèse.

I.3.2.4.2. Optimisation de la production d'oligomères de chitosane

Plusieurs méthodes ont été proposées pour préparer des oligomères de chitosane, notamment l'hydrolyse chimique avec acide inorganique (Domard *et al*., 1987) et l'hydrolyse enzymatique avec les enzymes chitosanolytiques, en particulier la chitosanase (Boucher *et al*., 1992). La préparation des oligomères de chitosane avec la chitosanase représente la méthode la plus utilisée car elle permet la production d'oligomères de chitosane avec un degré de polymérisation spécifique. La méthode conventionnelle pour produire les chito-oligomères consiste à solubiliser le chitosane par acidification chimique (utilisant l'acide acétique ou l'acide chlorhydrique à une concentration de 0,1 M ou 1% (Rinaudo *et al*., 1999)), puis en incubant la solution de chitosane avec la chitosanase et en arrêtant la réaction enzymatique par chauffage à 100°C (Li *et al*., 2005 ; Jeon et Kim, 2000).

Dans leurs premiers travaux, Lin Teng Shee *et al*. (2006; 2007) ont montré que l'acide nécessaire pour la solubilisation du chitosane pouvait être produit *in situ* par électro-acidification avec les MB (EDMB) au lieu de l'ajout d'acide chimique (**Figure I-36**). Il a aussi été démontré que la solubilisation électrochimique du chitosane par l'EDMB dépendait du type de membranes ioniques monopolaires, et du mode d'introduction du chitosane dans le système d'ED (Lin Teng Shee *et al.*, 2006). La meilleure configuration identifiée dans cette étude était constituée de membranes échangeuses d'anions avec un mode d'alimentation par étapes (« stepwise feeding mode »), menant à un rendement de solubilisation du chitosane de 91% en 60 minutes en utilisant une intensité de 20 mA/cm^2. Les configurations anioniques ont consommé moins d'énergie que les configurations cationiques, tandis que le mode d'ajout par étapes a amélioré la désagrégation du chitosane dans le système d'EDMB pour une meilleure protonation (Bazinet et Firdaous, 2010).

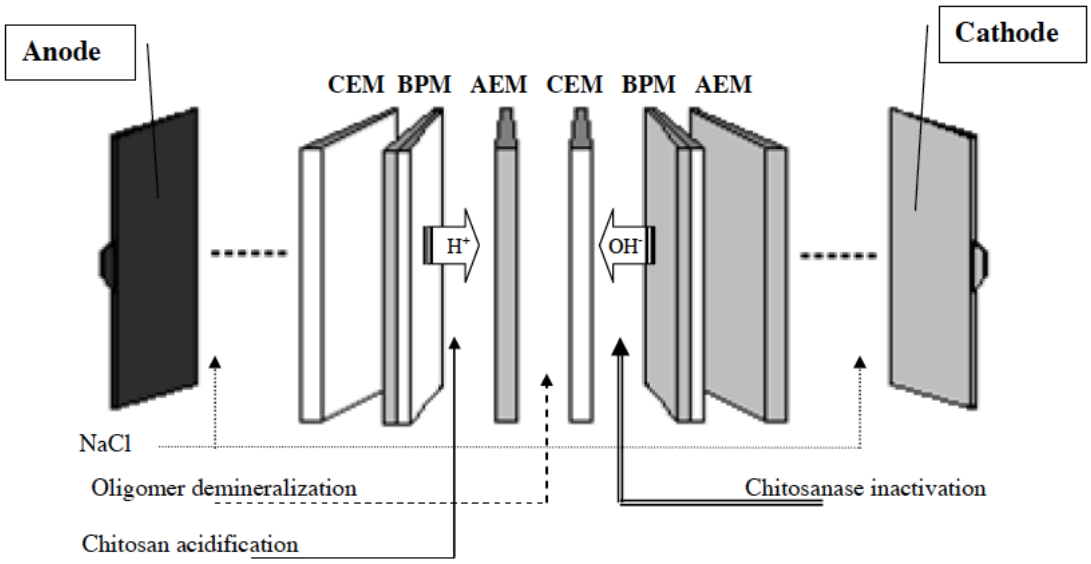


Figure I-36. Unité élémentaire utilisée dans la cellule d'électrodialyse à membrane bipolaire à trois compartiments. BPM, membrane bipolaire; AEM, membrane échangeuse d'anions; CEM, membrane échangeuse de cations (Bazinet et Firdaous, 2009).

Lin Teng Shee *et al.* (2008), ont également démontré que l'EDMB pouvait être utilisée pour terminer la réaction enzymatique du chitosane avec la chitosanase par électrobasification en utilisant la configuration membranaire bipolaire/cationique (**Figure I-36**). L'inhibition enzymatique a été obtenue par dénaturation à des pH très acides (pH < 3) ou très basiques (pH > 8). L'inhibition complète de la réaction enzymatique a été assurée à un pH extrêmement basique de la solution (pH > 11). Comme le montrent Lin Teng Shee *et al*. (2008), l'activité catalytique de la chitosanase pendant l'hydrolyse du chitosane a diminué après l'ajustement du pH par électrobasification. La vitesse de réaction a diminué de 50% de pH 5,5 à pH 6, alors que la réaction était complètement inhibée à pH > 7. Outre la dénaturation de la chitosanase, Li *et al*. (2005) ont montré que les valeurs de pH alcalin ont également un effet sur le chitosane, en précipitant le chitosane de haut poids moléculaire, tandis que les matériaux à faible poids moléculaire restent solubles. La diminution de la vitesse de réaction à des valeurs de pH alcalin était, par conséquent, due simultanément à l'insolubilité du chitosane et la dénaturation de la chitosanase. L'ajustement du pH à 7 suffissait pour arrêter la réaction sans dénaturer la chitosanase ; dans ce cas, l'enzyme peut être récupérée pour une nouvelle hydrolyse du chitosane. Autrement, si la chitosanase doit être complètement inactivée dans le produit final contenant les oligomères de chitosane, l'ajustement du pH à

une valeur supérieure à 9 serait nécessaire pour la destruction de la structure enzymatique (Bazinet et Firdaous, 2010).

Plus récemment, Lin Teng Shee *et al*. (2008) ont proposé un procédé intégré pour la transformation du chitosane en ses oligomères en utilisant une configuration d'EDMB à trois compartiments (bipolaire / anionique/ cationique / bipolaire). Chaque compartiment a été utilisé pour réaliser simultanément la solubilisation du chitosane, l'inactivation de la chitosanase et la déminéralisation des chito-oligomères (**Figure I-37**) (Bazinet et Firdaous, 2010) :

- Dans la première étape, le compartiment d'acidification est utilisé pour solubiliser la poudre de chitosane. La solution de chitosane résultante (pH 2) est retirée du système d'EDMB et le pH de la solution est ajusté au pH d'incubation de la chitosanase par ajout de la poudre de chitosane.
- Dans la deuxième étape, le compartiment de basification est utilisé pour terminer l'hydrolyse du chitosane en élevant le pH de la solution (pH > 7). Simultanément, le chitosane est solubilisé dans le compartiment d'acidification. La solution acidifiée obtenue est traitée comme décrit précédemment, en ajustant le pH et en l'incubant avec de la chitosanase.
- Dans la troisième étape, les trois compartiments fonctionnent simultanément. Les compartiments d'acidification et de basification sont utilisés pour la solubilisation du chitosane et l'inactivation de la chitosanase, respectivement. En parallèle, le compartiment de diluat situé entre les compartiments d'acidification et de basification est utilisé pour la déminéralisation de la solution basifiée pour produire des oligomères de chitosane purifiés.

Enfin, Lin Teng Shee *et al*. (2008) ont comparé le procédé d'EDBM à un procédé traditionnel utilisant un acide et une base chimique. L'un des avantages de l'EDMB par rapport à la méthode chimique est la possibilité d'éliminer les sels préalablement ajoutés lors de l'acidification et de la basification, de sorte qu'au bout d'une heure de traitement, la teneur en cendres revient à sa valeur initiale (environ 0,26%) (Bazinet et Firdaous, 2010). De plus,

aucun stockage et manipulation d'acides et de bases chimiques est nécessaire car les protons et les ions hydroxyles sont produits *in situ* dans le système d'EDMB, créant ainsi un environnement de travail sécuritaire (Bazinet et Firdaous, 2010). De plus, il n'y a aucune génération d'effluents puisque tous les flux dans les 3 compartiments sont utilisés pour produire les oligomères purifiés. Ce procédé innovant « 3-en-1 » de transformation du chitosane en oligomères présente un potentiel important pour l'application industrielle, puisqu'il est pratique, écologique et produit des oligomères à plus faible teneur en minéraux par rapport à la méthode conventionnelle. La consommation d'énergie relative a été estimée à 0,02 kWh/L de solution (Lin Teng Shee *et al*., 2008). De plus, l'énergie a été utilisée pour exécuter trois traitements simultanément, permettant une utilisation efficace du courant électrique (Bazinet et Firdaous, 2010).

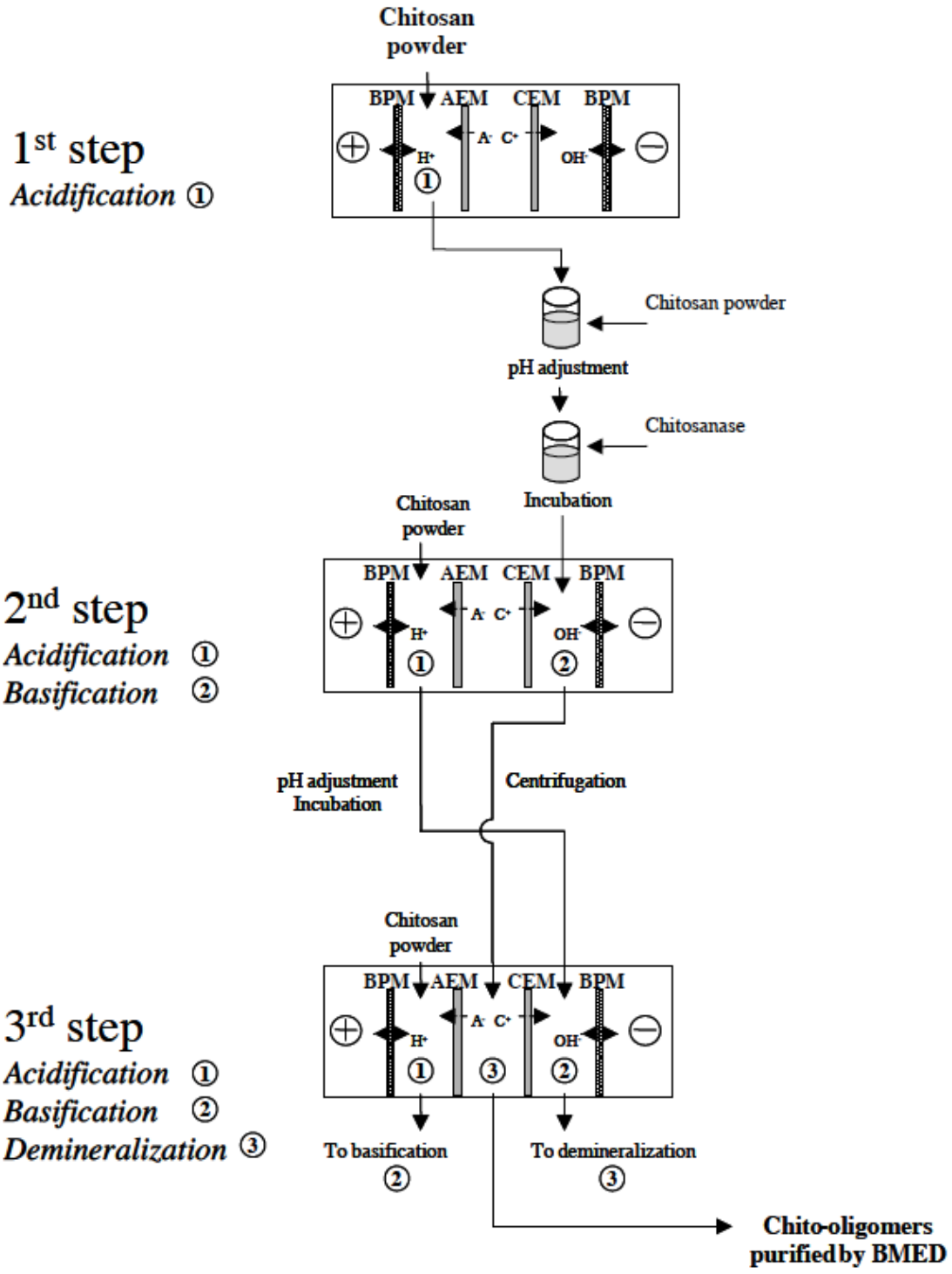


Figure I-37. Flux de processus intégré pour l'hydrolyse du chitosane en oligomères utilisant l'électrodialyse avec membranes bipolaires (Lin Teng Shee *et al*., 2008).

Ces exemples montrent que l'acidification et la basification des produits sans ajouts de produits chimiques sont possibles en utilisant l'électrodialyse avec membranes bipolaires.

D’autre part, les produits traités par EDMB sont très purs, car ils sont partiellement déminéralisés. L’EDMB est une technologie innovatrice et écologique pour la production de flux acides ou basiques à basse teneur en minéraux.

Au travers de cette synthèse bibliographique, il a été mis en exergue l’intérêt de produire des peptides bioactifs à partir de l’hémoglobine bovine, coproduit des abattoirs. Suite à la documentation, en se basant sur un grand nombre d’articles scientifiques, la plupart des hydrolysats sont produits par hydrolyse enzymatique selon des méthodes conventionnelles nécessitant l’ajout de plusieurs agents chimiques. De plus, la revue de littérature a montré l’importance des procédés électrodialytiques dans les applications alimentaires. La production des peptides bioactifs via des technologies innovatrices et vertes représente néanmoins un véritable défi.

Chapitre II

PROBLÉMATIQUE, HYPOTHÈSE ET OBJECTIFS

II. Problématique, hypothèse et objectifs

II.1. Problématique

Le cruor bovin, coproduit des abattoirs, est à l'heure actuelle une problématique environnementale pour l'industrie de la viande puisque dans certains pays du monde, il est rejeté dans la nature sans traitements efficaces induisant ainsi de la pollution. Afin d'être valorisé, plusieurs études ont démontré que l'hydrolyse enzymatique de l'hémoglobine bovine est une source importante de peptides bioactifs naturels. Ces peptides se positionnent comme des candidats potentiels en tant que conservateur alimentaire ou substitut pharmacologique de par leur multifonctionnalité. Cependant, la procédure la plus couramment utilisée pour générer ces peptides bioactifs à partir de l'hémoglobine est l'hydrolyse enzymatique conventionnelle. Elle comprend plusieurs étapes nécessitant l'ajout de produits chimiques et les hydrolysats produits contiennent des niveaux élevés de sels minéraux. La grande quantité de solvant utilisée constitue aussi un facteur d'impact sur l'environnement. Pour réduire les impacts environnementaux, l'électrodialyse avec membranes bipolaires (EDMB) a été utilisée avec succès ces dernières années pour plusieurs applications dans l'agroalimentaire. De l'ensemble de ces considérations découle une interrogation majeure : la technologie verte d'EDMB peut-elle servir de méthode alternative à l'hydrolyse enzymatique conventionnelle de l'hémoglobine pour obtenir des peptides bioactifs purifiés ? Les enjeux sont donc pluriels. Dans un premier temps, il convient de tester la faisabilité de l'EDMB pour l'hydrolyse enzymatique de l'hémoglobine bovine et de la comparer avec l'hydrolyse conventionnelle. Dans un second temps, il faut optimiser les paramètres permettant plusieurs opérations en simultané au sein de la même unité d'électrodialyse tout en prévenant et contrôlant le colmatage des membranes.

II.2. Hypothèse

L'hypothèse de travail est la suivante : L'électrodialyse avec membranes bipolaires permet de réaliser en simultané dans un système d'électrodialyse les différentes étapes nécessaires à

la production d'un hydrolysat (dénaturation et hydrolyse pepsique de l'hémoglobine bovine en milieu acide, inhibition de l'activité enzymatique à pH basique, déminéralisation et décoloration du produit final) sans ajout d'agents chimiques, sans colmatage sur les membranes, tout en aboutissant à un hydrolysat final à faible concentration en sels minéraux et ayant des activités antibactériennes, antifongiques et antioxydantes.

II.3. Objectifs

Les objectifs de cette thèse sont les suivants :

1) Étudier l'impact de la conductivité sur les performances du système, le colmatage membranaires et les paramètres de l'hydrolyse lors de la phase d'acidification de l'hémoglobine bovine par EDMB.

2) Étudier l'impact de l'empilement de membranes échangeuses d'anions ou de cations en tant que membranes homopolaires dans l'EDMB sur l'évolution du pH et de la conductivité et sur la formation de colmatage.

3) Optimiser le système d'EDMB en un procédé tout en un.

4) Quantifier la composition minérale pour chaque phase.

5) Identifier le mécanisme enzymatique impliqué dans l'hydrolyse lors de l'EDMB.

6) Caractériser la population peptidique obtenue après hydrolyse et identifier les peptides bioactifs issus de l'hydrolyse de l'hémoglobine dans les différentes conditions.

7) Étudier les activités biologiques (antibactériennes, antifongiques et antioxydantes) des hydrolysats obtenus à partir de l'EDMB.

Chapitre III

IMPACT DE LA CONDUCTIVITÉ SUR LES PERFORMANCES D'ÉLECTRO-ACIDIFICATION ET D'HYDROLYSE ENZYMATIQUE DE L'HÉMOGLOBINE BOVINE PAR ÉLECTRODIALYSE AVEC MEMBRANES BIPOLAIRES POUR LA PRODUCTION DE PEPTIDES BIOACTIFS.

III. Impact de la conductivité sur les performances d'électro-acidification et d'hydrolyse enzymatique de l'hémoglobine bovine par électrodialyse avec membranes bipolaires pour la production de peptides bioactifs.

III. Transition Contextuelle

La revue de la littérature a montré que la préparation des hydrolysats d'hémoglobine bovine se fait suite à une dénaturation et une hydrolyse enzymatique conventionnelle en milieu acide en premier lieu. L'électrodialyse avec membranes bipolaires (EDMB) est une technologie innovatrice qui n'a jamais été utilisée pour produire des peptides bioactifs issus de l'hémoglobine bovine. De plus, l'impact de la conductivité sur les performances du système et l'effet de sel sur l'activité de la pepsine n'ont jamais été rapportés dans la littérature. Dans ce contexte les objectifs de l'étude menée dans ce chapitre sont : 1) valider la faisabilité de la dénaturation et l'hydrolyse enzymatique de la solution d'hémoglobine par électro-acidification avec membranes bipolaires, 2) étudier l'effet du niveau de conductivité sur les paramètres d'EDMB et le colmatage des membranes, 3) identifier le mécanisme enzymatique impliqué dans l'hydrolyse de l'hémoglobine, 4) caractériser la population peptidique obtenue après hydrolyse, 5) identifier les peptides issus de cette hydrolyse de l'hémoglobine et enfin 6) comparer l'efficacité du procédé d'EDMB avec l'hydrolyse conventionnelle au niveau de l'hydrolyse et de la production des peptides bioactifs.

Ce chapitre a fait l'objet d'un article intitulé **« Impact of conductivity on the performances of electro-acidification and enzymatic hydrolysis phases of bovine hemoglobin by electrodialysis with bipolar membranes for the production of bioactive peptides »,** publié dans *Separation and Purification Technology*, 2021, 269, 118650. Les auteurs sont: Mira Abou-Diab (Candidate au Ph. D.: planification et réalisation des expériences, analyse des résultats et rédaction de l'article), Jacinthe Thibodeau (Professionnelle de recherche côté

canadien : supervision de l'étudiant pour les analyses d'électrodialyse et LC-MS, correction du manuscrit), Ismail Fliss (Co-directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit), Pascal Dhulster (Co-directeur de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit), Naima Nedjar (Directrice de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit) et Laurent Bazinet (Directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit).

III.1. Résumé

La production de peptides bioactifs à partir des déchets d'abattoirs contenant une concentration faible en minéraux par EDMB a récemment été démontrée comme une technologie innovante. Cependant, une limitation du processus d'électro-acidification a été remarquée en raison du colmatage sur les membranes. Le but du présent travail était d'identifier les conditions pour réduire le colmatage peptidique. Ainsi, deux niveaux de conductivité (0,9 mS/cm et 4,5 mS/cm) ont été étudiés en utilisant une configuration bipolaire/monopolaire et comparés à trois conditions d'hydrolyses conventionnelles différentes. L'impact du niveau de conductivité a été étudié sur le colmatage et les paramètres d'hydrolyse. Les résultats ont montré le même mécanisme enzymatique « one-by-one » pour toutes les conditions, mais une cinétique enzymatique ralentie pour l'EDMB à 0,9 mS/cm. L'EDMB-0,9 mS/cm a permis la production de peptides bioactifs, mais avec formation de colmatage sur la MCP tandis que l'EDMB-4,5 mS/cm a permis la production de peptides bioactifs sans colmatage. Enfin, l'optimisation de certaines conditions sera nécessaire.

Mots clés : Électrodialyse avec membranes bipolaires, Hémoglobine bovine, Hydrolyse enzymatique, Niveau de conductivité, Colmatage membranaire, Degré d'hydrolyse, Peptides bioactifs.

III.2. Abstract

Production of bioactive peptides from slaughterhouse wastes with a final low salt content by electrodialysis with bipolar membranes (EDBM) has recently been demonstrated as an innovative and efficient technology. However, a limitation in the electro-acidification process was noticed due to membrane fouling. In this context, the aim of the present work was to identify process conditions that could reduce peptide fouling in EDBM. Hence, two different conductivity levels (0.9 mS/cm and 4.5 mS/cm) were investigated in bipolar/monopolar (anionic and cationic) configuration and compared to conventional hydrolysis in the same conductivity conditions as well as a conventional hydrolysis using a buffer solution. The impact of such conductivity levels was studied during EDBM especially on membrane fouling and hydrolysis parameters such as: 1) kinetics of the enzymatic mechanism, 2) degree of hydrolysis and 3) peptide population. The results showed the same enzymatic mechanism «One-by-one» for all the conditions but a slower enzymatic kinetics for EDBM-0.9 mS/cm. EDBM-0.9 mS/cm allowed the production of bioactive peptides but with fouling formation on MCP while EDBM-4.5 mS/cm allowed the production of bioactive peptides without any fouling. Finally, optimizing certain conditions for further studies would lead to a promising green process to substitute synthetic additives used in food conservation by antimicrobial peptides from slaughterhouse blood.

Keywords: Electrodialysis with bipolar membrane, Bovine hemoglobin, Enzymatic hydrolysis, Conductivity level, Membrane fouling, Hydrolysis degree, Bioactive peptides.

III.3. Introduction

Nowadays, the needs and habits of food consumption generate alarming observations: the resources that can be transformed are disappearing and industrialization leads to an accumulation of waste (Segré and Gaiani, 2012; Gunders, 2012). Millions of tons of food waste are produced every year in the world. Their potential is however underestimated and underused and a lot of these wastes can today claim a second life. Indeed, due to their wealth of intrinsic compounds potentially active, they constitute new resources with high recovery

potential. Pfaltzgraff *et al*. (2013) mentioned that the recovery of waste is a key issue for moving towards a sustainable economy where minimizing the production of non-renewable waste would be a priority. However, the creation of new products from these wastes does not only concern non-food uses. Many wastes are still rich in protein (Goot *et al*., 2016) such as slaughterhouse wastes (particularly blood), fish wastes, etc. Through the hydrolysis by suitable proteases, these proteins can generate peptides having bioactivities (Castro and Sato, 2015; Hartmann and Meisel, 2007). Bioactive peptides are widely used today, whether for therapeutic or pharmaceutical applications (Agyei and Danquah, 2011; Danquah and Agyei, 2012; Saadi *et al*., 2015), in pet foods (Martinez-Alvarez *et al*., 2015) or for human consumption due to their various nutraceutical effects (Hartmann and Meisel, 2007; Lafarga and Hayes, 2014; Li-Chan, 2015). In addition, these naturally occurring compounds are generating a growing interest among consumers, who increasingly want to eat healthy, and avoid synthetic chemical additives whose long-term effects are sometimes little known or controversial (Agyei and Danquah, 2007; Bauer *et al*., 2001; Imaida *et al*., 1983; Lanigan and Yamarik, 2002). Finding new environmentally friendly (« green ») ways of producing peptides has never been of such critical interest, to meet consumer needs, to preserve the environment and to decrease the spoilages in industry.

Blood from slaughterhouses is an attractive source of proteins. Hemoglobin represents up to about 90% of total-cell-fraction proteins and is often an agricultural waste product causing environmental problems due to the large volumes recovered and its low valorization. Therefore, investigations have been carried out to transform this agricultural by-product by enzymatic hydrolysis into peptides with interesting biological activities (Adje *et al*., 2011a; 2011b; Gomes *et al*., 2010; Nedjar *et al*., 2008). The most commonly used method for the production of bioactive peptides from hemoglobin requires the use of chemical agents because of their proven effect on enzymatic hydrolysis. Chemical agents such as alcohols (ethanol, glycerol, etc.), urea, organic solvents (dimethylformamide), buffer (sodium acetate, sodium dodecyl sulfate), acids and bases have been used to improve the enzymatic hydrolysis of protein such as β-lactoglobulin (Dalgalarrondo *et al*., 1995), serum albumin (Houen *et al*., 1991), caseins (Tchorbanov and Iliev, 1993) or gluten (Hertmanni *et al*., 1991). Several authors have observed a modification of the peptide populations by alcohols such as ethanol

and glycerol (Tchorbanov and Iliev, 1993; Houen *et al*., 1991; Lignot *et al*., 1999; Nedjar *et al*., 2006; Dubois *et al*., 2005; Zouari *et al*., 2020) and explained these changes in protease selectivity by a modification of the conformation of the native proteins. Others have observed an improvement in antimicrobial peptide concentrations derived from bovine hemoglobin by using different kinds and concentrations of alcohols such as butanol, propanol, methanol, ethanol, etc. and explained these results by the effect of alcohols on modifying the tertiary and quaternary structure of hemoglobin while keeping intact the secondary structure (Adje *et al*., 2011a). Other studies reported the effect of the composition of ethanol and urea on the formation of two opioid peptides (LVVhaemorphin-7 and VV-haemorphin-7), neokyotorphin, VVhaemorphin-4 and the bradykinin-potentiating peptide in the course of the peptic hydrolysis of hemoglobin at pH 4.5 (Lignot *et al*., 1999). Furthermore, it was demonstrated that the pH value of the hemoglobin solution changed pepsin selectivity whether urea was present or not (Dubois *et al*., 2005). Indeed, the denaturation of the bovine hemoglobin is most often achieved by adding chemical denaturant (urea) and reducing the pH of hemoglobin by adding chemical acid (Nedjar *et al*., 2008; Lignot *et al*., 1999; Nedjar *et al*., 2006; Dubois *et al*., 2005; Zouari *et al*., 2020). Once the required pH is reached, the enzyme solubilized in sodium acetate buffer is added for hydrolysis and finally, the reaction is stopped by addition of chemical base to increase the pH and inactivate the enzyme. However, these chemicals increased the conductivity of the hemoglobin solution and subsequently the hydrolysates produced contain high levels of mineral salts and are considered as synthetic additives that no longer meet the consumer's needs.

Therefore, a green process (Mikhaylin *et al*., 2018), electrodialysis with bipolar membranes (EDBM) was recently demonstrated by Abou-Diab *et al.* (2020a; b) to be an effective process for the enzymatic hydrolysis of bovine hemoglobin without the use of chemicals, allowing a zipper mechanism and was considered as an alternative method to the conventional chemical enzymatic hydrolysis. This process used anion exchange membranes (AEM) and monovalent cation perm-selective membranes (MCP) to segregate the migration of ions and bipolar membranes (BM) to produce H^+ and OH^- ions under the application of an electric current. Hence, the decrease in pH needed for bovine hemoglobin denaturation and enzymatic hydrolysis as well as the maintaining of pH during hydrolysis was carried-out by H^+ ions

electrogenerated at the cationic interface of the BM. However, a fouling was present on the MCPs membranes due to a lack of ions in the hemoglobin solution despite the adjustment of the conductivity to an arbitrary initial value of 1.5 mS/cm to ensure a minimum conductivity. Indeed, the hemoglobin solution contains a very low amount of mineral salts, leading to a very high electrical resistance of the solution to be treated, incompatible with ED. Accordingly, the process should be optimized by studying the effect of different salt levels on enzymatic hydrolysis by pepsin during EDBM, which has never been reported in the literature, and evaluating its impact on the enzymatic mechanism, the peptide population, EDBM performances and fouling.

In this context, the specific objectives of this study were to 1) study the effect of conductivity level on EDBM parameters and membrane fouling, 2) identify the enzymatic mechanism involved during EDBM hydrolysis with pepsin, 3) characterize the peptide population obtained after hydrolysis, 4) identify the bioactive peptides resulting from this hydrolysis of hemoglobin and finally 5) compare the efficiency of EDBM processes with the conventional hydrolysis.

III.4. Materials and Methods

III.4.1. Materials

Hemoglobin. Purified hemoglobin powder from bovine blood, dark brown, H2625, was purchased from Sigma-Aldrich (Oakville, ON, Canada). Hemoglobin was stored at 4°C.

Pepsin. The pepsin lyophilized powder from porcine gastric mucosa was purchased from Sigma-Aldrich (P6887, Oakville, ON, Canada). Pepsin activity was measured at 3311.5 AU/mg of protein according to a protocol established by the supplier Sigma-Aldrich (Saint Quentin Fallavier, France). Pepsin was stored at -20°C.

III.4.2. Protocol

To test the impact of conductivity on the feasibility of EDBM for enzymatic hydrolysis, 5 different conditions were tested: two in EDBM with different initial conductivity (0.9 mS/cm-EDBM and 4.5 mS/cm-EDBM) and in comparison, two controls with the same initial conductivities without buffer (0.9 mS/cm-Control, 4.5 mS/cm-Control) and one control containing a mix of acetic acid and sodium acetate buffer (Fisher Scientific, ON, Canada). The two values of conductivities were chosen, since after preliminary tests, 0.9 mS/cm presents the minimum conductivity that an electrodialysis system can withstand to be able to carry out the process and 4.5 mS/cm presents the same conductivity value as the control when adding pepsin. The conventional hydrolysis with acetic acid/sodium acetate buffer (AA/AS) was considered as the control in this protocol, since several enzymatic hydrolysis studies were reported in the literature to allow the production of peptides with biological activities (Adje *et al*., 2011a; Gomes *et al*., 2010; Nedjar *et al*., 2008).

Preparation of Hemoglobin solution and Conventional Hydrolysis process

In Control, 2g of purified bovine hemoglobin were solubilized in 150 mL of distilled water. The solution was stirred (VWR, Hotplater/Stirrer from Fisher Scientific, OH, Canada) at 10°C overnight. The next day, the solution was centrifuged at 4000 rpm for 30 min (Avanti J-E Centrifuge, Beckman Coulter, IN, United States). The stock solution contained hemoglobin in its native form. To denature the hemoglobin, 25 mL of AA/AS buffer (0.5M) (Fisher Scientific, ON, Canada) was added. The pH was adjusted to 3.5 (optimal pH of pepsin activity) with concentrated HCl (Anachemia, VWR Company International, ON, Canada). The hydrolysis was carried out at pH 3.5 according to results reported in the literature (Tchorbanov and Iliev, 1993; Przybylski *et al*., 2016; Przybylski *et al*., 2020) in the presence of porcine pepsin solubilized in 25 mL of AA/AS buffer (0.1M), with an enzyme/substrate ratio of 1/11 (mole/mole). In this protocol, bovine hemoglobin solution was prepared at a concentration of hemoglobin (C_{Hb}) of 1% (w/v) and the temperature was maintained at 30°C.

In Control-0.9 mS/cm and Control-4.5 mS/cm, the same preparation as for control was done. But, since AA/AS buffer was not used in these conditions, the volume of the hemoglobin solution was adjusted to 200 mL with distilled water. The conductivity was then adjusted, with KCl powder, to 0.9 mS/cm in control-0.9 mS/cm and to 4.5 mS/cm in control-4.5 mS/cm (as initial conductivities). The pH was adjusted to 3.5 with HCl solution. The hydrolysis was carried out at pH 3.5 in the presence of porcine pepsin solubilized in water.

Preparation of hemoglobin solution and hydrolysis process using EDBM

In EDBM-0.9 mS/cm and EDBM-4.5 mS/cm, the 1% hemoglobin solution was prepared as previously, except that 1) the conductivity was adjusted to 0.9 mS/cm in EDBM-0.9 mS/cm and to 4.5 mS/cm in EDBM-4.5 mS/cm with KCl addition since the conductivity of the initial 1% HB solution was too low for ED treatment and 2) the pH adjusted to 3.5 by applying an electric current at a constant voltage of 15 V leading to the electrochemical production of protons H^+ at the cationic interface of the bipolar membrane in contact with the HB solution. When the pH value of 3.5 was reached, porcine pepsin solubilized in water was added to start the hydrolysis.

EDBM configuration, the module used was a Microflow-type cell (10 cm^2 of effective surface) equipped with a Dimensionally Stable Anode (DSA®), and a 316-stainless-steel cathode from ElectroCell AB (Täby, Sweden). The cell consisted of stacking one Food grade Neosepta AMX-SB anion-exchange membranes (AEM), two Food grade Neosepta monovalent cation perm-selective membrane (MCP) and two bipolar membranes (BM) purchased from Astom (Tokyo, Japan) (**Figure III-1**). Anion-exchange membranes intrinsically contain quaternary ammonium groups ($-N^+(CH_3)_3$) allowing the migration of anions oppositely charged. Monovalent ion permselective membranes are a category of ion-exchange membranes allowing the migration of oppositely charged monovalent ions while retaining the polyvalent ions. Monovalent cation permselective (MCP) membranes are made of resin, including negative charges coated with a thin positive charge layer. And bipolar membranes prepared in composite structure multilayer and formed of three parts: a cation-exchange membrane, an anion-exchange membrane and an intermediate hydrophilic layer in

the center, allowing the generation of H^+ and OH^- ions from water dissociation under an electrical field. This arrangement defines three circuits containing 1% bovine hemoglobin solution (200 mL), a 0.5 g/L corresponding to 0.9 mS/cm or 2.5 g/L corresponding to 4.5 mS/cm of KCl solution (200 mL; EMD Chemicals Inc, USA) and a 20 g/L of electrode-rinsing solution Na_2SO_4 (250 mL; Fisher Chemical, Belgium). Hemoglobin and KCl flow rates were 250 mL/min; Na_2SO_4 flow rate was controlled at 400 mL/min. Each closed loop was connected to a separate external reservoir allowing continuous recirculation for each compartment (**Figure III-1**). A constant voltage of 15 V was applied during all the electrodialysis with bipolar membranes (EDBM) experiments using a BK Precision power supply (model BK9110-ND, Vancouver, BC, Canada).

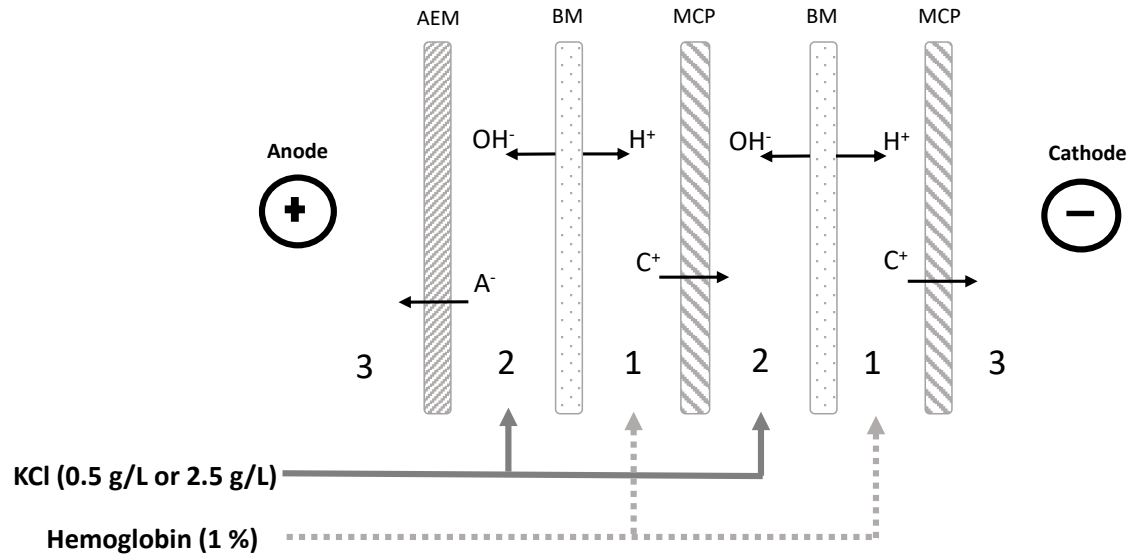


Figure III-1. Schematic representation of electrodialysis cell configuration for electro-acidification and hydrolysis of bovine hemoglobin. (1) Acidified compartment, (2) basified compartment, (3) Na_2SO_4 electrolyte; MCP: monovalent cation perm-selective membrane; AEM: anion exchange-membrane and BM: bipolar membrane.

During each experiment, the duration of the acidification was different depending on the condition, but for all, hydrolysis was conducted for 3 hours. Samples were taken at 0 min before adding the pepsin and then after pepsin addition at 30 min, 60 min, 120 min and 180 min during the hydrolysis step to follow the release of peptides. The reaction in each sample was stopped by addition of KOH (0.5M) (Fisher Scientific, New Jersey, United States) in

order to bring the sample to pH 9, allowing both to irreversibly inactivate the enzyme and to avoid any possible basic hydrolysis. The samples were stored at -20°C until further analyses. The current intensity, conductivities, pH and temperature, for each hemoglobin and KCl solutions, were recorded during EDBM treatment. Global resistance, energy consumption and characterization of membrane fouling were analyzed, as well as mineral composition to investigate the potential advantages of the EDBM method over the conventional chemical process. Moreover, the hydrolysis capacity and the enzymatic kinetics were compared by analyzing the degree of hydrolysis and mass spectra profiles between the different conditions. Three randomized repetitions of each EDBM and control conditions were carried out. For EDBM, after each treatment, only a cleaning in place (CIP) with water was performed according to the manufacturer's procedure. Then, the cell was dismantled and the membrane conductivity and thickness measured to evaluate their integrities. Also, the membranes were changed after each block of three repetitions. Hydrolysis mechanism and peptide identification were carried out on the samples collected during the hydrolysis at T_0, T_{30}, T_{60}, T_{120} and T_{180}.

III.4.3. Analyses

III.4.3.1. pH

pH of hemoglobin and KCl solutions was measured with a pH meter (model SP70, symphony, VWR international, Montreal, Canada) equipped with an electrode compensating for temperature variation. To stop the hydrolysis reaction in the hemoglobin hydrolysate samples, the pH was measured with a fine tip pH-electrode (InLab, micro, 3mm, Fisher Scientific, ON, Canada).

III.4.3.2. Conductivity

Conductivity of hemoglobin and KCl solutions was measured with an YSI conductivity meter (Model 3100, Yellow Springs Instrument, Yellow Springs, OH, USA) combined with an YSI 3252 electrode with a cell constant of 1 cm^{-1}.

III.4.3.3. Global resistance and energy consumption of EDBM

The global system resistance was calculated according to Ohm's law: R = U/I, where R is the global system resistance (Ω), I the current intensity (A) and U the voltage applied between the electrodes of the EDBM cell (V). The voltage and the current intensity were directly read on the power supply.

The energy consumption was calculated as follow:

$$E = \int U\, I\, dt/3600$$

where E is the energy consumption (Wh), U the voltage (V), I the current (A), and t the duration of the process (s).

III.4.3.4. Membrane thickness and electrical conductivity

Membrane thickness was measured with an electronic digital micrometer (Model Marathon Watch Company LTD., Richmond Hill, ON, Canada).
Membrane electrical conductance (G) was measured with a specially designed cell (Laboratoire des Matériaux Échangeurs d'Ions, Créteil, France) following the procedure described by Mikhaylin *et al.* (2016). The NaCl reference solution was 0.5 M. The membrane conductivity was calculated according to Lteif *et al*. (1999) and Lebrun *et al*. (2003):

$$R_m = R_{m+s} - R_s$$

where Rm is the electrical resistance of the membrane (in Ω), R_{m+s} the electrical resistance of the membrane and the reference solution measured together (in Ω), and R_s the electrical resistance of the reference solution (in Ω). The resistance R is equal to 1/G. The membrane conductivity (κ) was calculated as follow:

$$\kappa = L/R_m A$$

where L is the membrane thickness (in cm) and A is the electrode area (1 cm^2), these measurements were recorded before and after each replicate.

III.4.3.5. Mineral concentration measurement

The samples collected at 0 and 180 min were thawed at 4°C before their dilution 1/20 in Milli-Q water. Potassium and sodium concentrations were determined by optical emission spectrometry with inductively coupled plasma as atomisation and excitation source (ICP-OES Agilent 5110 SVDV Agilent Technologies, Victoria, Australia), using the following wavelengths (in nm): 766.491 (K), 588.995; 589.592 (Na). The analyses for both ions were carried out in axial and radial view.

III.4.3.6. Determination of the bovine hemoglobin degree of hydrolysis

The degree of hydrolysis (DH) is defined as the ratio between the number of peptide bonds cleaved by an enzyme and the number of total substrate bonds (protein), according to the following equation:

$$DH = 100 \times \frac{h}{h_0}$$

where DH is the degree of hydrolysis (%), h is the number of peptide bonds hydrolyzed on the substrate and h_0 is the number of total peptide bonds of the protein substrate.

DH was quantified by the ortho-phthaldialdehyde (OPA) technique, adapted from Church *et al*. (1985) and Spellman *et al*. (2003). In order to determine the number of hydrolyzed bonds h, the following equation was used:

$$h = \frac{\Delta Abs. M. DF}{\varepsilon. C_{HB}}$$

where ΔAbs is the difference between the absorbance at 340 nm of the hydrolyzed sample and the absorbance of the unhydrolyzed sample, *M* the molar mass of the protein (for bovine hemoglobin: 64500 g.mol^{-1}), DF the dilution factor of the sample, ε the molar extinction coefficient of OPA reagent at 340 nm (6000 mol^{-1}. cm^{-1} according to Church *et al.* (1985)) and C_{HB} the concentration of bovine hemoglobin.

The reagent was prepared at least one hour before use and stored in the dark. For a final volume adjusted to 50 mL, its composition was 25 mL of sodium tetraborate solution (0.1M) (Fisher Chemicals, Belgium), 5 mL of 10% (w/v) SDS (Fisher Scientific, New Jersey, United States), 10 µL of β-mercaptoethanol (Sigma-Aldrich, Oakville, ON, Canada) and 80 mg of OPA (Sigma-Aldrich, Oakville, ON, Canada) dissolved in 1 mL of methanol (Fisher Chemicals, Belgium). The linearity of the reagent was verified by assaying L-leucine (Sigma-Aldrich, Oakville, ON, Canada). 50 µL of sample to be tested were added to 1 mL of reagent. The absorbance was read at 340 nm after incubation for 2 min at room temperature.

III.4.3.7. RP-UPLC analyses

All samples were analyzed by UPLC-QTOF to identify the hydrolysis mechanism and the peptide sequences. A 1290 Infinity II UPLC (Agilent Technologies, Santa Clara, CA, USA) system was used for the RP-UPLC analyses. The equipment consisted of a binary pump (G7120A), a multisampler (G7167B), an in-line degasser and a variable wavelength detector (VWD G7114B) adjusted to 214 nm. A Poroshell 120 EC-C18 column (2.1 × 100 mm i.d., 2.7 micron, Agilent, Santa Clara, USA) was chosen for sample injections (0.25 µL). Samples were diluted 1:2 before being filtered (0.22 µm PVDF filter) into a glass vial. The mobile phases were LC-MS grade water with 0.1% formic acid as eluent A and LC-MS grade ACN with 0.1% formic acid as eluent B. The flow rate was 400µL/min at 45°C. A linear gradient with solvent B increasing from 1% to 65% in 25 min, ramping to 95% and holding until 30 min was applied. Each experiment was done in triplicate and a sample of each was injected on the UPLC system for statistical evaluation of technical reproducibility.

III.4.3.8. Mass spectrometry analyses

A hybrid ion mobility quadrupole time of flight mass spectrometer (6560 high definition mass spectrometry (IM-Q-TOF), Agilent, Santa Clara, USA) was used to identify and quantify the relative abundances of the peptides present in the hydrolysates. All LC-MS/MS experiments were acquired using Q-TOF. Signals were recorded in positive mode at Extended Dynamic Range, 2Ghz, 3200m/z with a scan range between 100-3200m/z. Nitrogen was used as the drying gas (13.0 L/min and 150°C) and as nebulizer gas (30 psig). The capillary voltage was set at 3500 V, the nozzle voltage at 300 V and the fragmentor at 400 V. ESI-L low concentration tuning mix (Agilent Technologies, Santa Clara, CA, USA) was used to calibrate the instrument. Data acquisition and analysis were performed using the Agilent Mass Hunter Software package (LC/MS Data Acquisition, Version B.08.00 and Qualitative Analysis for IM-MS, Version B.07.00 Service Pack 2 with BioConfirm Software). UPLC-MS/MS combination was used to analyze the peptides by comparing them with the known hemoglobin alpha and beta sequences with the BioConfirm software as recently described by Abou-Diab *et al.* (2020a). Then bioactive peptides were identified by comparing them to the literature (Nedjar *et al*., 2008; Dalgalarrondo *et al*., 1995; Zhao *et al*., 1995; Vercaigne-Marko *et al*., 2000; Takagi *et al*., 1982; Chang *et al*., 1980; Piot *et al*., 1992).

III.4.3.9. Statistical analyses

Data were subjected to one-way analyses of variance (ANOVA). Tukey tests were also performed on data using SigmaPlot software (Version 14.0) to determine which treatment was statistically different from the others at a probability level p of 0.05.

III.5. Results and Discussion

III.5.1. Process parameters

III.5.1.1. During conventional hydrolysis process

pH

During acidification process, in control, hemoglobin solution was denatured by addition of acetic acid/sodium acetate buffer (0.5M). After adding the buffer, the pH decreased from 6.71 ± 0.24 to 4.65 ± 0.19. To reduce the pH to 3.5, HCl was added. In comparison, in control-0.9 mS/cm and control-4.5 mS/cm, only HCl solution was added to reduce the pH to 3.5. *During hydrolysis process*, after the addition of pepsin, for the three control conditions, a small increase in pH was noticed, due to chemical reactions between the enzyme and the substrate releasing molecules of OH^-. The pH was then adjusted and maintained at 3.5 for 3 hours by addition of HCl solution.

Conductivity

Statistical analysis demonstrated that the conductivity of hemoglobin solution significantly changed between T_0 and T_{180} in control, control-0.9 mS/cm and control-4.5 mS/cm ($P < 0.001$) and this conductivity change was different according to the 3 conditions of controls at T0 and at T180 ($P < 0.001$) (**Table III-1**)) : conductivity increased by 1.58, 0.88 and 1.41 mS/cm from T_0 to T_{180} for control, control-0.9 mS/cm and control-4.5 mS/cm respectively. This difference in initial conductivity values was due to the addition of buffer to hemoglobin solution in the control and the addition of KCl powder for adjusting the conductivity at a different level between 0.9 and 4.5 mS/cm. Further change in conductivity was due to the addition of HCl solution to maintain the pH at 3.5 in the three controls during the hydrolysis phase. Higher HCl solution was added in control to adjust the pH due to the presence of buffer.

Tableau III-1. Conductivity value of hemoglobin solution in control, control-0.9 mS/cm and control-4.5 mS/cm at T_0 (before adding the pepsin) and T_{180} (after 3 hours of hemoglobin hydrolysis).

Conditions	T_0	T_{180}
Control	$4.00 \pm 0.06^{aA*}$	5.58 ± 0.14^{bA}
Control-0.9 mS/cm	1.39 ± 0.10^{aB}	2.27 ± 0.10^{bB}
Control-4.5 mS/cm	5.06 ± 0.05^{aC}	6.47 ± 0.12^{bC}

*a-b : Values with different lowercase letter within the same condition are significantly different between T_0 and T_{180} ($P < 0.001$, Tukey).

A-C : values with different capital letter within the same time are significantly different between the conditions ($P < 0.001$, Tukey).

During hydrolysis by EDBM

pH and conductivity evolution in bovine Hemoglobin compartment

In EDBM-0.9 mS/cm and EDBM-4.5 mS/cm, as expected, the electro-acidification processes allowed to decrease the pH of the hemoglobin solution, from 6.6-6.8 to 3.5 (**Figure III-2-a** and **c**) by generation of H^+ ions from water dissociation at the bipolar membrane cationic interface. The averaged times required to electro-acidify the 1% HB solution were 135 ± 5 min and 15 ± 1 min in EDBM-0.9 mS/cm and EDBM-4.5 mS/cm respectively. A two-step pH variation was observed for EDBM-0.9 mS/cm: a linear decrease from 0 to 60 min and then a slow decrease afterwards until 135 min (**Figure III-2-a**) while in EDBM-4.5 mS/cm a rapid linear decrease in pH was observed from 0 to 15 min (**Figure III-2-b**). A shorter acidification time was observed for EDBM-4.5 mS/cm than EDBM-0.9 mS/cm since more protons were available to react with hemoglobin in solution. Indeed, after a certain duration of treatment in EDBM-0.9 mS/cm, a leakage of protons appeared through the cation-exchange membrane to compensate for the non-sufficient concentration of mobile ions such as K^+ (as can be observed via the low conductivity of the solution, around 0.75 mS/cm). Those protons, migrating to compensate for this lack of mobile ions, did not participate anymore to the decrease in pH and then delay reaching pH 3.5. Such a decrease in electrical efficiency linked to a loss of electrogenerated H^+ through the cationic membrane, due to a lack of sufficiently mobile potassium ions, was previously observed by Bazinet *et al*. (2000), for a two-compartment EDBM process during electroacidification of milk, and Lin Teng Shee *et al*. (2006), for a two-compartment EDBM process during chitosan solubilization by acidification. After the addition of pepsin (*hydrolysis phase)* in EDBM, a small increase of the pH was also observed but was adjusted and controlled at 3.5 during the 3 hours by applying the electric current: for EDBM-0.9 mS/cm: the power remained on for 3 hours since

it was difficult to readjust and maintain the pH at 3.5. For EDBM-4.5 mS/cm, the current was applied only 10 seconds every 30 min to maintain the targeted pH value. The difficulty of maintaining the pH at 3.5 in EDBM-0.9 mS/cm could be explained by the lack of H^+ ions in the hemoglobin compartment making difficult the protonation of HB or by possible fouling on MCP or BM.

During electro-acidification and hydrolysis phase, conductivity was maintained as much as possible and feasible at 0.9 mS/cm and 4.5 mS/cm respectively in EDBM-0.9 mS/cm (**Figure III-2-a**) and EDBM-4.5 mS/cm (**Figure III-2-c**) by addition of KCl. It was more difficult to maintain the conductivity at a low conductivity of 0.9 mS/cm than 4.5 mS/cm. Indeed, when the conductivity was low, the migration of K^+ was very quick in such a cell configuration as demonstrated previously by Bazinet *et al*. (2000) and Lin Teng Shee *et al*. (2006) during milk and chitosan electro-acidification.

pH and conductivity evolution in basified compartment

In KCl solution, where pH was not controlled, the statistical analyses demonstrated significant changes in pH between the beginning and the end of acidification in EDBM-0.9 mS/cm and EDBM-4.5 mS/cm ($P < 0.001$): it increased from 5.37 ± 0.18 to 10.74 ± 0.08 (after 135 min) in EDBM-0.9 mS/cm and from 5.49 ± 0.37 to 11.09 ± 0.08 (after 15 min) in EDBM-4.5 mS/cm. There was also a significant difference between pH values obtained at the end of acidification phase between EDBM-0.9 mS/cm and EDBM-4.5 mS/cm ($p<0.05$). During the hydrolysis phase the pH was stable ($P = 0.238$ and $P = 0.097$ respectively for EDBM-0.9 mS/cm and EDBM-4.5 mS/cm): after 3 hours of hydrolysis, it varied of 0.06 and 0.10 unit respectively. In KCl solution, the pH was increase due to the OH^- electrogenerated at the anionic interface of the bipolar membrane. In the basified compartment, the pH increase was quicker and the range of pH change was higher since the KCl solution presented a very low buffer capacity in comparison with the hemoglobin solution, by the presence of protein able to fix the protons generated (Cheftel *et al*., 1985; Altschul and Wilcke, 1985; Prakash and Narasinga, 1990). Indeed, the same number of OH^- and H^+ were generated by the bipolar membrane.

In KCl solution, the conductivity, also not controlled, significantly changed during acidification ($p<0.05$) and hydrolysis ($P < 0.001$) phases and in a different range: it increased of 1.11 and 0.54 mS/cm during acidification and of 0.45 and 1.01 mS/cm during hydrolysis respectively for EDBM-0.9 mS/cm and EDBM-4.5 mS/cm respectively (**Figure III2-b** and **III-2-d**). During acidification phase, higher conductivity variation was observed for EDBM-0.9 mS/cm than for EDBM-4.5 mS/cm, this was due to the longer acidification time (135 min) in EDBM-0.9 mS/cm and therefore, K^+ ions migrated and H^+ leaked for a longer period, from the hemoglobin solution compartment to the KCl solution compartment. This difference can also be explained by the accumulation of OH^- ions in KCl compartment and the depletion of Cl^- ions from KCl compartment to electrolyte compartment for a longer duration in EDBM-0.9 mS/cm. Indeed, OH^- ions are more conductive than Cl^- ions: 198.3 and 76.4 $S.cm^2.mol^{-1}$ respectively (Selley, 1977). During the hydrolysis phase, a higher conductivity variation was observed for EDBM-4.5 mS/cm: more K^+ migrated during EDBM-4.5 mS/cm from hemoglobin compartment to KCl compartment due to the large amount of K^+ in addition to the generation of OH^- ions by bipolar membranes and their accumulation in the basified compartment. Lower conductivity variation in EDBM-0.9 mS/cm can be explained by the fact that the power remained on for 3 hours and for only 1 min in EDBM-4.5 mS/cm; therefore, more H^+ ions were generated by bipolar membranes in EDBM-0.9 mS/cm. Then a migration of protons appeared through the cation-exchange membrane from hemoglobin solution compartment to KCl compartment to compensate for the non-sufficient concentration of K^+. Those protons migrating, neutralized OH^- electrogenerated ions and produced water, resulting in a lower conductivity variation in the basified compartment for EDBM-0.9 mS/cm.

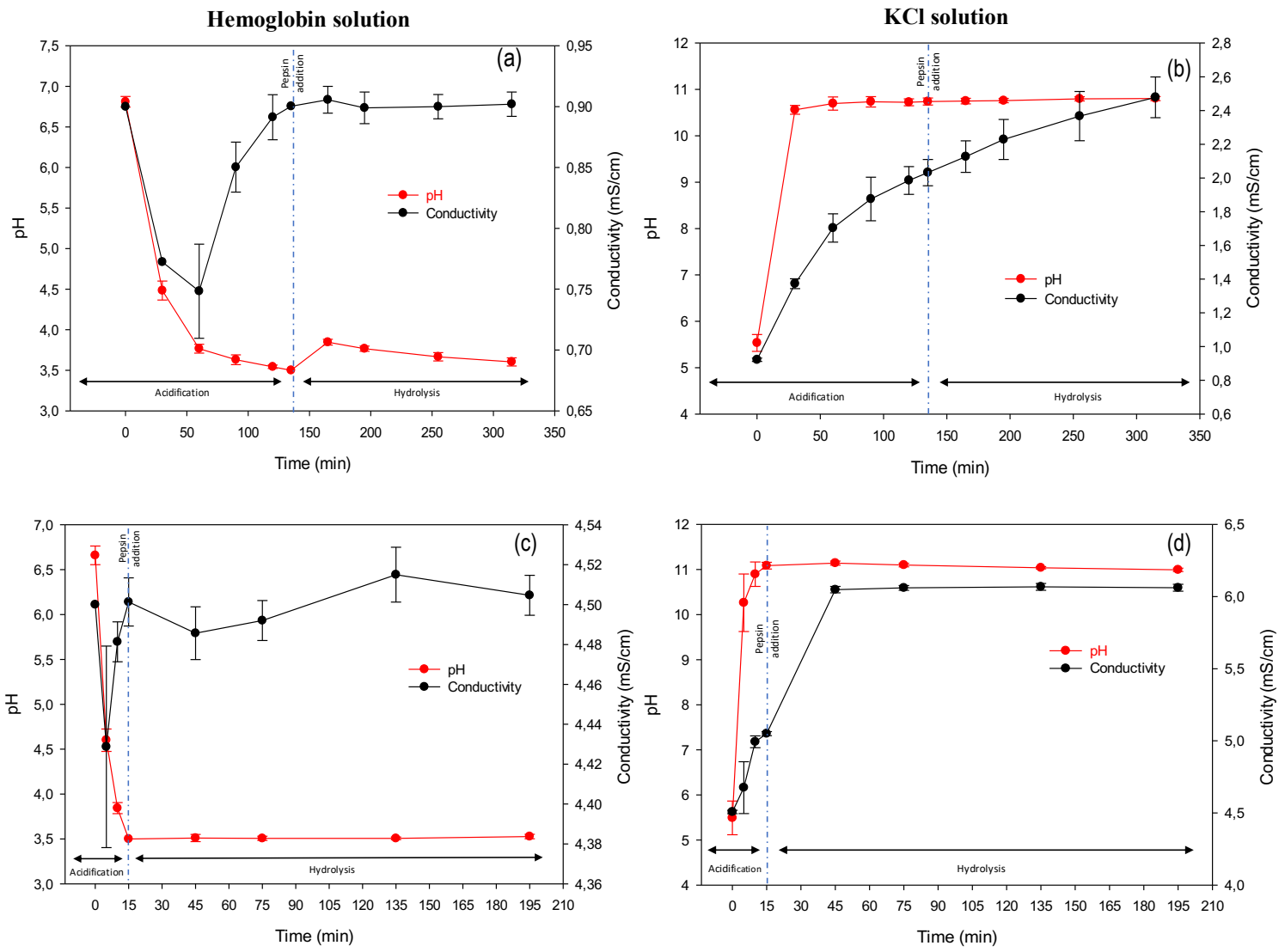


Figure III-2. Evolution of pH and conductivity in hemoglobin (a;c) and KCl (b;d) solutions during electro-acidification and hydrolysis in EDBM-0.9 mS/cm (a;b) and EDBM-4.5 mS/cm (c;d) before and after adding the pepsin.

Global resistance

The evolution of EDBM cell global resistance was significantly different between EDBM-0.9 mS/cm and EDBM-4.5 mS/cm during acidification ($P < 0.001$) as well as hydrolysis phase ($P < 0.001$). During the acidification phase, the global resistance increased by 6 folds and 1.5-fold for EDBM-0.9 mS/cm and EDBM-4.5 mS/cm respectively (**Figure III-3-a** and **III-3-b**). However, a two-step increase appeared in the global resistance for EDBM-0.9 mS/cm, but this trend was less marked for EDBM-4.5 mS/cm. The overall resistance

increased gradually and linearly during the first 60 minutes and then continues to increase more quickly and linearly until the end of acidification in EDBM-0.9 mS/cm, while in EDBM-4.5 mS/cm, the overall resistance increased very rapidly and linearly.

During the hydrolysis phase, after adding the pepsin, the overall resistance decreased and then increased gradually and linearly. The same trend was observed for the two treatments, but it was less pronounced for EDBM-4.5 mS/cm; these variations were due to the pH variation after pepsin addition releasing new ions that affected the global resistance. A higher global resistance variation was observed for EDBM-0.9 mS/cm than EDBM-4.5 mS/cm during hydrolysis due to the higher pH variation noticed and explained previously (section 3.1.2.) for EDBM-0.9 mS/cm.

The shorter time needed to reach pH 3.5 (15 min vs. 135 min) and the overall low resistance in EDBM-4.5 mS/cm can be explained by the abundant presence of minerals while for EDBM-0.9 mS/cm, the longest duration needed to reach the targeted pH and the high global resistance can be due to mineral depletion as described by Bazinet *et al.* (2000) and Jaime-Ferrer *et al.* (2009). However, such a high increase in global resistance could not be fully explained by just a mineral depletion, and it is possible that the integrity of the membranes was changed or a fouling was present. In order to confirm or not the possible change in membrane integrity or potential fouling, the membranes were characterized after EDBM.

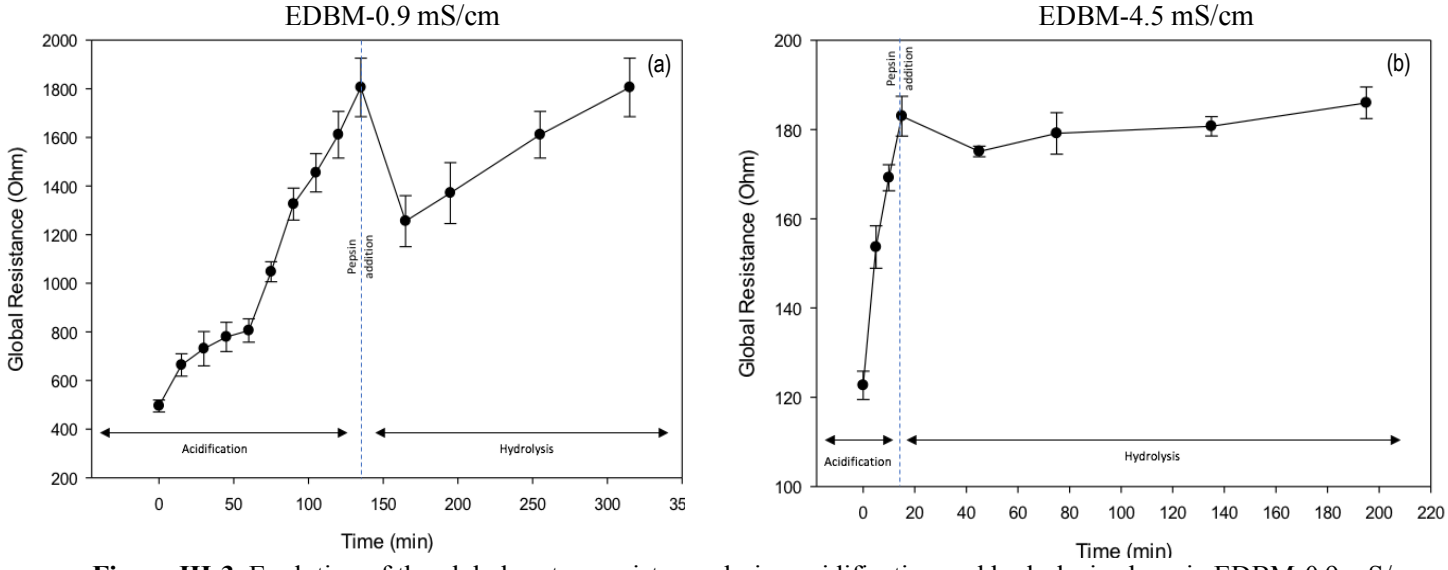


Figure III-3. Evolution of the global system resistance during acidification and hydrolysis phase in EDBM-0.9 mS/cm (a) and EDBM-4.5 mS/cm (b).

Membrane characterization

Membrane Photographs

To evaluate whether there is formation of fouling on membranes, after each ED run, photographs of each membrane were taken (**Figure III-4a; b**). **Figure III-4a** showed that, in the EDBM-0.9 mS/cm condition, the monovalent cationic perm-selective membranes (MCPs) exhibited a major fouling, while anionic and bipolar membranes did not present any fouling. This confirmed the previous hypothesis concerning the integrity change of the MCP due to a fouling formation. In comparison, **Figure III-4b** showed that, in EDBM-4.5 mS/cm, MCPs, anionic and bipolar membranes exhibited no significant visual fouling.

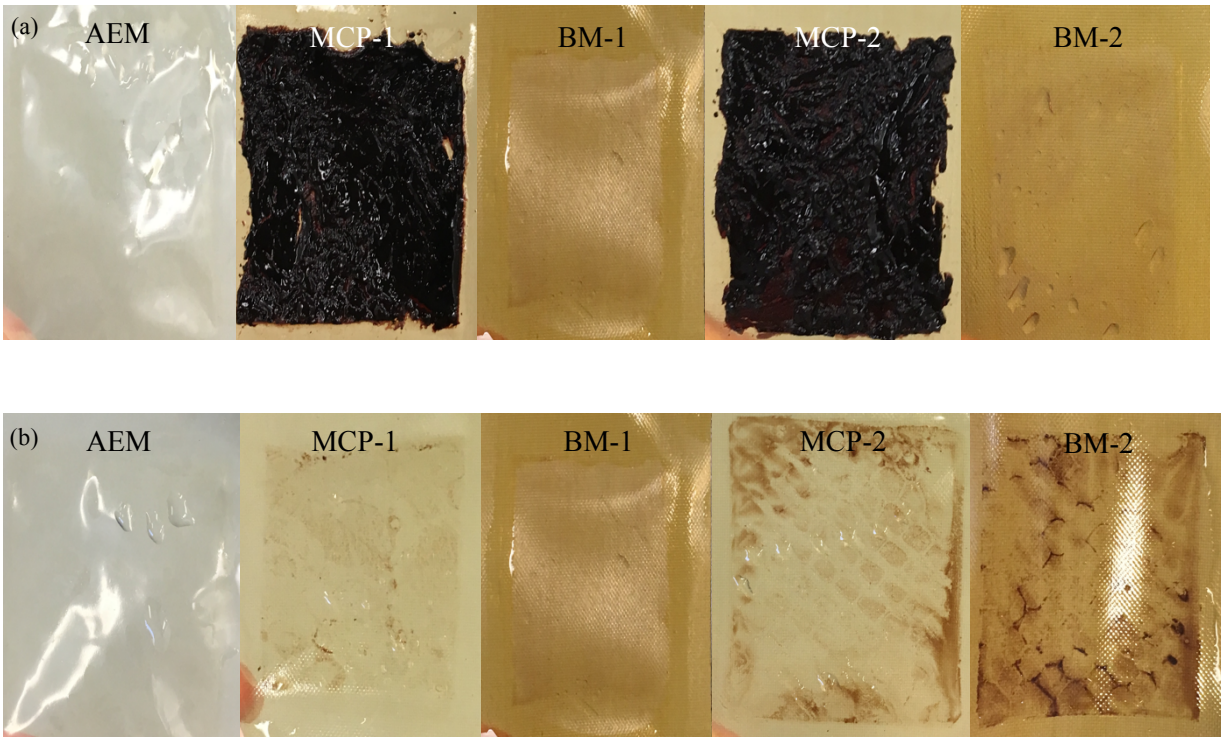


Figure III-4. Hemoglobin fouling on EDBM-0.9 mS/cm (a) and EDBM-4.5 mS/cm (b) membranes at the end of the treatment.

Membrane Thickness and Conductivity

Membranes were also characterized by measuring their thickness (mm) and conductivity (mS/cm) before and after each treatment. Regarding membrane thickness, the AEMs, BM-1 and BM-2 remained similar before and after EDBM treatment for both conditions ($p>0.05$). The MCPs remained similar before and after EDBM-4.5 mS/cm while a statistical difference was observed ($p<0.05$) for MCPs before and after EDBM-0.9 mS/cm treatment. Regarding membrane conductivity, statistical analysis demonstrated that electrical conductivity of AEM, BM-1 and BM-2 did not significantly ($p > 0.05$) change before and after the process for EDBM-0.9 mS/cm and EDBM-4.5 mS/cm (Table III-2). However, the conductivity of MCPs decreased by 80 % ($p < 0.05$) and 62 % ($p < 0.05$) for EDBM-0.9 mS/cm and EDBM-4.5 mS/cm, respectively (**Tableau III-2**). These data also confirmed the integrity changes of MCP membranes. However, the variation in conductivity of MCPs before and after the treatment did not affect the course of treatment in EDBM-4.5 mS/cm while in EDBM-0.9 mS/cm it did. In addition, the highest difference in electrical conductivity and the fouling observed for MCPs in EDBM-0.9 mS/cm also explained the difficulty of reducing the pH and consequently the longer acidification duration.

Tableau III-2. Conductivity (mS/cm) of MCP (monovalent cation permselective membrane), AEM (anion exchange membrane) and BM (bipolar membrane) before and after treatment in EDBM-0.9 mS/cm and EDBM-4.5 mS/cm.

Membrane type	EDBM-0.9 mS/cm		EDBM-4.5 mS/cm	
	Before	After	Before	After
AEM	$5.09 \pm 0.47^{a*}$	5.07 ± 0.35^{a}	4.76 ± 0.19^{a}	4.9 ± 0.42^{a}
MCP-1	4.06 ± 0.31^{a}	0.73 ± 0.073^{b}	4 ± 0.12^{a}	1.86 ± 0.41^{b}
MCP-2	4.13 ± 0.35^{a}	0.9 ± 0.09^{b}	4.26 ± 0.1^{a}	1.23 ± 0.2^{b}
BM-1	5.93 ± 0.35^{a}	5.78 ± 0.45^{a}	5.84 ± 0.53^{a}	5.87 ± 0.32^{a}
BM-2	5.9 ± 0.38^{a}	5.87 ± 0.36^{a}	5.9 ± 0.6^{a}	5.89 ± 0.5^{a}

$*^{a-b}$: Values with different lowercase letter before and after the treatment for each type of membranes within the same condition, are significantly different ($P < 0.001$, Tukey).

As mentioned above, photographs and values of thickness and conductivity were taken at the end of the ED process after the hydrolysis phase. So, the fouling on the cationic perm-selective membranes (MCPs) in EDBM-0.9 mS/cm was probably due to electrostatic interactions between peptides (positively charged) and the MCP (negatively charged), as described, for conventional cation-exchange membrane by Persico et Bazinet (2018). However, the fact that the overall resistance increased in the electro-acidification phase would mean that the fouling formation started rapidly during the acidification phase with protein-hem interactions and continued during hydrolysis with peptide-hem interactions. At the beginning, before acidification phase, hemoglobin was still a native protein composed of two hem-containing α- and β-globin dimers (αβ) arranged in a tetrahedral dimer fashion (Eaton *et al*., 1999; Perutz, 1970) with a high global molecular weight of 64,500 Da and gradually dissociated throughout the acidification phase by reducing the pH (Boys et al., 2007). As can be seen, the presence of the thin layer oppositely charged on the surface of the MCP, used to modify the possible electrostatic interactions between protein and/or peptides and the membrane surface did not completely avoid or limit fouling in EDBM-0.9 mS/cm. Indeed, some protein and/or peptides-hem were still able to interact with the matrix underneath as described by Abou-Diab *et al*. (2020a). The greater fouling observed for EDBM-0.9 mS/cm was due to the lower conductivity value of the solution and the lack of mobile ions such as K^+. Those mobiles did not interact anymore with the membrane interface by electrostatic links and then allowed the protein and/or peptides (positively charged) to take their place. In comparison, due to the higher conductivity in EDBM-4.5 mS/cm, more K^+ were therefore available to interact electrostatically with the membrane interface preventing the protein and/or peptide-membrane bonds by decreasing the electrostatic interaction. This concept was studied by Langevin et Bazinet, (2011) who described for CEM membranes soaked in NaCl solution, the replacement of peptides adsorbed electrostatically at the membrane interface by Na^+ ions and by Persico *et al*. (2017) who soaked ion-exchange membranes in 2.0% (w/w) NaCl solution of 30 mL for peptide desorption by increasing the conductivity. In addition, EDBM-4.5 mS/cm, having a higher ionic strength, was more effective to desorb high molecular weight chains from the membrane interface than EDBM-0.9 mS/cm. This result would confirm those reported by Langevin et Bazinet, (2011), who observed that a higher NaCl concentration was more efficient to desorb high molecular

weight peptides because they are susceptible to present more charges on their surface. It is important to mention, that in addition to the effect of salt on protein/peptide-hem fouling, the slow decrease in pH due to low conductivity in EDBM-0.9 mS/cm may have caused more fouling because the hemoglobin solution remained in contact with the membrane for a longer time than during EDBM-4.5 mS/cm.

On the other hand, difference in electrical conductivity noticed for MCP before and after the treatment in EDBM-4.5 mS/cm may be due to monovalent counter-ion replacement inside the membrane by divalent ions migrating through the membrane. Such a replacement would decrease the conductivity of the membrane without necessarily being a fouling, as previously reported by Dufton *et al.* (2018) for CEM: those authors did not observe any scaling or fouling on both side of the membranes during acid whey deacidification however a decrease in conductivity after the treatment was noticed due to a mineral replacement by less conductive divalent ions in the membranes.

Energy consumption

The analysis of variance showed a significant difference ($P < 0.001$) between the energy consumed during the acidification and hydrolysis phases of the different conditions. During the acidification phase, the energy consumption of EDBM-0.9 mS/cm and EDBM-4.5 mS/cm were 0.548 ± 0.024 Wh and 0.358 ± 0.0079 Wh, while during the hydrolysis phase these energy consumption values were 0.451 ± 0.029 Wh and 0.022 ± 0.0005Wh, respectively (**Figure III-5**). Considering both phases of the process, EDBM-0.9 mS/cm globally consumed 3 times more energy than EDBM-4.5 mS/cm, this can be explained by the fact that the current remained on for a longer duration during the treatment in EDBM-0.9 mS/cm to generate H^+ ions necessary to maintain the pH at a constant value due to the lack of protons and the loss of efficiency related with the fouling issues.

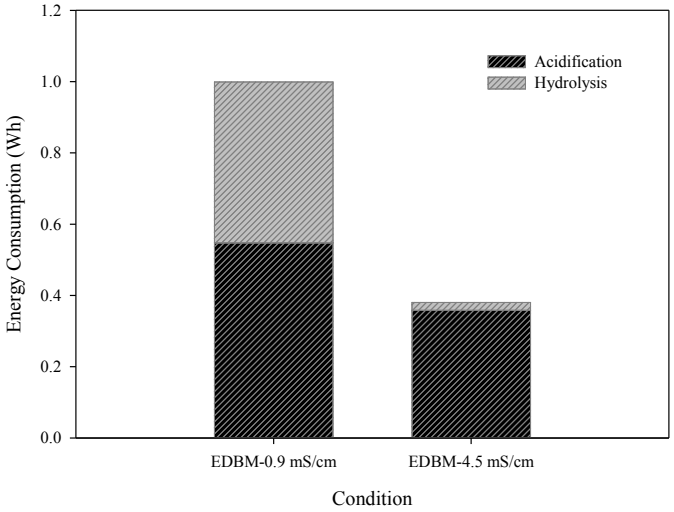


Figure III-5. Evolution of the energy consumption during acidification and hydrolysis phase in EDBM-0.9 mS/cm and EDBM-4.5 mS/cm.

III.5.2. Mineral composition

The determination of the potassium and sodium composition in the hemoglobin solution during the process under the five different conditions was important to test the feasibility of the ED configuration to maintain or decrease the salt concentration in comparison with conventional hydrolysis. The concentrations of these minerals (mg/L) are shown in **Figure III-6** and **III-7**.

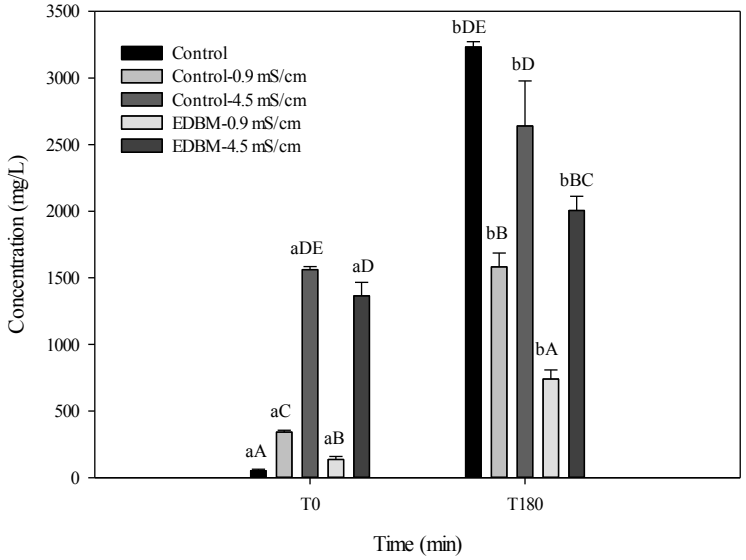


Figure III-6. Potassium concentrations (mg/L) in hemoglobin solution at the beginning (T_0: before adding the pepsin) and the end (T_{180}: after 3 hours of hydrolysis and after stopping the reaction of hydrolysis with KOH (0.5M) in the different conditions tested. Values with different lowercase letter (a-b) within the same condition are significantly different between T_0 and T_{180}; values with different capital letter (A-D) within the same time of hydrolysis (T_0 or T_{180}) are significantly different between conditions ($P < 0.001$, Tukey test).

The potassium concentration of the hemoglobin solution significantly changed between T_0 and T_{180} ($P < 0.001$) and was different according to the condition tested (**Figure III-6**). Hence, the concentration of potassium according to the conditions tested increased in the following order: EDBM-0.9 mS/cm (+605 mg/mL) < EDBM-4.5 mS/cm (+641 mg/ml) < Control-4.5 mS/cm (+1080 mg/mL) < Control-0.9 mS/cm (+1241mg/mL) < Control (+3180 mg/mL). Higher potassium concentrations were observed at T_{180} than at T_0: this was normal because KOH solution was added at T_{180} to stop the hydrolysis reaction. A higher K^+ concentration was observed at T_{180} in Control since it was necessary to add a larger amount of KOH to stop the reaction in the presence of the buffer.

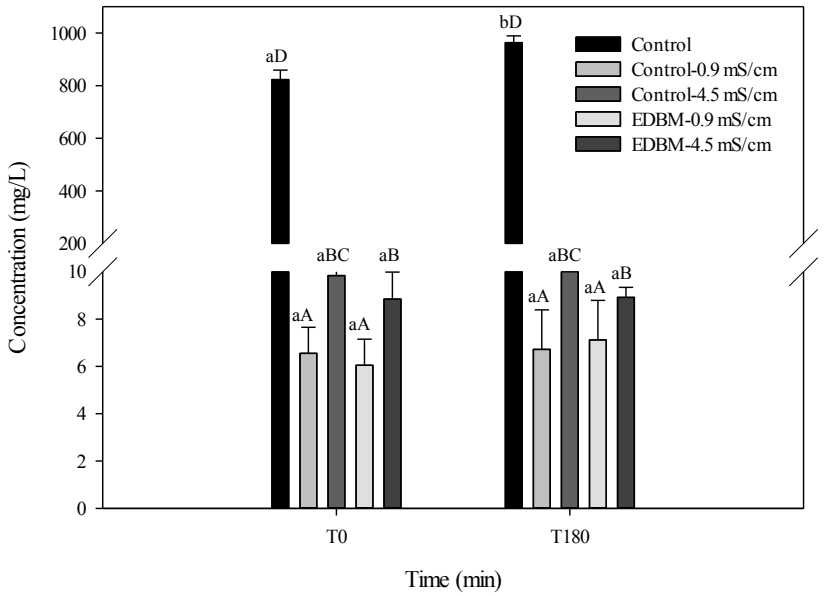


Figure III-7. Sodium concentrations (mg/L) in hemoglobin solution at the beginning (T_0: before adding the pepsin) and the end (T_{180}: after 3 hours) of hydrolysis in the different conditions tested. Values with different lowercase letter (a-b) within the same condition are significantly different between T_0 and T_{180}; values with different capital letter (A-D) within the same time of hydrolysis (T_0 or T_{180} are significantly different between conditions ($P < 0.001$, Tukey test).

The sodium concentration of hemoglobin solution significantly changed between T_0 and T_{180} ($p < 0.05$) in control and in EDBM-0.9 mS/cm (**Figure III-7**). Hence, the concentration of sodium according to these two conditions tested increased in the following order: EDBM-0.9 mS/cm (+1.07 mg/mL) < Control (+140.55 mg/mL). In the contrary, sodium concentration values in control-0.9 mS/cm, control-4.5 mS/cm and EDBM-4.5 mS/cm were very similar, the difference of concentration between T_0 and T_{180} was negligible (0.17, 0.27 and 0.07 mg/mL respectively). Moreover, the sodium concentration (mg/L) of hemoglobin solution significantly changed ($P < 0.001$) between control and all the conditions at T_0 and T_{180}. The higher Na^+ concentration observed in control was due to the addition of acetic acid/sodium acetate buffer. For all the other conditions, the low concentration in sodium was probably due to its presence in the hemoglobin powder at the origin since all along the treatment there was no addition of sodium supplement or to a possible minor leakage from the electrode rinsing solution (Na_2SO_4) through the extremity membranes in EDBM conditions.

III.5.3. Comparative hydrolysis kinetics and hydrolysate characterization

III.5.3.1. Chromatogram and enzymatic mechanism involved

This part was dedicated to understand the different enzymatic kinetics and to identify the enzymatic mechanism of bovine hemoglobin hydrolysis obtained in EDBM and control by UPLC-QTOF. Chromatographic profiles in the different hydrolysis conditions as a function of time are shown in **Figure III-8**. For all the mass spectra at T_0, before adding the pepsin, three peaks are shown: α-chain, β-chain and heme. After 30 min, in each of the conditions, the α-chain and β-chain persisted meaning that the hemoglobin was not completely denatured. The α- and β-chains disappeared at T_{60} min in control, control-0.9 mS/cm, control-4.5 mS/cm and EDBM-4.5 mS/cm, while they persisted in EDBM-0.9 mS/cm after 120 min. The enzymatic kinetics were slightly different between the conditions, since the profiles are not exactly the same. However, the same enzymatic mechanism called «one-by-one», described for the first time by Linderstrom-Lang, (1953), was observed in all conditions but a more pronounced one-by-one mechanism was noticed in EDBM-0.9 mS/cm than the others. This mechanism allowed molar digestion per mole of the substrate, one mole of enzyme degrades one mole of substrate at a time. As a result, throughout proteolysis, the peptide concentrations increased in a proportional and limiting manner over time and peptide population did not change, they were all present in a qualitatively identical manner. The same mechanism was observed in other studies for conventional hydrolysis and described by Dubois *et al.* (2005) who studied the kinetics of native hemoglobin hydrolysis and showed that it followed the one-by-one mechanism. The appearance of the same fractions can be observed over the course of peptic hydrolysis, but their concentrations change. No appreciable amounts of intermediate products were present: the reaction mixture was only composed of native protein and final products.

The one-by-one mechanism appeared in all conditions because the hemoglobin was not completely denatured even in presence of a buffer. This may be due to the value of pH of 3.5 which was probably not sufficiently low to change the native globular form of hemoglobin into a more exposed linear form for enzymatic cleavage. However, Dubois *et al.* (2005),

studied the effect of pH on the denaturation of hemoglobin and observed a zipper mechanism at pH 3.5 in presence or absence of urea denaturant and a one-by-one mechanism at higher pH (4.5 and 5.5). The more pronounced one-by-one mechanism observed in EDBM-0.9 mS/cm may be due to fouling on MCP caused by the low conductivity slowing down the denaturation of the protein attached to the membrane by electrostatic interactions making difficult its protonation and/or to the difficulty to adjust the pH to 3.5 during the hydrolysis phase affecting consequently the efficiency of pepsin. Furthermore, at the same pH of 3.5, the slightly different enzymatic kinetic profiles observed may be due to another factor affecting the pepsin activity and/or hemoglobin stability. This hypothesis is in line with a previous work reported by Adje *et al*., (2011a), who described that the stability of the native conformation, structures and characteristics of proteins depends on several variables such as temperature, pH, ionic strength and solvent composition. In order to confirm or not this hypothesis, the following section is dedicated to the determination of the degree of hydrolysis.

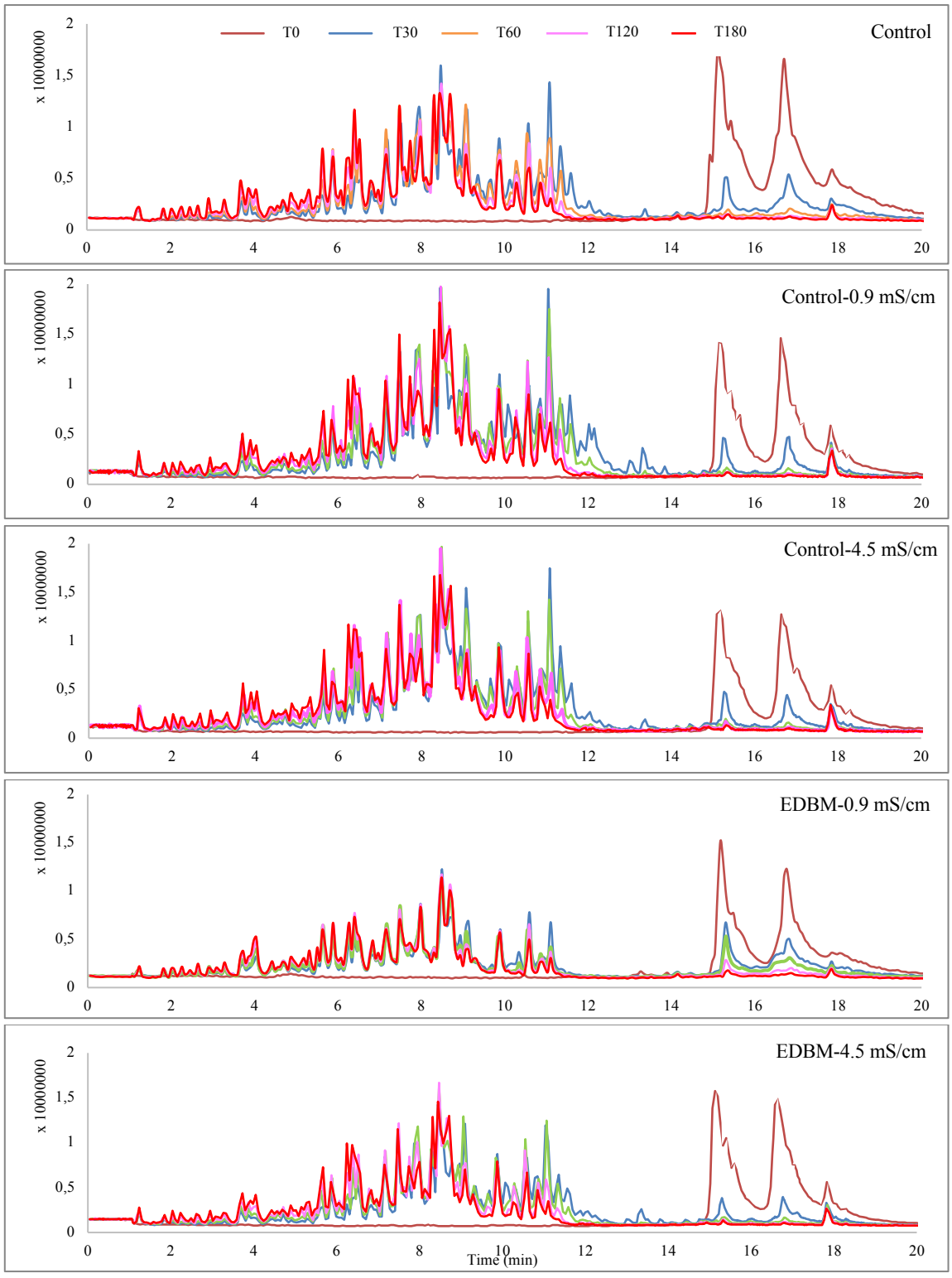


Figure III-8. Overlapping chromatographic profiles of hydrolysis of bovine hemoglobin in the five different conditions of hydrolysis as a function of time at 214 nm by UPLC-QTOF, analyzed by C18 column at different hydrolysis degrees for 3 hours (pH 3.5, 30°C, E/S = 1/11, C_{BH} = 1% w/v).

III.5.3.2. Determination of the degree of hydrolysis

The degree of hydrolysis increased over time whatever the condition tested but did not reach the same final values (**Figure III-9**). Hydrolysis of bovine hemoglobin was characterized by a rapid increase in the degree of hydrolysis during the first 30 min for all conditions, while at T_{60} min the rate of enzymatic hydrolysis increased slightly, and then it continued to increase quite linearly until 180 min. The highest DH was observed for Control-0.9 mS/cm and the lowest for Control. The degree of hydrolysis was statistically different between all conditions ($P < 0.05$): final DH of 4.57 ± 0.18 %, 5.76 ± 0.55 %, 5.53 ± 0.26 %, 4.95 ± 0.94 % and 5.35 ± 0.15 % for Control, control-0.9 mS/cm, control-4.5 mS/cm, EDBM-0.9 mS/cm and EDBM-4.5 mS/cm respectively after 3 hours of hydrolysis. These results confirmed the slightly different enzymatic kinetics involved observed previously by chromatographic profiles. A higher DH for control-0.9 mS/cm compared to control-4.5 mS/cm can be explained by the lower salt concentration in control-0.9 mS/cm. According to Beauchamp et Khajehpour, (2010), the nature and concentration of salt may impact the activity of the enzyme. These authors tested the effect of salt concentration ranging from 0 to 1M of different salt species such as KCl, NaCl, LiCl, $CaCl_2$, NH_4Cl and $MgCl_2$, on catalytic activity of ribonuclease t1 by characterizing the enzyme specificity constant K_{cat}/K_m, the Michaelis-Menten parameter that is sensitive to both enzyme affinity and catalytic ability. The results of our study using KCl salt and being in the same range of concentration (0 to 0.1M) are in line with this previous work. Beauchamp et Khajehpour demonstrated that salt had an effect on enzyme activity based on 4 possibilities: (1) salt effect on pKa values of active-site residues, (2) salt species weaken coulombic interactions between the substrate and enzyme (counterion effect), (3) metal cations bind directly to the enzyme, thereby inhibiting the enzyme, (4) salt species changing the hydrogen bonding properties of the water molecules of the protein solvent shell, thus indirectly stabilizing a less active conformation of the enzyme. In our study, based on possibility (1): two aspartic acid (Asp^{32} et Asp^{215}), as well as three isoleucine (Ile^{30}, Ile^{120} and Ile^{213}), one serine (Ser^{39}), two alanine (Ala^{124} and Ala^{301}) and two threonine (Thr^{218} and Thr^{305}) have been identified as the major active sites of porcine pepsin and constituted a location where the substrate is lodged (Davies, 1990). Increasing the salt concentration increase pK_a values of those amino acids (Beauchamp et Khajehpour, 2010).

Indeed, at pH 3.5, this increase in pK_a could change the protonation state, thus affecting the K_{cat}/K_m constant. Based on (2), electrostatic interactions are the major contributors to the pepsin-hemoglobin bonding (Raufmann, 2020) following the recognition of a structural pattern and whose nomenclature was established by Schechter et Berger, (1967). Salts could decrease the substrate-enzyme affinity that will strongly affect the K_{cat}/K_m ratio. Based on (3), it is very likely that the conformational change was mediated by cations (K^+ and Na^+) or anion (Cl^-) binding to a specific active site on the pepsin and thus leading to a less active compact form, as noticed in Control-4.5 mS/cm, EDBM-4.5 mS/cm and Control. Lower cations and anions amount in control-0.9 mS/cm caused lower binding and thus lower pepsin inhibition. Finally, based on (4), the protein hydration is important to improve the enzymatic activity. According to Zhao, (2005), and following the kosmotrope order of cations and anions in the Hofmeister series, it can be suggested that the presence of Cl^-, a chaotropic anions with a β-coefficient of -0.005 (negative β-coefficients for weakly hydrated ions) may destabilize the pepsin by perturbing the enzyme hydration layer and thereafter, decreasing the enzyme efficiency. It was important to mention that kosmotropic anions and chaotropic cations stabilize enzymes while chaotropic anions and kosmotropic cations destabilize them. Kosmotropes usually have positive β-coefficients since strongly hydrated ions exhibit a larger change in viscosity with concentration, while chaotropes have negative β-coefficients due to the weak hydration (Zhao, 2005). Hence, the higher Cl^- ion concentration, due to the higher amount of KCl in control-4.5 mS/cm and EDBM-4.5 mS/cm, affected the water hydrogen bonds of pepsin more than in control-0.9 mS/cm containing less ions; consequently, the degree of hydrolysis exhibited lower values under the conditions at 4.5 mS/cm comparing to control-0.9 mS/cm. On the other hand, the lowest DH observed in control in comparison with all conditions can be explained by the presence of Na^+ from sodium acetate buffer considered as kosmotropic cation having a strongest destabilizing effect than K^+ because of its strongly hydrated ions represented by a higher β-coefficient of 0.085 (positive β-coefficients for strongly hydrated ions) in addition to the higher amount of Cl^- ions. Indeed, more HCl solution was added in control than control-0.9 mS/cm and control-4.5 mS/cm to adjust and maintain the pH to 3.5 because of the presence of sodium acetate buffer and therefore more Cl^- was present in control affecting the pepsin and explaining the lower DH. However, the order of ions in stabilizing and destabilizing various enzymes may

be different depending on the specific application, the charge of the enzyme, the concentration of ions and the overall enzyme-medium-substrate relationship as described by Zhao, (2005). For comparison purpose, trypsin is a type of proteolytic enzyme secreted by the digestive system in order to digest proteins like pepsin. It was mentioned that neutral salts at high concentrations (0.3 to 3.0 M) inhibit the activity of trypsin in an order of increasing effectiveness of inhibition for anions: $CH_3COO^- < Cl^- < NO_3^- < Br^- < I^- < SCN^- < ClO_4^-$, with ClO_4^- having the higher inhibition effect and in an order of increasing effectiveness for cations: $(CH_3)_4N^+ < Cs^+ < K^+ < Na^+ < Li^+$, with Li^+ having the higher inhibition effect due to its larger β-coefficient comparing to other cations tested (Zhao, 2005). Moreover, despite the lower salt level in EDBM-0.9 mS/cm than EDBM-4.5 mS/cm, the DH in EDBM-0.9 mS/cm was lower, this was due to the fouling on MCP.

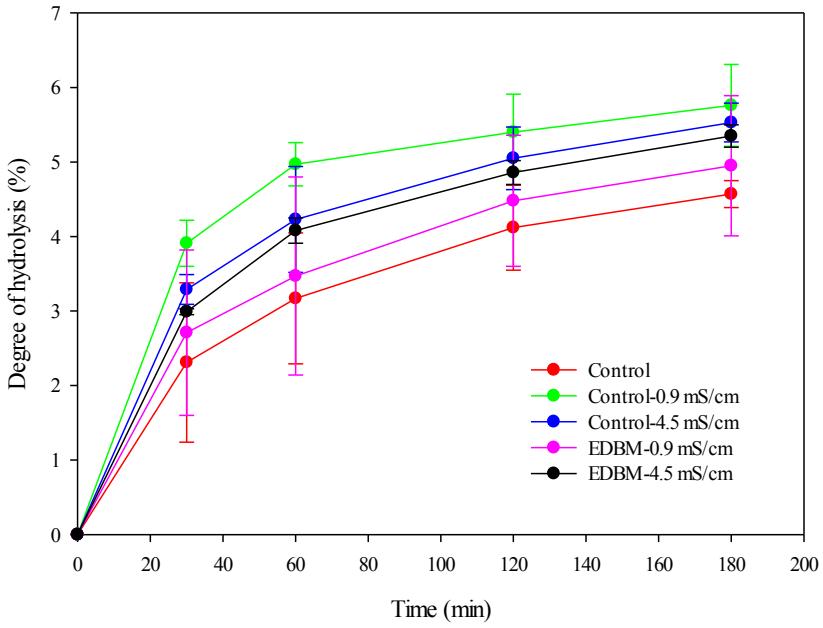


Figure III-9. Evolution of the degree of hemoglobin hydrolysis (%) as a function of time of hemoglobin hydrolysis in the five conditions tested.

III.5.3.3. Peptide population and identification of bioactive peptides

In our study, mass spectrometry identified peptide populations obtained with «One-by-one» mechanism in the different conditions. After data analysis, it appeared that 183, 202, 198, 190, and 193 peptides were generated by Control, Control-0.9 mS/cm, Control-4.5 mS/cm,

EDBM-0.9 mS/cm and EDBM-4.5 mS/cm, respectively. Venn diagram showed all possible population relations between the five conditions. As can be seen in **Figure III-10**, 113 peptides were in common between all conditions. It also showed differences between the different conditions. Higher peptide population was observed for Control-0.9 mS/cm and lower population was observed for Control, this confirmed the result obtained previously for the degree of hydrolysis. Different salt levels and species affected the activity of pepsin thus obtaining a weakly varied peptide population. Moreover, in this study the enzyme was solubilized in water for control-0.9 mS/cm, control-4.5 mS/cm, EDBM-0.9 mS/cm and EDBM-4.5 mS/cm while it was solubilized in sodium-acetate buffer for control, the use of an organic solvents, a non-aqueous medium, would have an effect on the selectivity of pepsin in the case of bovine hemoglobin hydrolysis (Dalgalarrondo *et al*., 1995; Welinder, 1988; Dalgalarrondo *et al*., 1993). By way of comparison with recent work studied by Abou-Diab *et al*., (2020a; 2020b) a larger peptide population was generated after enzymatic hydrolysis using bipolar membranes with zipper mechanism: 218, 233 and 216 peptides were generated in control, EDBM-MCP and EDBM-AEM respectively. The different peptide populations generated between these studies and the present one would be mainly due to the different enzymatic mechanisms, linked to the different pH. Indeed, zipper mechanism obtained by Abou-Diab *et al.* (2020a) at pH 3 generated more peptides than the one-by-one mechanism since with zipper mechanism the alpha and beta chains were completely denatured and hydrolyzed allowing the apparition of intermediate peptides that generated new peptides. However, according to the results observed in this study, this difference could also be due to the effect of salts on pepsin influencing its stability and thus impacting its selectivity. The salts may have impacted the hemoglobin structure, by rendering less accessible certain cleavage areas available for pepsin or inducing its partial precipitation.

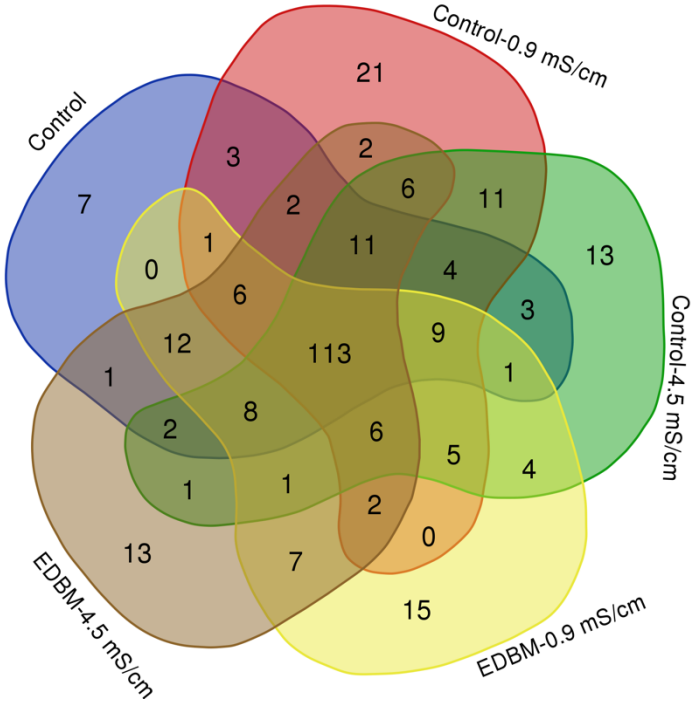


Figure III-10. Venn diagram showing the different peptide populations produced in the different conditions tested.

Bioactive peptides

The identification of the bioactive peptides obtained following the hydrolysis of bovine hemoglobin within different conditions was important. Bioactive peptide sequences identified by UPLC-MS/MS are presented in **Tableau III-3**.

Tableau III-3. Bioactive peptide sequences identified by UPLC-MS/MS resulting from hydrolysis of bovine hemoglobin for the five different hydrolysis processes tested.

Biological activity	Position	Sequence	Control	Control-0.9 mS/cm	Control-4.5 mS/cm	EDBM-0.9 mS/cm	EDBM-4.5 mS/cm
Antimicrobial	α1-29	VLSAADKGNVKAAWGKVGGHAAEYGAEAL	+	+	+	+	+
	α1-28	VLSAADKGNVKAAWGKVGGHAAEYGAEA	+	+	+	+	+
	α1-27	VLSAADKGNVKAAWGKVGGHAAEYGAE	+	+	+	+	+
	α1-23	VLSAADKGNVKAAWGKVGGHAAE	+	+	+	+	+
	α34-66	**LSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAAL**	-	+	-	-	-
	α34-65	**LSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAA**	-	+	-	-	-
	α33-45	**FLSFPTTKTYFPH**	-	-	-	+	+
	α34-46	LSFPTTKTYFPHF	+	+	+	+	+
	α37-46	PTTKTYFPHF	+	+	+	+	+
	α137-141	TSKYR	+	+	+	+	+
	β1-13	**MLTAEEKAAVTAF**	-	-	+	-	-
	β140-145	QADFQKVVAGVANALAHRYH	+	+	+	+	+
	β126-145	LAHRYH	+	+	+	+	+
	α99-106	KLLSHSLL	+	+	+	+	+
	α99-105	KLLSHSL	+	+	+	+	+
Opioid	α137-141	TSKYR	+	+	+	+	+
	β31-40	LVVYPWTQRF	+	+	+	+	+
	β32-41	**VVYPWTQRFF**	+	+	-	-	-
	β32-40	**VVYPWTQRF**	-	+	+	+	+
	β32-37	**VVYPWT**	-	+	+	+	-
Antihypertensive	α99-105	KLLSHSL	+	+	+	+	+
Hematopoietic	β2-12	**LTAEEKAAVTA**	-	+	+	-	-
	β2-11	**LTAEEKAAVT**	-	+	+	+	-
Potentiator of bradykinin	α110-125	ASHLPSDFTPAVHASL	+	+	+	+	+
	α129-134	LANVST	+	+	+	+	+
Dipeptidyl peptidase IV inhibitors	α130-134	**ANVST**	-	-	-	+	-
Antioxidant	α137-141	TSKYR	+	+	+	+	+

Among this large peptide population, numerous bioactive peptides were found in the five different conditions after 3h of hydrolysis. The peptides identified by UPLC-MS/MS and compared with the literature show several bioactivities: antimicrobial (Adje *et al.,* 2011a; Adje *et al*., 2011b; Nedjar *et al*., 2008; Gomes *et al*., 2010; Nedjar *et al*., 2008; Lignot *et al*., 1999; Nedjar *et al*., 2006; Dubois *et al*., 2005), hematopoietic Vercaigne-Marko *et al*., 2000, opioid (Lignot *et al*., 1999; Zhao and Piot, 1997), potentiator of bradykinin (Lignot *et al*., 1999; Piot *et al*., 1992), coronaroconstrictor (Barkhudaryan *et al*., 1992), antihypertensive (Adje *et al*., 2011b), dipeptidyl peptidase IV inhibitors (Caron *et al*., 2016) and antioxidant (Przybylski *et al*., 2016). The majority of the identified bioactive peptides were roughly the same under all conditions. The five hydrolysis processes produced 14 peptides in common; 11 antimicrobial peptide, 2 opioid peptides, 1 antihypertensive, 2 potentiator of bradykinin, and 1 antioxidant peptide. Higher number of bioactive peptides was identified in Control-0.9 mS/cm and lower number of bioactive peptides was identified in Control, this confirmed the results obtained previously (Sections 3.3.2 and 3.3.3). The α137-141 which has

antimicrobial, opioid and antioxidant activity appeared in all conditions. Despite the « one-by-one » mechanism, its production was fast and appeared after only a few minutes from the beginning of hydrolysis. It thus accumulated during hydrolysis, because it's a final peptide, not being hydrolyzed in other shorter peptides. The α34-66 and α34-65 appeared in Control-0.9 mS/cm and did not appear in the other conditions. The presence of the final peptide α37-46 in addition to the two intermediate peptides α34-66 and α34-65 derived from α2 family (α33- 98) in Control-0.9 mS/cm confirmed the higher DH obtained for Control-0.9 mS/cm. Furthermore, the exclusive presence of the three peptides β32-41, β32-40 and β32-37 derived from β2 family (β31-40) together in Control-0.9 mS/cm also confirmed the highest DH obtained by the presence of a greater number of peptides. The α33-45 appeared in EDBM-0.9 mS/cm and EDBM-4.5 mS/cm and did not appear in all other conditions and the β1-13 appeared in control-4.5 mS/cm and did not appear in all other conditions because of the impact of salt on pepsin selectivity favoring one peptide to another in the cleavage zone available on hemoglobin. The α130-134 derived from α3 family (α107-141) is a final peptide because of its low molecular weight, appeared in EDBM-0.9 mS/cm and did not appear in all other conditions: this could be due to the pronounced one-by-one mechanism observed in this specific condition which would have cut directly the alpha chain into the final peptides. However, the « one-by-one » mechanism makes it possible to produce many bioactive peptides but the peptide concentration was less significant at pH 3.5 since the alpha and beta chains are rapidly hydrolyzed by pepsin before being completely denatured. Moreover, by comparing the area under the curve of peptide identified in this study with « one-by-one » mechanism to the area under the curve of peptides identified by Abou-Diab *et al*., (2020a) with « zipper » mechanism using the same UPLC-MS/MS method and at the same concentration, it was noticed that the peptide area was 2 and 3 times greater with « zipper » mechanism than « one-by-one » mechanism. This result confirms previous study by Dubois et al. (2005), who showed that the increase in active peptide amount was related to hemoglobin denaturation (« zipper » mechanism).

III.6. Conclusion

This study demonstrated that the conductivity has major impacts on EDBM and bovine hemoglobin hydrolysis parameters. Indeed, conductivity affected the acidification duration, the global resistance, the energy consumption and the fouling on MCP membranes. Different salt levels impacted the hydrolysis degree, the enzymatic mechanism and the production of peptides due to their likely effect on the enzyme activity and hemoglobin structure. Therefore, EDBM-4.5 mS/cm can be considered as the best condition allowing rapid electro-acidification with a low mineral content compared to conventional hydrolysis, producing bioactive peptides without membrane fouling and involving minimal global resistance and consequently a lower energy consumption.

Furthermore, it was demonstrated that hydrolysis at pH 3.5 in EDBM has the same enzymatic mechanism as the conventional hydrolysis, the «one-by-one» mechanism. This mechanism produced a large peptide population in all conditions whatever the conductivity but with a low concentration of peptides due to incomplete denaturation of hemoglobin. Further experiments will focus on optimizing the process by 1) adjusting the pH to the level of acidity to completely denature the hemoglobin, thus, more accessible for the pepsin, 2) inducing demineralization in EDBM and 3) minimizing peptide fouling on the monovalent perm-selective membrane.

This new environmentally process of EDBM based on converting co-products from waste into other resources will be a promising technology to fight a real environmental and health problem.

III.7. Acknowledgements

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III. Atteinte des objectifs et avancement des connaissances

Cette étude a démontré la capacité de l'électrodialyse avec membranes bipolaires à dénaturer et maintenir le pH de la solution d'hémoglobine au cours de l'hydrolyse enzymatique grâce aux protons électro-générés par les membranes bipolaires suite à la dissociation des molécules d'eau sous l'effet d'un champ électrique.

Les résultats obtenus dans ce chapitre ont permis d'atteindre l'objectif visant à étudier l'effet du niveau de conductivité sur les paramètres d'EDMB et le colmatage des membranes. Ces résultats ont montré que la conductivité a des impacts majeurs non seulement sur l'EDMB mais sur les paramètres d'hydrolyse de l'hémoglobine bovine aussi. En effet, la conductivité a affecté la durée d'acidification, la résistance globale, la consommation d'énergie et le colmatage des membranes MCP. À un niveau de conductivité de 4,5 mS/cm, un plus court temps d'acidification et une plus faible résistance globale ont été observés en comparaison avec le niveau de conductivité de 0,9 mS/cm. De plus, à 4,5 mS/cm, les membranes n'ont présenté aucun colmatage visuel. Enfin, les résultats obtenus dans cette partie de l'étude ont permis de comprendre la formation du colmatage sur la MCP et d'identifier la nature de ce colmatage. Ce colmatage a pour effet d'augmenter la résistance éclectique des membranes au cours du temps et de ralentir la migration des espèces ioniques.

Ces résultats ont aussi concouru à répondre à l'objectif concernant l'étude de l'impact de sel sur les paramètres d'hydrolyse. Les différents niveaux de sel ont eu un impact sur le degré d'hydrolyse, le mécanisme enzymatique et la production de peptides en raison de leurs effets probables sur l'activité enzymatique et la structure de l'hémoglobine. Par conséquent, EDMB-4,5 mS/cm peut être considérée comme la meilleure condition permettant une électro-acidification rapide avec une faible teneur en minéraux par rapport à l'hydrolyse

conventionnelle, produisant des peptides bioactifs sans colmatage des membranes et impliquant une résistance globale minimale et par conséquent une consommation d'énergie plus faible. De plus, il a été démontré que l'hydrolyse à pH 3.5 dans l'EDMB a le même mécanisme enzymatique que l'hydrolyse conventionnelle, le mécanisme « One-by-one ». Ce mécanisme a produit une grande population de peptides dans toutes les conditions quelle que soit la conductivité mais avec une faible concentration de peptides due à une dénaturation incomplète de l'hémoglobine. Enfin, cette étude a démontré la faisabilité de l'électrodialyse avec membranes bipolaires comme procédé alternatif à la méthode conventionnelle pour produire des peptides bioactifs.

Ces résultats ont contribué à un avancement des connaissances sur la compréhension des phénomènes membranaires au cours de l'hydrolyse enzymatique en EDMB, l'impact de la conductivité et l'effet du pH sur le mécanisme enzymatique. Selon la littérature, il est privilégié de se placer dans des conditions permettant un mécanisme de type « Zipper » là où l'hémoglobine est complètement dénaturée, au détriment d'un mécanisme de type « One-by-one », pour pouvoir influencer la population peptidique totale, tant d'un point de vue qualitatif (nombre et variété des peptides présents dans l'hydrolysat) que d'un point de vue quantitatif (concentrations relatives des peptides entre eux). Par conséquent, d'autres expériences se concentreront sur l'optimisation du processus en 1) ajustant le pH au niveau d'acidité pour dénaturer complètement l'hémoglobine, 2) en induisant la déminéralisation dans l'EDMB et 3) en minimisant le colmatage peptidique sur la membrane cationique permsélective.

Chapitre IV

OPTIMISATION DU NOUVEAU PROCÉDÉ VERT POUR LA PRODUCTION DE NÉOKYOTORPHINE (α137-141) ET DES PEPTIDES BIOACIFS DÉRIVÉS DE L'HYDROLYSE ENZYMATIQUE DE L'HÉMOGLOBINE BOVINE UTILISANT L'ÉLECTRODIALYSE AVEC MEMBRANES BIPOLAIRES

IV. Optimisation du nouveau procédé vert pour la production de néokyotorphine (α137-141) et des peptides bioacifs dérivés de l'hydrolyse enzymatique de l'hémoglobine bovine utilisant l'électrodialyse avec membranes bipolaires

IV. Transition Contextuelle

Dans le chapitre précèdent, le but de l'étude était de démontrer la faisabilité de l'électrodialyse avec membranes bipolaires pour la dénaturation et l'hydrolyse enzymatique de l'hémoglobine bovine en milieu acide en fonction de la conductivité de la solution d'hémoglobine. Il a été démontré qu'à basse conductivité, un colmatage peptidique était observé sur la MCP, tandis qu'à une conductivité plus élevée, aucun colmatage n'était présent. Afin que le procédé d'électro-acidification de l'hémoglobine bovine soit fonctionnel et acceptable économiquement, il est essentiel de réduire, voire d'éliminer le colmatage mais aussi de diminuer la teneur en sel autant que possible.

Dans ce contexte, les objectifs spécifiques de cette étude étaient de : 1) étudier l'impact de l'empilement de membranes échangeuses d'anions ou de cations en tant que membranes homopolaires dans l'EDMB sur l'évolution du pH et de la conductivité, 2) identifier le mécanisme enzymatique impliqué dans l'hydrolyse de l'EDMB, 3) caractériser la population peptidique obtenue après hydrolyse, 4) quantifier la production de l'α137-141 et enfin 5) étudier les activités biologiques des hydrolysats obtenus à partir de l'EDMB.

Ce chapitre a fait l'objet de deux articles : Le premier intitulé « **Bovine Hemoglobin Enzymatic Hydrolysis by a New Ecoefficient Process - Part I: Feasibility of Electrodialysis with Bipolar Membrane and Production of Neokyotophin (α137-141)** », publié dans *Membranes,* 2020, 10, 257. Les auteurs sont : Mira Abou-Diab (Candidate au

Ph. D.: planification et réalisation des expériences, analyse des résultats et rédaction de l'article), Jacinthe Thibodeau (Professionnelle de recherche côté canadien : supervision de l'étudiant pour les analyses d'électrodialyse et LC-MS, correction du manuscrit), Barbara Deracinois (collaboratrice scientifique pour les études bioinformatiques de LC-MS), Christophe Flahaut (collaborateur scientifique pour les études bioinformatiques de LC-MS), Ismail Fliss (Co-directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit), Pascal Dhulster (Co-directeur de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit), Naima Nedjar (Directrice de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit) et Laurent Bazinet (Directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit).

Le second ayant pour titre : « **Bovine Hemoglobin Enzymatic Hydrolysis by a New Eco-Efficient Process - Part II: Production of Bioactive Peptides** », publié dans *Membranes*, 2020, 10, 268. Les auteurs sont: Mira Abou-Diab (Candidate au Ph. D.: planification et réalisation des expériences, analyse des résultats et rédaction de l'article), Jacinthe Thibodeau (Professionnelle de recherche côté canadien : supervision de l'étudiant pour les analyses d'électrodialyse et LC-MS, correction du manuscrit), Barbara Deracinois (collaboratrice scientifique pour les études bioinformatiques de LC-MS), Christophe Flahaut (collaborateur scientifique pour les études bioinformatiques de LC-MS), Ismail Fliss (Co-directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit), Pascal Dhulster (Co-directeur de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit), Laurent Bazinet (Directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit) et Naima Nedjar (Directrice de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit).

IV. *Premier Article*

IV.1. Résumé

La néokyotorphine est reconnue comme un peptide antimicrobien. Il est produit par hydrolyse conventionnelle de l'hémoglobine bovine. Cependant, lors de l'hydrolyse conventionnelle, l'ajout d'agents chimiques est nécessaire pour réguler le pH de la solution et la teneur en minéraux de l'hydrolysat final est élevée. Pour produire ce peptide avec une faible concentration en sel, l'électrodialyse avec membranes bipolaires (EDMB), a été étudiée avec deux configurations membranaires différentes (membranes cationique (MCP) et anionique (AEM)). L'hydrolyse en EDMB a montré le même mécanisme enzymatique, « zipper », et a permis la génération de la néokyotorphine. L'EDMB-MCP a permis la production d'hydrolysats contenant une faible concentration en sels, mais avec un colmatage sur la MCP, tandis que l'EDMB-AEM a permis la production d'un hydrolysat sans colmatage, mais avec une concentration plus élevée en sel. À notre connaissance, c'est la première fois que l'EDMB est démontrée comme une technologie faisable pour produire des hydrolysats peptidiques.

Mots clés : Hémoglobine bovine, Électrodialyse avec membranes bipolaires, Hydrolyse enzymatique, Néokyotrophine.

IV.2. Abstract

Neokyotorphin (α137-141) is recognized as an antimicrobial peptide and a natural meat preservative. It is produced by conventional enzymatic hydrolysis of bovine hemoglobin, a major component of cruor, a by-product of slaughterhouses. However, during conventional hydrolysis, chemical agents are necessary to adjust and regulate the pH of the protein solution and the mineral salt content of the final hydrolysate is consequently high. To produce this peptide of interest without chemical agents and with a low salt concentration, electrodialysis with bipolar membranes (EDBM), an electro-membrane process recognized as a green process, with two different membrane configurations (cationic (MCP) and anionic (AEM)

membranes) was investigated. Hydrolysis in EDBM showed the same enzymatic mechanism, « Zipper », and allowed the generation of α137-141 in the same concentration as observed in conventional hydrolysis (control). EDBM-MCP allowed the production of hydrolysates containing a low concentration of mineral salts but with fouling formation on MCP, while EDBM-AEM allowed the production of hydrolysates without fouling but with a similar salt concentration than the control. To the best of our knowledge, this is the first time that EDBM was demonstrated as a feasible and innovative technology to produce peptide hydrolysates from enzymatic hydrolysis.

Keywords: Bovine hemoglobin; Electrodialysis with bipolar membrane; Enzymatic hydrolysis; Neokyotorphin

IV.3. Introduction

Blood from slaughterhouses is an inevitable part of the meat production but also a natural bioresource produced in very large quantities. Its current use is mainly limited to the animal food chain (controversial concept in the context of prevention of animal diseases) or intended for landfills. Hence, only 30% of the blood is valorized (Gatnau *et al*., 2001), while it has a high protein content and is an ideal substrate for proteolysis. For this reason, in the context of the circular economy, of which the objective is to produce goods while strongly limiting the consumption of raw materials and non-renewable energy sources, the agri-food industry is turning today towards the recovery of this waste and seeking to develop products with high added value.

After centrifugation, the slaughter cattle blood is essentially subdivided into two parts. The colorless part, plasma, equivalent to around 60% of the volume of blood, can be used in pharmaceuticals due to its high content of thrombin, fibrinogen or albumin serum (Toldrà *et al*., 2012). The second part, cruor, responsible for the red color of the blood, is considered as waste and represents up to 40% of the blood volume. However, cruor contains mainly hemoglobin (90%). Bovine hemoglobin is a 64,500 Da protein, consisting of four polypeptide chains, two α chains and two β chains (Sasakawa, 1961). The hydrolysis of

bovine hemoglobin with pepsin is a source of many peptides with biological activities such as opioid (Lignot *et al*., 1999; Zhao and Piot, 1997a; 1997b), hematopoietic (Vercaigne-Marco *et al.,* 2000) and antihypertensive (Adje *et al*., 2011a). Nevertheless, the most described aspect remains the antimicrobial activity (Daoud *et al*., 2005; Nedjar *et al*., 2008; Choisnard *et al*., 2002; Przybylski *et al.,* 2016). One of these antimicrobial peptides, the α137-141 peptide (neokyotorphin, 653 Da, pI of 10.5), has recently been reported as a natural preservative to protect meat during its storage or distribution. α137-141 showed a 60% reduction in the rancidity time and inhibited microbial growth after 14 days in the refrigerator with effects close to butylhydroxytoluene (BHT), commonly used as a meat preservative (Przybylski *et al.,* 2016).

The most commonly used procedure to generate the α137-141 peptide from hemoglobin is conventional enzymatic hydrolysis (Adje *et al*., 2011b; Gomes *et al*., 2010; Nedjar *et al.,* 2006). It includes several steps consisting of hemoglobin denaturation by chemical acidification, hydrolysis with pepsin enzyme and pH adjustment and inactivation of enzymatic reaction by chemical basification. However, the hydrolysates produced contain high levels of mineral salts due to the addition of chemicals. Therefore, in this study, it is proposed to apply a green technology (Mikhaylin *et al*., 2018), named electrodialysis with bipolar membrane (EDBM), as an alternative method to the conventional enzymatic hydrolysis of hemoglobin to obtain α137-141. EDBM consists in combining homopolar membranes with bipolar membranes, whose membranes generate H^+ ions from water dissociation under an electrical field inducing acidification of the solution at the cationic interface of the bipolar membrane (BM). One major advantage of EDBM reported by Bazinet *et al.* (1997) is the possibility to acidify and demineralize a stream simultaneously, so that the electro-acidified product has higher purity than a chemically acidified product. EDBM technology was previously used for solubilization of chitosan before its enzymatic hydrolysis using bipolar membrane electroacidification (Lin Teng Shee *et al.,* 2006). Recently, EDBM was applied to produce deacidified cranberry juice, with a drastic energy consumption reduction and ecoefficiency improvement (Faucher *et al.,* 2018). Consequently, by using EDBM, the whole traditional process necessary for the production of peptide α137-141 from hemoglobin in a single technology without the addition of acids, to acidify the protein

solution before its hydrolysis and subsequently to regulate the pH of the reaction medium, would be simplified and more sustainable. Eventually, the hydrolysates produced would contain low levels of mineral salts since no acid would be added and the solution would be demineralized. However, EDBM has never been tested for hemoglobin hydrolysis and the optimal configuration has yet to be determined.

In this context, the present study aimed to prove the feasibility of enzymatic hydrolysis of bovine hemoglobin by EDBM to obtain α137-141. The specific objectives of this study were to 1) study the impact of stacking anion or cation-exchange membranes as homopolar membranes in EDBM on pH and conductivity evolution, 2) identify the enzymatic mechanism involved in EDBM hydrolysis, 3) characterize the peptide population obtained after hydrolysis, 4) quantity the production of α137-141 and finally 5) compare the efficiency of EDBM processes with the conventional hydrolysis.

IV.4. Materials and Methods

IV.4.1. Materials

Hemoglobin. The purified hemoglobin powder from bovine blood, dark brown, was purchased from Sigma-Aldrich (H2625, Oakville, ON, Canada). Its composition is given in **Tableau IV-1.** Hemoglobin was stored at 4 °C before use.

Tableau IV-1: Hemoglobin composition (%w/w).

Protein (%)	**≃98**
Ash (%)	≤2
Water (%)	≤8
Iron (Fe) (%)	0.25–0.35

Pepsin. The pepsin lyophilized powder from porcine gastric mucosa was purchased from Sigma-Aldrich (P6887, Oakville, ON, Canada). The activity of pepsin was measured at 3250

AU/mg of protein according to a protocol established by the supplier Sigma-Aldrich (Saint-Quentin Fallavier, France). Pepsin was stored at -20 °C.

IV.4.2. Electrodialysis cell configuration

The electrodialysis (ED) experiments were carried out using a Microflow-type cell (10 cm^2 of effective membrane surface). The anode was a plate dimensionally stable electrode (DSA-O_2, Ti/IrO_2 coating) and the cathode a 316-stainless-steel electrode from ElectroCell AB (Taby, Sweden). Two ED configurations were tested: the ED configuration named « EDBM-AEM » (**Figure IV-1a**) formed by stacking three anion-exchange membranes (AEM, Astom, Tokyo, Japan) and two bipolar membranes (BM, Astom, Tokyo, Japan) and the ED configuration named « EDBM-MCP » (**Figure-IV-1b**) stacking one anion-exchange membrane (AEM, Astom, Tokyo, Japan), two monovalent cation permselective membranes (MCP, Japan Food grade Neosepta, Astom, Tokyo, Japan) and two bipolar membranes (BM, Astom, Tokyo, Japan) (**Figure IV-1b**). Monovalent ion permselective membranes are a category of ion-exchange membranes allowing the migration of oppositely charged monovalent ions while retaining the polyvalent ions. Monovalent cation permselective (MCP) membranes are made of resin, including negative charges coated with a thin positive charge layer (Abdu *et al*., 2014; Van der Bruggen *et al*., 2004). Such layers on both membrane interfaces are able to separate ions of the same charge but with different valences. With this particular layer on its interface, the MCP may increase its resistance to peptide fouling, as demonstrated recently by Persico *et al*. (2018). This was the reason for choosing this type of membrane for our study. In both cases, the ED configuration defined three closed loops containing 200 mL of hemoglobin solution (1% Hb w/v), 200 mL of salt ion recovery (KCl 1 g/L (EMD Chemicals Inc, Port Wentworth, GA, USA)) and 250 mL of electrode-rinsing solution (Na_2SO_4 20 g/L (ACP Inc., Montréal, QC, Canada)).

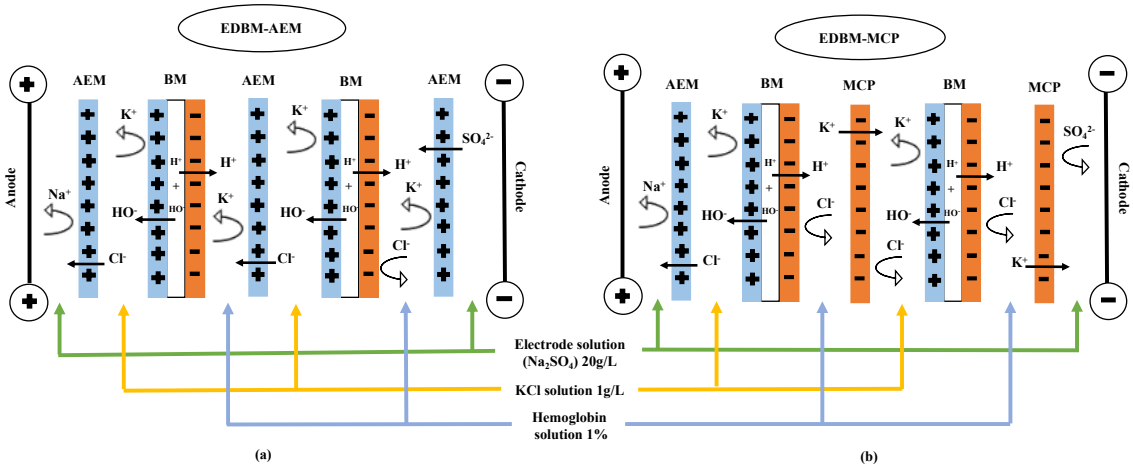

Figure IV-1. Schematic representation of electrodialysis cell configurations for electro-acidification and hydrolysis of bovine hemoglobin in electrodialysis with bipolar and anion exchange membranes (EDBM-AEM (a)) and in electrodialysis with bipolar and monovalent cation permselective membranes (EDBM-MCP (b)). MCP: monovalent cation permselective membrane; AEM: anion exchange membrane; BM: bipolar membrane.

IV.4.3. Protocol

To assess the feasibility of EDBM for enzymatic hydrolysis of bovine hemoglobin by pepsin, three different conditions were tested: conventional hydrolysis in beaker, hydrolysis by EDBM-MCP and hydrolysis by EDBM-AEM. The conventional hydrolysis in beaker was considered as the « control » since it has been demonstrated in several enzymatic hydrolysis studies to allow the production of α137-141 peptide with various biological activities (Nedjar *et al.*, 2008; Przybylski *et al.*, 2016; Takagi *et al.*, 1982; Catiau *et al.*, 2011). Hence, all results were compared to this control.

IV.4.3.1. Conventional hydrolysis

Stock solution preparation

A stock solution was prepared by adding 15 g of purified bovine hemoglobin (BH) to 100 mL of ultrapure water. After centrifugation (6000x*g* for 30 min (Eppendorf AG, 22331 Hamburg, Centrifuge 5804 R, Brinkmann Instruments, Westbury, N.Y., USA)), the

supernatant was recovered and the real BH concentration (C_{BH}) was determined according to Drabkin's method (Crosby *et al*., 1954). From the concentration C of the stock solution, a solution of purified BH was prepared by dilution to a 1% (w/v) precise concentration of hemoglobin C_{BH}. The pH of the 1% hemoglobin solution was 6.94 ± 0.1 and its conductivity value 58.36 ± 1.33 µS/cm.

Hydrolysis process

Since the stock solution was composed of hemoglobin in its native form, the pH was first adjusted to 3 with HCl (2M, Anachemia, VWR Company International, ON, Canada) to denature hemoglobin. Then, the hydrolysis was carried out at pH 3 in the presence of porcine pepsin (Sigma-Aldrich, Oakville, ON, Canada) from porcine gastric mucosa (EC 3.4.23.1, 3200-4500 units mg^{-1} protein) solubilized in ultrapure water, with an enzyme/substrate ratio equal to 1/11 (mole/mole). Pepsin is an enzymatic protein belonging to the aspartyl proteases family (Dunn, 2002). This endopeptidase catalyzes the hydrolytic cleavage of peptic hydrophobic or aromatic amino acid residues.

IV.4.3.2. Hydrolysis by EDBM-MCP and EDBM-AEM

The 1% hemoglobin solution was prepared as previously, except that 1) the conductivity was adjusted to 1.5 mS/cm with KCl addition since the 1% HB solution conductivity was too low for ED experiments and 2) the pH was adjusted to 3 by applying an electric current, leading to the electrochemical production of protons H^+ at the cationic interface of the bipolar membrane in contact with the HB solution. When the pH value of 3 was reached, porcine pepsin was added to start the hydrolysis. The protocol for both conventional and EDBM hydrolysis is presented in **Figure IV-2**.

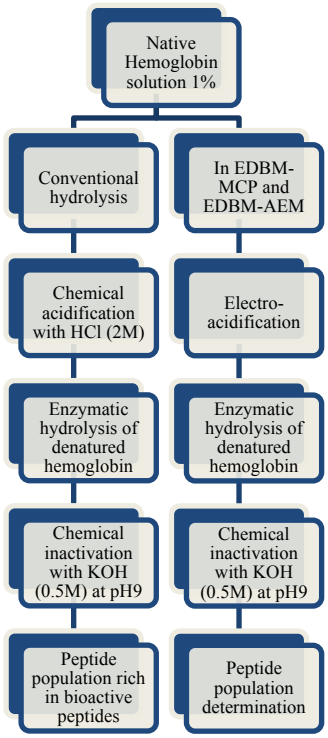

Figure IV-2. Diagram illustrating the acidification and hydrolysis of bovine hemoglobin in conventional hydrolysis (control) and by EDBM.

The flow rate of the electrode-rinsing solution was set at 450 mL/min and the flow rates of 1% HB and KCl solutions were set at 400 mL/min. Each closed loop was connected to a separate external reservoir, allowing a continuous recirculation of each compartment (**Figure IV-1).** A constant voltage of 15 V, corresponding at the beginning of the process to a current density of 5.3 mA/cm^2, was applied during the EDBM experiments using a BK Precision power supply (Model BK9110-ND, Vancouver, BC, Canada). During each experiment, the hydrolysis was conducted for 3 h at 30°C. Samples were taken at 0 min before adding the pepsin and then after pepsin addition at 2.5, 30, 60, 120 and 180 min during the hydrolysis step, corresponding to different degrees of hydrolysis. The reaction in each sample was stopped by addition of KOH (0.5M) (Fisher Scientific, New Jersey, USA) in order to bring the sample to pH 9, allowing both to irreversibly inactivate the enzyme and to avoid any possible basic hydrolysis. The samples were stored at -20 °C until further analyses. The current intensity, conductivities, pH and temperature, for both 1% HB and KCl solutions, were recorded every 30 min during EDBM treatments. Three randomized repetitions of each EDBM and control condition were carried out. For EDBM, after each treatment, a cleaning

in place (CIP) was performed according to the manufacturer's procedure, the cell was dismantled and the membrane conductivity and thickness measured to evaluate their integrities. The membranes were changed for new membranes after each repetition. Hydrolysis mechanism and peptide identification were carried out on samples collected during hydrolysis (T_0, $T_{2.5}$ min, T_{30} min, T_{60} min, T_{120} min and T_{180} min).

IV.4.4. Analyses

IV.4.4.1. Determination of hemoglobin concentration: Drabkin's Method

Drabkin's reagent is a spectrophotometric method used for the quantitative determination of hemoglobin concentration in whole blood. Briefly, 40 µL of the sample was added to 10 mL of the Drabkin's reagent (D5941, Sigma-Aldrich, Oakville, ON, Canada) for 15 min at room temperature. Then, the absorbance was read at a wavelength of 540 nm by a UV spectrophotometer (ChemStation 8453A UV-Vis Spectrophotometer, Agilent Technologies, Santa Clara, CA, USA) and reported into the calibration curve. From the concentration C of the stock solution, solution of purified bovine hemoglobin was prepared by dilution to precise concentrations of hemoglobin C_{BH}: 1% (w/v).

IV.4.4.2. pH

The pH levels of the 1% HB and KCl solutions were measured with a pH meter (model SP70, symphony, VWR international, Montreal, QC, Canada) equipped with an automatic temperature compensation (ATC) pH probe. To stop the hydrolysis reaction in the hemoglobin hydrolysate samples, the pH was measured with a fine tip pH-electrode (Mettler Toledo, LE422, micro, 3 mm, United Kingdom).

IV.4.4.3. Conductivity

Conductivity of the 1% HB and KCl solutions was measured with a YSI conductivity meter (Model 3100, Yellow Springs Instrument, Yellow Springs, OH, USA) combined with a YSI 3252 electrode with a cell constant of 1 cm^{-1}.

IV.4.4.4. EDBM global resistance

The global system resistance was calculated according to Ohm's law: R = U/I, where R is the global system resistance (Ω), I the current intensity (A) and U the voltage applied between the electrodes of the ED cell (V). The voltage and the current intensity were directly read on the power supply.

IV.4.4.5. Membrane thickness and electrical conductivity

Membrane thickness was measured with an electronic digital micrometer (Model Marathon Watch Company LTD., Richmond Hill, ON, Canada).

Membrane electrical conductance (G) was measured with a specially designed cell (Laboratoire des Matériaux Échangeurs d'Ions, Créteil, France) following the procedure described by Mikhaylin *et al*. (2016). The NaCl reference solution was 0.5 M. The membrane conductivity was calculated according to Lteif *et al*. (1999) and Lebrun *et al*. (2003) equation:

$$R_m = R_{m+s} - R_s$$

where R_m is the electrical resistance of the membrane (in Ω), R_{m+s} the electrical resistance of the membrane and the reference solution measured together (in Ω) and R_s the electrical resistance of the reference solution (in Ω). The resistance R is equal to 1/G. The membrane electrical conductivity (κ) was calculated according to the following equation:

$$\kappa = L/R_m A$$

where L is the membrane thickness (in cm) and A is the electrode area (1 cm^2). These measurements were recorded before and after each replicate.

IV.4.4.6. Total solid and ash contents

The total solid content was determined by drying hemoglobin solution samples at 95 °C overnight. The ash content was measured by heating the dried samples at 550 °C for 20 h according to the AOAC international method 945.46 (AOAC, 1990).

IV.4.4.7. Mineral concentration measurement

The samples collected after 180 min of hydrolysis were thawed at 4 °C before their dilution 1/20 in Milli-Q water. Potassium, sodium and sulfur concentrations were determined by optical emission spectrometry with inductively coupled plasma as atomization and excitation source (ICP-OES Agilent 5110 SVDV Agilent Technologies, Victoria, Australia), using the following wavelengths (in nm): 766.491 (K), 588.995; 589.592 (Na), 181.972; 180.669 (S). The analyses for all ions were carried out in axial and/or radial view. For chlorides, the samples were analyzed with Flow Injection Analysis (FIA) (Quikchem 8500 series 2, Zellweger Analytic, inc., Lachat Instruments Division, Milwaukee, WI, USA) with Quikchem method 10-117-07-1-C: Determination of chloride by Flow Injection Analysis colorimetry.

IV.4.4.8. Determination of the degree of hydrolysis of bovine hemoglobin

The degree of hydrolysis (DH) is defined as the ratio between the number of peptide bonds cleaved by an enzyme and the number of total substrate bonds (protein), according to the following equation:

$$DH = 100 \times \frac{h}{h_0}$$

where DH is the degree of hydrolysis (%), h is the number of peptide bonds hydrolyzed in the substrate and h_0 is the number of total peptide bonds of the protein substrate.

DH was quantified by the ortho-phthaldialdehyde (OPA) technique, adapted from Church *et al*. (1983) and Spellman *et al*. (2003). In order to determine the number *h* of hydrolyzed bonds, the following equation was used:

$$h = \frac{\Delta Abs.\, M.\, DF}{\varepsilon.\, C_{HB}}$$

where ΔAbs is the difference between the absorbance at 340 nm of the hydrolyzed sample and the absorbance of the unhydrolyzed sample, *M* the molar mass of the protein (for bovine hemoglobin: 64,500 g.mol^{-1}), DF the dilution factor of the sample, ε the molar extinction coefficient of OPA reagent at 340 nm (6000 $mol^{-1}.\, cm^{-1}$ according to Church *et al*. (1985)) and C_{HB} the concentration of bovine hemoglobin (10g/L equivalent to 1%). The reagent was prepared at least one hour before use and stored in the dark. For a final volume adjusted to 50 mL, its composition was 25 mL of sodium tetraborate solution (0.1M) (Fisher Chemicals, Belgium), 5 mL of 10% (w/v) SDS (Fisher Scientific, New Jersey, USA), 10 µL of β-mercaptoethanol (Sigma-Aldrich, Oakville, ON, Canada) and 80 mg of OPA (Sigma-Aldrich, Oakville, ON, Canada) dissolved in 1 mL of methanol (Fisher Chemicals, Belgium). The linearity of the reagent was verified by assaying L-leucine (Sigma-Aldrich, Oakville, ON, Canada). A volume of 50 µL of sample to be tested was added to 1 mL of reagent. The absorbance was read at 340 nm after incubation for 2 min at room temperature.

IV.4.4.9. RP-UPLC Analyses

Identification of Hydrolysis Mechanism and α137-141 Peptide: Reversed phase ultra-performance liquid chromatography (RP-UPLC) analyses were performed on all samples used a 1290 Infinity II UPLC (Agilent Technologies, Santa Clara, CA, USA). The RP-UPLC apparatus was composed of a binary pump (G7120A), a multisampler (G7167B), an in-line degasser and a variable wavelength detector (VWD G7114B) adjusted to 214 nm. Then, 0.22µm polyvinylidene difluoride (PVDF) filters were used to filter hydrolysis fractions into a glass vial. Samples were loaded (0.25µL) into a Poroshell 120 EC-C18 column (2.1 × 100mm i.d., 2.7 micron, Agilent, Santa Clara, CA, USA). A flow rate of 500 µL/min at 23

°C was used to operate the column. A gradient was applied for the mixture of solvent A (LC-MS grade water with 0.1% formic acid) and solvent B (LC-MS grade ACN with 0.1% formic acid) with solvent B increasing from 1% to 13% in 6 min, to 35% until 25 min and to 100% until 35 min and holding until 45 min, then back to initial conditions. Each sample analysis was performed in triplicate for ensuring technical reproducibility.

To identify and quantify the relative abundances of the peptides present in the samples, a hybrid ion mobility quadrupole time of flight mass spectrometer 6560, IM-Q-TOF, Agilent, Santa Clara, CA, USA) was used. All LC-MS/MS experiments were acquired using Q-TOF. A positive mode at Extended Dynamic Range, 2 Ghz, 3200m/z with a scan range of 100–3200 m/z was used to record the MS/MS signals. The drying gas was nitrogen at 13.0 L/min and 150 °C and was also used as nebulizer gas at 30 psig. The capillary voltage was set at 3500 V while the nozzle voltage at 300 V and the one of the fragmentors at 400 V. The instrument calibration was carried out by using an ESI-L low concentration tuning mix (G1969-85000, Agilent Technologies, Santa Clara, CA, USA). Data acquisition and analysis were performed with the Agilent Mass Hunter Software package (LC/MS Data Acquisition, Version B.08.00 and Qualitative Analysis for IM-MS, Version B.07.00 Service Pack 2 with BioConfirm Software).

UPLC-MS/MS coupling was used to analyze the α137-141 peptide. This coupling offered the advantage of a complementarity between UV profile from the UPLC and the masses of the corresponding compounds for each point of the chromatogram. This also gives the amino acid composition of each compound. Several parameters were taken into account to ensure good identification of the α137-141 peptide: the retention time, the monoisotopic molecular mass and the second-order derivative spectral analysis of peptides testified of the presence (or absence) of aromatic amino acids (Zhao *et al*., 1995). The α137-141 peptide was identified by comparing it with alpha hemoglobin sequence for bovine hemoglobin hydrolysis by pepsin.

Identification and characterization of peptide population: After sample drying, peptides were dissolved in H_2O / 0.1% TFA at 0.5 mg/mL and centrifuged for 5 min at 10,000x*g*.

Then, 10 µL was separated by chromatography at 30 °C on an ACQUITY UPLC system (Waters Corporation) equipped with a Kinetex 2.6 µm C18 100Å (150 mm × 4.6 mm) column (Phenomenex). Acetonitrile gradient was set at 0.5 mL/min (the mobile phases were composed of solvent A (0.1% (v/v) formic acid/99.9% (v/v) water) and solvent B (0.1% (v/v) formic acid/99.9% (v/v) acetonitrile): from 5% to 15% solvent B during the first 30 min, from 15% to 30% over 60 min, from 30% to 50% over 10 min and then maintained for 10 min at 95% solvent B. The eluate was then directed into the electrospray ionization source of the qTOF Synapt G2-Si™ (Waters Corporation). The analysis was performed in sensitivity, positive ion and data-dependent analysis (DDA) modes. The source temperature was at 150 °C and the capillary and cone voltages were fixed, respectively, at 3,000 and 60 V. Data for m/z values were collected in the range of 100 to 2000 Da with a scan time of 0.2 sec. A maximum of 10 precursor ions were chosen for MS/MS analysis with an intensity threshold of 100,000. A collision-induced dissociation (CID) fragmentation mode and a scan time of 0.1 sec were chosen for MS/MS data collection.

PEAKS Studio (version 8.5, Bioinformatics Solutions Inc., Waterloo, Canada) software and its database search tools were used for peptide identification. The UniProt database restricted to Bos Taurus was used. A mass tolerance of 35 ppm, 3 missing cleavage sites and an MS/MS tolerance of 0.2 Da were allowed while a variable methionine oxidation was considered. Protein and peptide identity relevance was judged according to their score in the research software (p value of 0.05 ($p < 0.05$), False Discovery Rate < 1%). For the search of peptides from the pepsic hydrolysate by Peaks® software, the Bos taurus bovine hemoglobin alpha and beta chain sequences were taken as model proteins. In addition, heat maps were generated to visualize peptide abundance. The results were expressed using a color code where high, low and no occurrence of an individual amino acid were represented in red, yellow and white, respectively.

Progenesis QI for proteomics (Version 4.0, Nonlinear Dynamics, England) was also used to analyze mass spectrometry data. These data were subjected to the following successive processing steps: (i) alignment of the peptide maps with a quality control corresponding to an equivolume mixture of each of the analyzed samples, (ii) peak picking, detection of

isotopic massif passing the software intensity threshold, (iii) data standardization to carry out a statistical principal component analysis (PCA). The variables used were derived from the comparison of peptide maps, i.e., the position of the isotopic massifs and their intensity.

Quantification of α137-141 in the Hydrolysates by Mass Spectrometry: A α137-141 standard was weighed out and dissolved in H_2O to prepare stock solution at 5 mg/mL. The solution was centrifuged for 5 min at 10,000x*g*. A standard curve at concentrations ranging between 0.40 and 12.5 µg/mL was established by injection of the α137-141 standard. Dried hydrolysates were dissolved at 0.5 mg/mL in H_2O. The concentration of α137-141 in the samples was determined by the same chromatographic procedure and equipment as previously for the characterization of the sample peptide population. The mobile phases were 0.1% (v/v) formic acid/99.9% (v/v) water (solvent A) and 0.1% (v/v) formic acid/99.9% (v/v) acetonitrile (solvent B). The acetonitrile gradient was set at 0.5 mL/min: 1% solvent B for 1.50 min, from 1% to 20% solvent B over 8.50 min, from 20% to 50% solvent B over 1 min followed by a washing step and an equilibrating procedure, respectively, with 95% and 1% solvent B for 2 min. The eluate was directed as previously and the MS analysis performed in sensitivity, with positive ions. As previously for the identification and characterization of the peptide population, the source temperature, capillary, cone voltages m/z value range and scan time of 0.2 sec were set as the same values.

MassLynx V4.1 software (Waters Corporation, Guyancourt, France) was used to analyze the LC-MS data and, in these conditions, the retention time for α137-141 was 3.20 min. Extracted chromatograms from α137-141 ($(M+H_2)^{2+}$ 327.7) were generated at a mass precision of 0.1 Da. After integration, quantification of α137-141 was performed according to peak areas after validation of isotopic clusters and elution time. To establish a linear relation between the α137-141 concentration in the sample and the area surface from each spectrum, the areas under the mass peak were used, for any hydrolysate, by using the following equation (R^2 = 0.999):

$$A_{\alpha 137\text{-}141} = 5935612.61.C_{\alpha 137\text{–}141}\text{-}1042.65$$

where $C_{\alpha 137\text{-}141}$ is the α137-141 concentration (µg/mL) and $A_{\alpha 137\text{-}141}$ is the area peak.

IV.4.4.10. Statistical analyses

All analyses were performed in triplicate and three independent repetitions were carried out for each condition. Data were subjected to one-way or two-way analyses of variance (ANOVA). Tukey tests were also performed on data using SigmaPlot software (Version 14.0, Systat Software, San Jose, CA, USA) to determine which treatment was statistically different from the others at a probability level p of 0.05.

IV.5. Results and Discussion

To test the feasibility of EDBM, to understand the different results obtained and to compare them with the control, the section below will present the parameters of the three conditions: conventional hydrolysis without membranes (control), hydrolysis in electrodialysis with bipolar and cationic membranes configuration (EDBM-MCP) and hydrolysis in electrodialysis with bipolar and anionic membranes configuration (EDBM-AEM). The EDBM hydrolysis reaction is due to the characteristics of the bipolar membranes allowing the generation of the necessary ions without the use of chemical solvents.

IV.5.1. Conventional hydrolysis (Control): Evolution of pH and conductivity

To denature hemoglobin, a solution of HCl was added to decrease the pH from 6.81 ± 0.01 to 3. In parallel, conductivity increased from 0.058 ± 0.001 to 1.49 ± 0.013 mS/cm. Once the pH was adjusted to 3, pepsin was added. The hydrolysis phase is shown in **Figure IV-3**. Throughout the 3 h of hydrolysis, the pH was maintained at 3 by adding HCl if needed. In parallel, conductivity continued to increase, to reach 2.33 ± 0.032 mS/cm at 180 min. The increase in conductivity was representative of the addition of salts to the solution to adjust the pH.

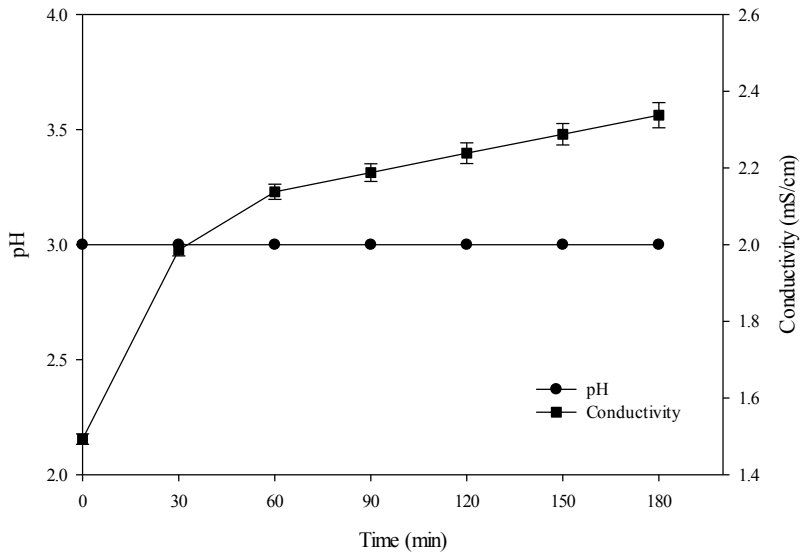

Figure IV-3. Evolution of pH and conductivity during conventional hydrolysis.

IV.5.2. Hydrolysis in EDBM: Evolution of pH, conductivity, global resistance and membrane characterization

IV.5.2.1. pH

Hemoglobin electro-acidification process was carried out to decrease the solution pH from 7.02 ± 0.01 to 3 by bipolar membranes which generate the H^+ ions from water dissociation needed for pH decrease. The mean times required to electro-acidify the 1% HB solution were 390 ± 20 min and 60 ± 2 min in EDBM-MCP and EDBM-AEM, respectively, while the global process durations (electro-acidification + hydrolysis) were 570 ± 20 min and 240 ± 2 min in EDBM-MCP and EDBM-AEM, respectively (**Figure IV-4-a; b**). The shorter duration of the acidification step observed in EDBM-AEM was explained by the fact that H^+ ions generated by the bipolar membrane were retained in the hemoglobin compartment by the AEM, not allowing the migration of the H^+ due to the similar charges of its positive fixed groups ($N^+(CH_3)_3$, as described by Persico *et al*. (2016) repulsing the H^+ by Donnan's effect. Consequently, the H^+ ions generated were globally all used to decrease the pH of the solution. However, in EDBM-MCP, two phenomena appeared as a function of time. During the first part of the process, the 1% HB solution was depleted in K^+ migrating through the MCP to the base recovery compartment to form KOH. K^+ ions were able to cross the cationic

membrane due to the opposite charges of its negative fixed groups (SO_3^-, as described by Persico *et al*. (2017)) allowing their transfer; the migration of K^+ ions ensures the electroneutrality of the solution since H^+ ions were generated in the solution. As observed previously by Bazinet *et al*. (2000) for a two-compartment EDBM process during electroacidification of milk, and Lin Teng Shee *et al*. (2006) for a two-compartment EDBM process during chitosan solubilization by acidification, a decrease in electrical efficiency was observed due to a loss of electrogenerated H^+ through the cationic membrane, linked to a lack of sufficiently mobile ions such as potassium. Hence, in our case, once the main part of the K^+ present in the 1% HB solution was migrated, there was a lack of available cations to transport the current and ensure the electroneutrality. Then, the H^+ has to migrate through the MCP to compensate for this lack of available positively charged species. Those H^+ ions migrating did not participate anymore in the decrease in pH and then delayed reaching pH 3. During the hydrolysis phase, after adding the solubilized pepsin in water, pH increased normally, as shown in **Figure IV-4**. This increase was also observed in the control, due to the change in the medium caused by the chemical reaction between the enzyme and the substrate releasing new molecules. Furthermore, it is important to note that, during the hydrolysis phase in EDBM-MCP, it was more difficult to maintain the pH at 3. Despite the fact that the current remained on throughout the hydrolysis phase, the value remained constant around 3.67 ± 0.03 (**Figure IV-4a**). While it was easy to reduce and maintain the pH in EDBM-AEM, the current was applied for a total of 30 min over the 3 h of hydrolysis to maintain the pH constant at 3.0. The difficulty of maintaining the pH at 3.0 in EDBM-MCP can be explained by the lower proton gradient in the hemoglobin compartment in EDBM-MCP than in EDBM-AEM, making difficult the protonation of HB as described for lower pH values in the chitosan compartment with AEM configuration than CEM by Lin Teng Shee *et al*. (2006), and also the difficulty of maintaining the pH can be explained by possible fouling of MCP or BM.

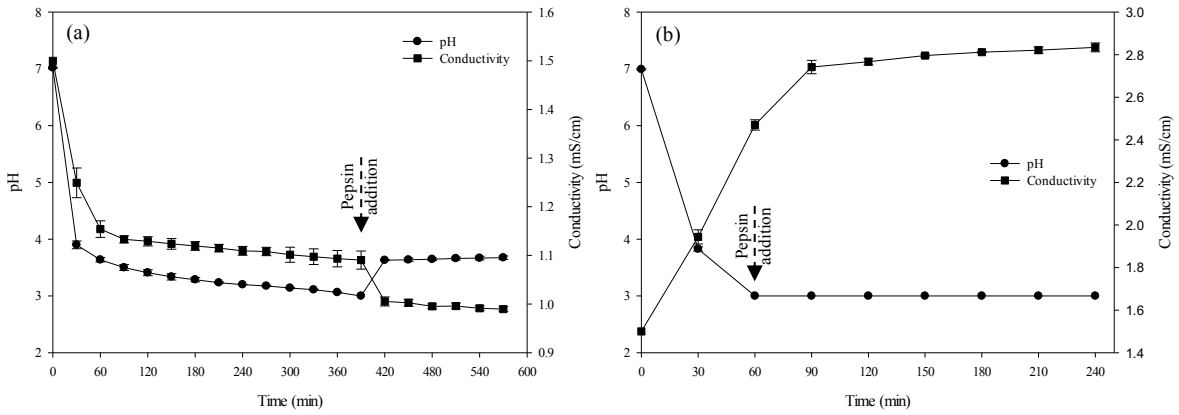

Figure IV-4. Evolution of pH and conductivity as a function of time during electro-acidification and hydrolysis in EDBM-MCP (a) and in EDBM-AEM (b).

IV.5.2.2. Conductivity

During EDBM-MCP, conductivity dropped from 1.5 to 0.98 ± 0.004 mS/cm (**Figure IV-4a**), which corresponds to a 34% demineralization of the 1% HB solution, while in EDBM-AEM, conductivity increased from 1.5 to 2.83 ± 0.02 mS/cm, corresponding to a mineralization rate of 88% (**Figure IV-4b**). The statistical analysis demonstrated that the values of conductivity were significantly different ($p < 0.05$) at the end of the global process of hydrolysis between control (2.33 ± 0.032 mS/cm), EDBM-MCP (0.98 ± 0.004 mS/cm) and EDBM-AEM (2.83 ± 0.02 mS/cm). Conductivity decreased for EDBM-MCP configuration due to the partial demineralization caused by the migration of K^+ through the MCP (Bazinet *et al*., 1997; Lin Teng Shee *et al*., 2005) and increased for EDBM-AEM configuration due to the partial mineralization caused by migration of Cl^- anions through the AEM from the basified KCl solution (Lin Teng Shee *et al*., 2006; Vera *et al*., 2003).

IV.5.2.3. ED system global resistance

At a constant voltage, the current varried throughout the process and was inversely proportional to global resistance **(Figure IV-5)**. In EDBM-MCP, during the electro-acidification phase, the global resistance increased rapidly from 281.33 ± 6.16 Ω to 1083.33 ± 144.33 Ω, corresponding to pH values between 7.02 and 3.5. Thereafter, it slowly increased

to reach 1301.28 ± 178.68 Ω at the end of the acidification phase (T_{390min}) when, in parallel, the pH continued to decrease to 3. In comparison, in EDBM-AEM, the global resistance decreased rapidly from 288.22 ± 39.69 Ω to 196.73 ± 18.22 Ω (T_{60min}). Furthermore, in EDBM-MCP, after the addition of pepsin, the global resistance decreased slightly to a value of 849.2 ± 143.51 Ω, followed by a slight increase to 1086.3 ± 156.78 Ω after 3 h of hydrolysis (T_{570min}) (**Figure IV-5a**) while in EDBM-AEM, it was constant at a value of 198.29 ± 37.79 Ω (T_{240min}) (**Figure IV-5b**). The increase in global resistance observed for the cationic configuration was explained by the conductivity decrease in the acidified compartment due to the migration of free cations, as observed by Lin Teng Shee *et al*. (2005). Furthermore, as described in several studies, the ionic equilibrium is important during an electrodialytic process since an important impoverishment of one compartment in ion (low ash content of the hemoglobin solution) would increase the global system resistance and consequently will affect the current efficiency (Bazinet *et al*., 2000; Jaime-Ferrer *et al*., 2009). However, such a high increase in global resistance could not be fully explained by just the demineralization, and it is possible that the integrity of the MCP was changed or fouling was present on this membrane. For EDBM-AEM, the decrease in overall resistance was explained by an increase in conductivity of the acidified compartment due to migration of Cl^- anions from the KCl basified solution. In order to confirm or not the possible change in MCP integrity or potential fouling, the following section is dedicated to the characterization of the membranes after EDBM in terms of membrane thickness and electrical conductivity, as well as presenting photographs.

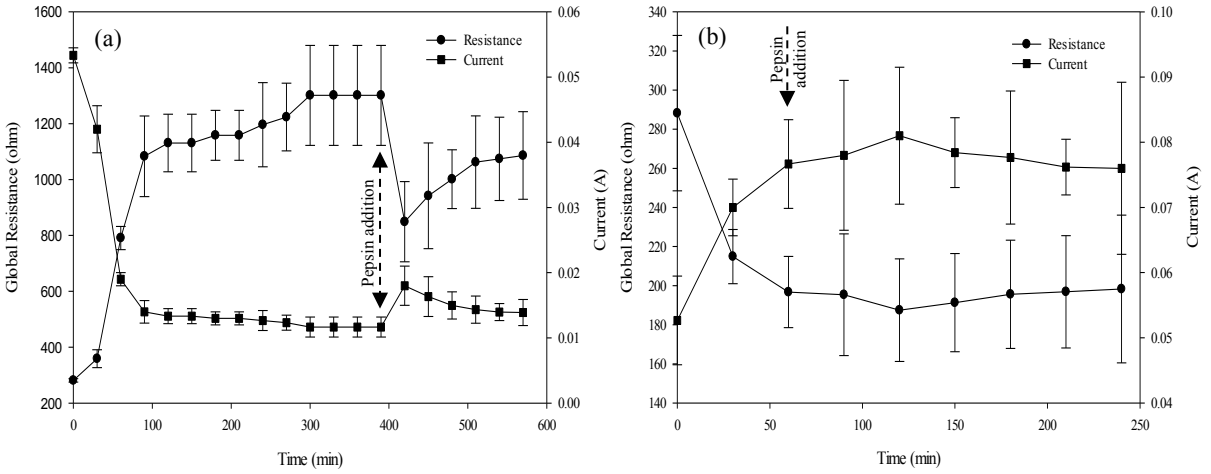

Figure IV-5. Evolution of the system global resistance and current as a function of time during electrodialysis process in EDBM-MCP (a) and in EDBM-AEM (b).

IV.5.2.4. Membrane characterization

Membranes were first characterized by measuring their thickness (cm) and conductance (Ohm) before and after each treatment as well as the conductance of the NaCl solution (0.5M), then the conductivity of the membrane (mS/cm) was calculated and presented in **Tableau V-2**. In EDBM-MCP, a configuration formed by stacking one AEM, two MCPs and two BMs, statistical analysis demonstrated that conductivity of AEM did not significantly change before and after the treatment, with an averaged value of 5.3 ± 0.0 mS/cm ($p > 0.05$). However, statistical analyses demonstrated that conductivity of MCP-1, MCP-2, BM-1 and BM-2 significantly changed before and after the treatment ($p < 0.05$ for all membrane types). Concerning MCPs, a large variation in conductivity from an averaged value of 4.26 ± 0.03 before and 0.96 ± 0.10 mS/cm after the treatment was observed, while it was quite negligible for BM. In comparison, in EDBM-AEM, formed of three AEMs and two BMs, statistical analyses demonstrated that conductivity of AEM-1, AEM-2 and AEM-3 did not significantly change before and after the treatment ($p > 0.05$). However, conductivity values of BM-1 and BM-2 significantly changed before and after the treatment ($p < 0.05$). These data confirmed the absence of integrity change on AEM membranes and changes or presence of fouling on MCP membranes. The slight differences in conductivity observed for BM membranes could be due to a change in ash content after EDBM and/or to peptide precipitation on BM

membranes based on electrostatic or hydrophobic interactions, as proposed by Kravtsov *et al*. (2020) on whey deacidification. In addition, the highest difference in electrical conductivity observed for BMs in EDBM-MCP in combination with the decrease in conductivity of MCP can probably explain the difficulty of reducing and maintaining the pH and consequently the longer acidification duration.

Tableau IV-2. Conductivity (mS/cm) of MCP (monovalent cation perm-selective membrane), AEM (anion exchange membrane) and BM (bipolar membrane) before and after treatment in EDBM-MCP and EDBM-AEM.

Process	**EDBM-MCP**					**EDBM-AEM**				
Conductivity (mS/cm)	**AEM**	**MCP-1**	**MCP-2**	**BM-1**	**BM-2**	**AEM-1**	**AEM-2**	**AEM-3**	**BM-1**	**BM-2**
Before	5.3±0.05^{a}	4.3±0.1^{a}	4.23±0.05^{a}	5.8±0.05^{a}	5.7±0.2^{a}	5.3±0.15^{a}	5±0.2^{a}	4.9±0.2^{a}	5.9±0.2^{a}	5.8±0.15^{a}
After	5.3±0.15^{a}	0.86±0.11^{b}	1.06±0.25^{b}	5.06±0.05^{b}	5.13±0.15^{b}	5.1±0.05^{a}	4.76±0.25^{a}	5.06±0.2^{a}	5.33±0.2^{b}	5.4±0.11^{b}

Population means within each column with different letters are significantly different $p < 0.05$ (Tukey).

Figure IV-6 shows that, in the EDBM-MCP, the MCPs exhibited fouling, while bipolar and anionic membranes did not present any fouling. This confirmed the previous hypothesis concerning the integrity change of the MCP. In comparison, **Figure IV-7** shows that, in EDBM-AEM, the anionic and bipolar membrane exhibited lower or even no fouling. Only the last anionic membrane had a fine deposit that may be due to hem or high protein concentration, but this did not affect the course of treatment.

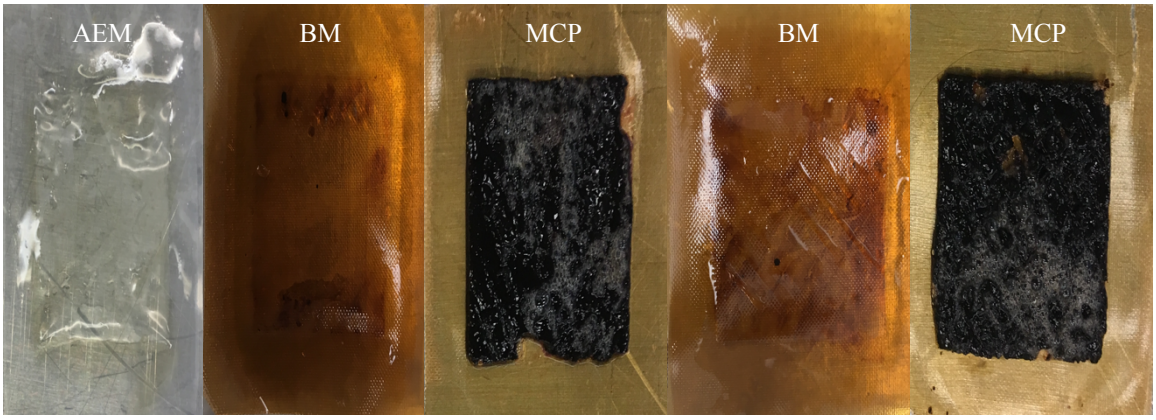

Figure IV-6. Hemoglobin fouling on EDBM-MCP membranes at the end of the treatment.

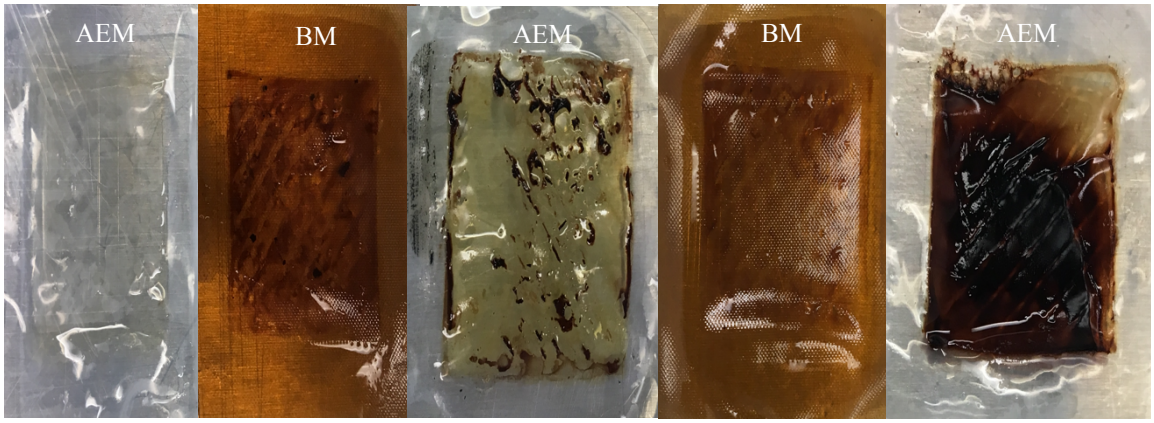

Figure IV-7. Hemoglobin fouling on EDBM-AEM membranes at the end of the treatment.

The pH of the hydrolysate solution was close to 3.0 this allows electrostatic interactions between peptides and the MCP which may be responsible for its fouling. Indeed, at pH 3, the peptides were mainly positively charged and since the sulfonic groups of the MCP are negatively charged (SO_3^-), electrostatic bonds were formed, leading to peptide fouling, as described for conventional cation-exchange membrane by Persico et Bazinet, (2018). However, the presence of a thin layer oppositely charged on the surface of the MCP matrix, to render it selective to monovalent cations, should have avoided the possibility of electrostatic interactions between peptides and the membrane surface to avoid or limit peptide fouling. It appeared that this thin layer repulsed the main part of positively charged peptides with high molecular weights, due to the restriction of the cavity size and/or highly charged positively peptides, due to the electrostatic repulsion of the thin layer, but some were able to interact with the matrix underneath. Indeed, Persico and Bazinet, (2018) observed that the presence of a positively charged layer at the interfaces of the same MCP as the one used in these experiments decreased drastically peptide fouling by 95-100% but did not completely avoid it. They also demonstrated with rhodamine B, a positively charged fluorescent compound, that rhodamine B was only detected in scattered cavities (probably crosslinking polymers) of MCP. However, this was not consistent with 1) a potential decrease in the peptide fouling expected since fouling on the MCP was major, 2) the dark red color of the fouling and 3) the fact that after hydrolysis, soaking fouled membranes in NaCl solution, as proposed by Persico *et al*. (2017) to eliminate peptide fouling on ion-exchange membranes, was not enough to remove the entire red layer. So, this fouling was probably composed not only of peptides but also of hem, a molecule tightly attached to hemoglobin at

neutral pH and responsible for its red color. Hence, according to the pH of the solution and the release of hem alone or protein fragments containing hem and peptides during hydrolysis, peptides may have first interacted electrostatically (positive residues) into the scattered cavities of the thin layer, where the negatively fixed group of the MCP matrix was reachable and, afterwards, hem or the protein fragments containing hem interacted with these already adsorbed peptides via hydrophobic interactions. Persico *et al*. (2017) developed this hypothesis for a conventional cationic membrane (CMX-SB) when they observed that at pH 6, after fouling, four sequences of LIVTQTMK, ALPMHIR, TKIPAVFK and IPAVFK were present in both salt (decrease electrostatic interaction) and SDS solutions (competition with hydrophobic interactions). They demonstrated that a first layer was formed by positively charged peptide reacting by electrostatic interactions and a second one due to hydrophobic interaction through their respective hydrophobic residues.

Earlier ESI-MS experiments sought to gain insights into the HB assembly and disassembly mechanism by studying the protein at increasing acid concentrations. Simmons *et al*. (2004) and Boys *et al*. (2007) suggested major differences in the behavior of alpha and beta-globin during pH denaturation. They studied the gradual changes in HB from pH 6.8 to 2.1 in a manner that can be described by a stepwise sequential unfolding mechanism inducing the separation of α chain, β chain and hem. Based on both structural considerations and experimental results, Lebrun *et al*. (1998) highlighted the predominant role of the hydrophobic interactions in the formation of the hem-peptide complexes at acidic pH after denaturation, for which hem alone is completely insoluble. Nonetheless, this explanation could be proposed in our study: those positive peptides may have interacted firstly electrostatically with the negative sulfonic groups of the membranes and secondly through peptide-hem associations. Furthermore, it is important to mention that demineralization in EDBM-MCP could have also increased fouling. Decreasing in overall conductivity and low ionic strength may probably increase the electrostatic interaction of the peptide–membrane bonds due to a lack of potassium which could interact electrostatically with the membrane and avoid another peptide interaction.

On the other hand, absence of or low fouling in EDBM-AEM (**Figure IV-7**) is related to the pH of the solution. The lower the pH, the more positively charged the peptides and,

consequently, the weaker the ability of peptides to establish electrostatic interactions with the AEM (positively charged) leading to less fouling. The positively charged functional groups of the anionic membrane led to electrostatic repulsions with peptides (positively charged). This explains the absence of or low fouling on the anionic membranes (Persico *et al*., 2016; Langevin and Bazinet, 2011). However, very thin red fouling was observed on the latest AEM, which may be due to possible hydroxyl ion leakage, which causes precipitation of hem or peptide-hem associations, as described for chitosan precipitation at the interface of the anionic membranes by Lin Teng Shee *et al*. (2007).

IV.5.3. Ash content and mineral composition

IV.5.3.1. Evolution of ash content

The ash content evolution, from the 1% initial HB solution to the end of the three processes (conventional hydrolysis (control), EDBM-MCP and EDBM-AEM), is shown in **Figure IV-8.**

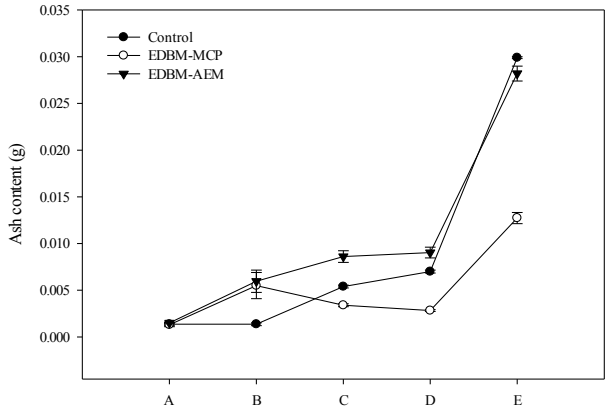

Figure IV-8. Ash content (g) evolution during three different processes. Letters A to E refer respectively to hemoglobin solution, conductivity adjustment (only for EDBM-MCP and EDBM-AEM), end of acidification, end of hydrolysis and reaction stop.

For 1% HB solution, at the beginning of the treatment, as expected, the ash content was the same whatever the conditions, with an average value of 0.0015 ± 0.0000 g. After conductivity adjustment for EDBM-MCP and EDBM-AEM, which corresponds to the addition of

potassium chloride to ensure a minimum conductivity of the hemoglobin solution before its treatment in ED, as expected, the ash content increased. Since the same quantity of KCl was added (0.18 g) for both ED treatments, the final amount of ash reached an averaged value of 0.0057 ± 0.0002 g. Since no adjustment was carried out for conventional hydrolysis, the amount of ash remained the same. At the end of acidification, the ash content was significantly different ($p < 0.05$) between the three conditions. It increased to 0.0054 ± 0.0001 and 0.0086 ± 0.0006 g for control and EDBM-AEM, respectively, while it decreased to 0.0034 ± 0.0001 g in EDBM-MCP. At the end of the hydrolysis phase, the ash content was also significantly different ($p < 0.05$) between the three conditions. It continued to increase for control and EDBM-AEM to reach, respectively, 0.007 ± 0.0001 and 0.009 ± 0.0005 g while it still decreased for EDBM-MCP to reach 0.0028 ± 0.0001 g. After the reaction was stopped, the final ash content was significantly different ($p < 0.05$) between the three conditions. For all three processes, the amount of ash logically increased due to the addition of KOH (0.5 M) to stop the reaction at pH 9, but the final ash content was different between the three conditions. The highest amount of ash was reported for the control, with a final ash content of 0.0291 ± 0.0005 g in comparison to 0.0282 ± 0.0004 and 0.0127 ± 0.0001 g for EDBM-AEM and EDBM-MCP, respectively. During the hydrolysis phase in EDBM-MCP, the pH was at 3.67 ± 0.03, while it was maintained at 3 in control and EDBM-AEM, hence the need for a lower volume of KOH to adjust the pH at 9 in EDBM-MCP.

IV.5.3.2. Mineral composition of the 1% Hb solution after hydrolysis

The determination of the mineral composition in the hemoglobin solution at the end of hydrolysis under the three different conditions was important to test the feasibility of the ED configuration to reduce the salt concentration in comparison with conventional hydrolysis. The minerals identified are chloride, potassium, sulfur and sodium. The concentrations of these minerals (mg/L) are shown in **Tableau IV-3**.

Tableau IV-3. Mineral concentration (mg/L) of hemoglobin solution at the end of the hydrolysis.

Conditions	Cl	K	S	Na
Control	388.3 ± 5.03^{a}	3.73 ± 0.34^{a}	61.35 ± 1.31^{a}	2.85 ± 0.11^{a}
EDBM-MCP	197.6 ± 4.04^{b}	6.48 ± 0.15^{a}	41.37 ± 0.98^{b}	1.68 ± 0.8^{a}
EDBM-AEM	235.6 ± 4.16^{c}	364.76 ± 3.8^{b}	313.69 ± 3.87^{c}	23.35 ± 2.06^{b}

P = probability level for the difference in mineral concentrations between control, EDBM-MCP and EDBM-AEM ($p < 0.05$); Population means within each column with different letters are significantly different $p < 0.05$ (Tukey).

Chloride concentration (mg/L) of hemoglobin solution after 3 h of hydrolysis (before stopping the reaction with KOH) was significantly different ($p < 0.05$) between control, EDBM-MCP and EDBM-AEM (**Tableau IV-3**). The higher chloride concentration observed in the control in comparison with EDBM conditions was due to the addition of hydrochloric acid (HCl) throughout the process to adjust and maintain the pH at 3. In EDBM, the pH was adjusted and maintained by the bipolar membrane without addition of chemicals. Although KCl was added, for EDBM experiments, to adjust the conductivity of the solution, the final concentration was lower than the one of the control. Furthermore, the lower Cl^- ion concentration in EDBM-MCP was due to the migration through the AEM of Cl^- ions from the basified KCl solution to the hemoglobin compartment to keep it electroneutral, while the MCP did not allow the migration of Cl^- ions, so they remained in the hemoglobin compartment, as discussed previously (Section § 3.2.2).

Potassium concentration (mg/L) of hemoglobin solution was significantly different ($p < 0.05$) for control vs. EDBM-AEM and EDBM-MCP vs. EDBM-AEM, but no difference was observed between control and EDBM-MCP. The higher K^+ concentration observed in EDBM-AEM was due to the fact that K^+ were retained in hemoglobin compartment by the AEM, while K^+ migrated through the MCP, as explained previously (Section § 3.2.1). For the control, its low concentration in potassium is related to its low concentration in the hemoglobin powder since no potassium was added throughout this treatment.

Sulfur concentration (mg/L) significantly changed ($p < 0.05$) between the three treatments. The higher sulfur content in EDBM-AEM than in control and EDBM-MCP was due to the migration of sulfate ions (SO_4^{2-}) from the electrode rinsing solution (Na_2SO_4) through the

AEM. For control and EDBM-MCP, since, throughout the treatment, there was no addition of sulfur, the low concentration of sulfur observed is probably due to its presence in the starting hemoglobin powder.

Finally, sodium concentration (mg/L) significantly changed ($p < 0.05$) for control vs. EDBM-AEM and EDBM-MCP vs. EDBM-AEM, and no significant difference appeared between control and EDBM-MCP. Since no addition of sodium was carried out, the concentration of sodium observed is probably due to its presence in the starting hemoglobin powder for the three conditions.

IV.5.4. Enzymatic kinetics and mechanism of action of the new process

IV.5.4.1. Determination of the degree of hydrolysis

The degree of hydrolysis (DH) has an identical tendency to increase over time in control, EDBM-MCP and EDBM-AEM (**Figure IV-9**). The shape of the curves was identical to those previously published in the literature (Przybylski *et al.*, 2016). For control and EDBM-AEM, the DH evolution was identical, while a statistical difference was observed ($p < 0.05$) between the control and EDBM-MCP. A higher DH was observed for the control compared to EDBM-MCP and EDBM-AEM. Hydrolysis of bovine hemoglobin was characterized by a high rate of hydrolysis during the initial 30 min for the three conditions. At T_{60min}, the rate of enzymatic hydrolysis increased slightly, and then it continued to increase quite linearly until 180 min. At 180 min, the degree of hydrolysis was not significantly different ($p > 0.05$) between control (7.25 ± 0.03) and EDBM-AEM (7.12 ± 0.08), while there was a significant difference ($p < 0.05$) between control (7.25 ± 0.03) and EDBM-MCP (6.7 ± 0.076) as well as between EDBM-MCP (6.7 ± 0.076) and EDBM-AEM (7.12 ± 0.08). The lower DH was observed in EDBM-MCP, meaning that there was a slow-down in enzymatic kinetics during the hydrolysis of hemoglobin in this special condition. This lower final DH could be due to the fouling of MCP containing peptides that were not hydrolyzed and/or to the inability to adjust the pH to 3 during the hydrolysis phase, affecting consequently the efficiency of pepsin.

Indeed, it was demonstrated that pH 3 was the most efficient pH for hemoglobin hydrolysis by pepsin while keeping the solution homogeneous (Lignot *et al.*, 1999).

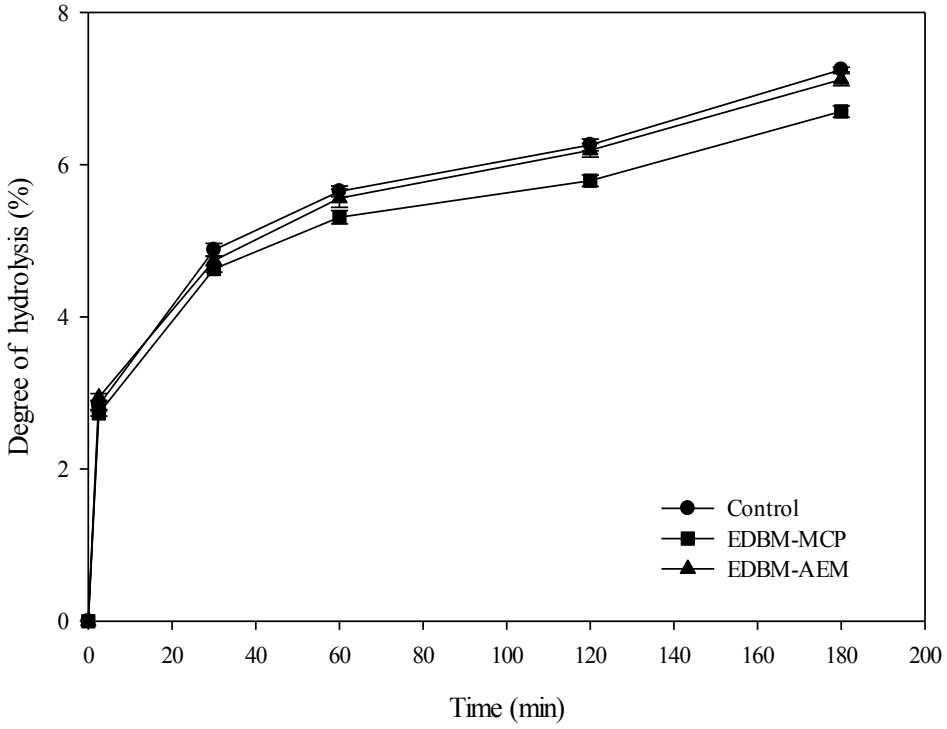

Figure IV-9. Hydrolysis curves of hemoglobin hydrolysis in control, EDBM-MCP and EDBM-AEM treated with pepsin for 3 h. Hydrolysis was conducted at the following conditions: enzyme (U)/protein(mg) ration of 1/11, pH 3 and at 30 °C.

IV.5.4.2. Reaction mechanism of bovine hemoglobin hydrolysis by pepsin and identification of α137-141 Peptide

To understand the different enzymatic kinetics and to identify the enzymatic mechanism of bovine hemoglobin hydrolysis obtained in EDBM and control, the UPLC-QTOF chromatographic profiles obtained as a function of time for the three conditions were analyzed. Chromatographic profiles of hydrolysis in control, EDBM-MCP and EDBM-AEM are shown, respectively, in **Figures IV-10, IV-11** and **IV-12**. The most striking fact was that the chromatograms of control, EDBM-MCP and EDBM-AEM were almost the same.

Three peaks are shown at T0 before adding the pepsin: α-chain, β-chain and hem (**Figures IV-10, IV-11** and **IV-12).** After adding pepsin, the hemoglobin subunits were rapidly

hydrolyzed to intermediate peptides which were converted to final peptides, smaller and generally more hydrophilic than intermediate peptides. This enzymatic mechanism, called « Zipper », was described for the first time by Linderstrom-Lang, (1953). The enzymatic hydrolysis of bovine hemoglobin in EDBM-MCP and EDBM-AEM allowed a « Zipper » mechanism identical to the one obtained in conventional hydrolysis (control). When hemoglobin is completely denatured by adjusting the pH to 3, it allows the complete opening of its globular chain. This privileges the enzymatic mechanism « Zipper » at the expense of the « one-by-one » mechanism, observed for higher pH (Dubois *et al*., 2005). This mechanism allowed us to obtain α137-141 peptide that was identified by UPLC-MS/MS. The same mechanism was observed in other studies for conventional hydrolysis of hemoglobin and described by Nedjar-Arroume *et al*. (2008), Nedjar-Arroume *et al*. (2006) and Dubois *et al*. (2005). α137-141 appeared identically in the three conditions from the beginning of hydrolysis and its concentration increased throughout the course of proteolysis. The α137-141 production was fast-it appeared after only a few minutes from the start of hydrolysis. It thus accumulated during hydrolysis, meaning that it was a final peptide, not being hydrolyzed in other shorter peptides under the conditions of hydrolysis used here (30 °C; pH 3, C_{BH} of 1% w/v). This confirmed what was previously described by Lignot *et al*. (1999). Moreover, these conditions have shown an important accumulation of α137-141 peptide, explained by the « Zipper » mechanism. The peptide α137-141 represents the last five amino acids at the C-terminal of the α chain of bovine hemoglobin, namely Thr-Ser-Lys-Tyr-Arg (TSKYR), which has a molar mass of 653 Da and has an isoelectric point of 10.5. It is thus charged +2 at a pH of 7 (Catiau *et al*., 2011).

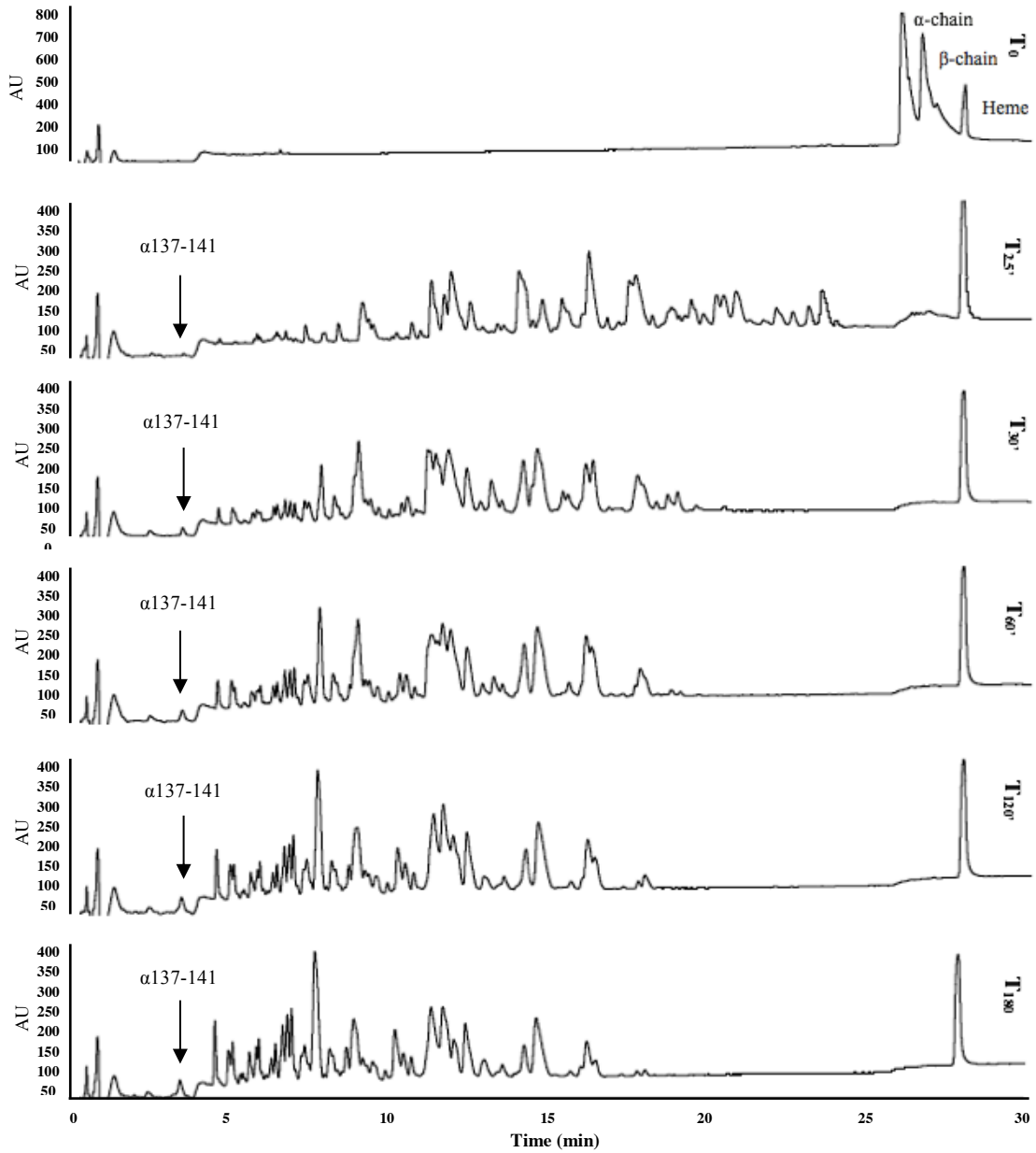

Figure IV-10. Chromatographic profiles of hydrolysis of bovine hemoglobin in control at 214 nm by UPLC-QTOF, analyzed by C18 column at different hydrolysis degrees for 3 hours (pH 3, 30°C, E/S = 1/11, CBH = 1%, w/v).

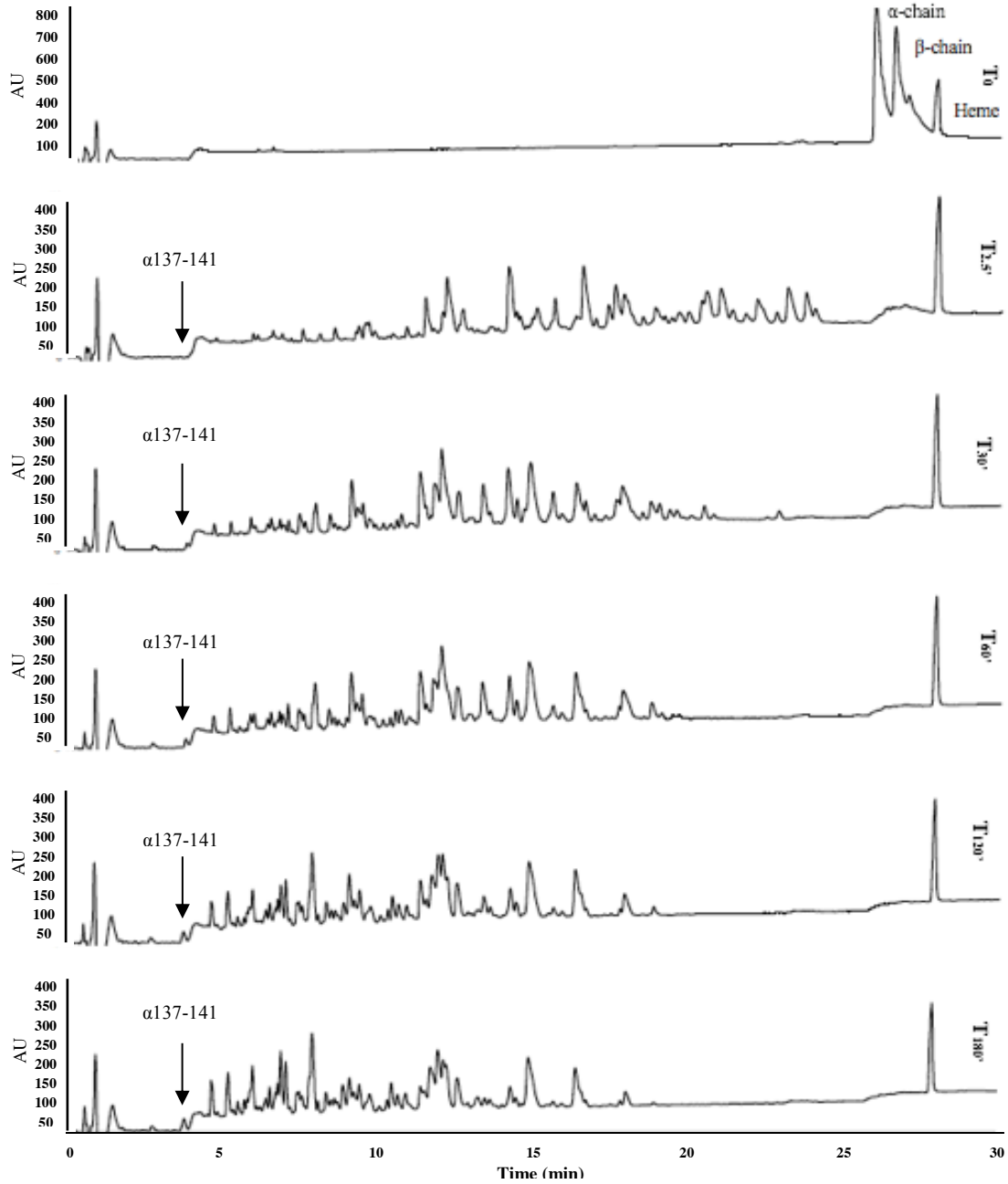

Figure IV-11. Chromatographic profiles of hydrolysis of bovine hemoglobin in EDBM-MCP at 214 nm by UPLC-QTOF, analyzed by C18 column at different hydrolysis degrees for 3 hours (pH 3, 30°C, E/S = 1/11, CBH = 1%, w/v).

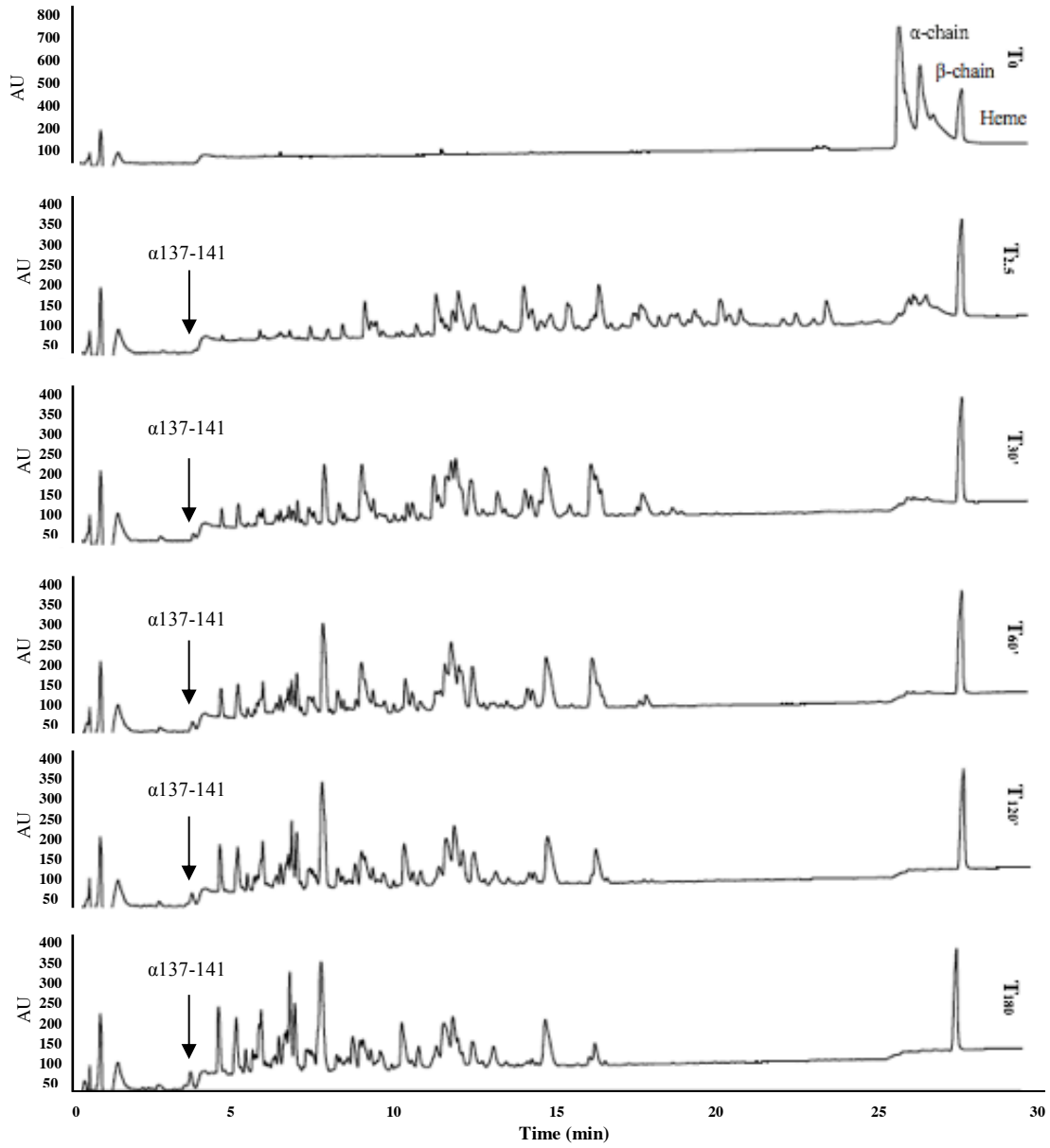

Figure IV-12. Chromatographic profiles of hydrolysis of bovine hemoglobin in EDBM-AEM at 214 nm by UPLC-QTOF, analyzed by C18 column at different hydrolysis degrees for 3 hours (pH 3, 30°C, E/S = 1/11, CBH = 1%, w/v).

IV.5.4.3. Characterization of the peptide population

Principal Component Analysis (PCA) and Heat Maps

In order to investigate the different hydrolysis conditions, hydrolysates obtained following 3 h of hydrolysis were selected for peptide identification by LC-MS/MS. PCA was performed on the mass spectrometry data after importation of raw data in Progenesis QI for proteomics software (version 4.1). Peptic maps, representing retention time of the ions according to the m/z ratio of the ions and the intensity of the ions, obtained for all hydrolysates, were automatically and manually aligned thanks to the quality control used and peak picking of mass signals was constrained (§ M&M section). The PCA was done using the detected peptides and showed the peptide population obtained by considering all the mass signals (**Figure IV-13A**). The first two dimensions in the PCA explained 54.4% of the variance (detected ions corresponding to variables). The more distant the groups were, the more different they were in terms of ion population. The PCA clearly showed a similar ion population and thus a similar peptide population between the three repetitions of each condition, meaning that it was reproducible at the condition level. Additionally, PCA showed a slight difference in ion population and thus a slight difference in peptide population between control, EDBM-MCP and EDBM-AEM. Moreover, the divergence between the different identified peptides in control, EDBM-MCP and EDBM-AEM were subsequently presented in the form of a heat map via Peaks Studio Software (Version 8.5, Bioinformatics Solutions Inc., Waterloo, Canada)) (**Figure IV-13B**).

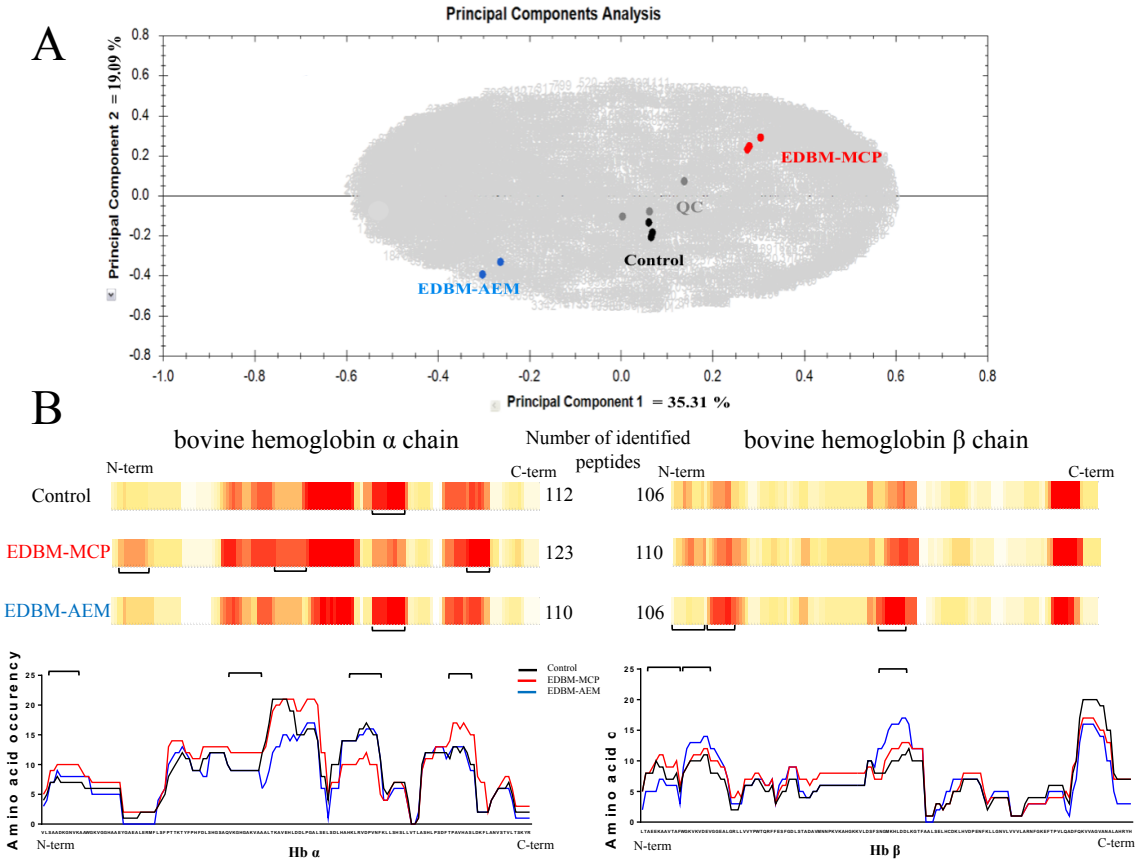

Figure IV-13. (A) Principal component analysis based on comparison of mass spectrometry detected ions in bovine hemoglobin hydrolysates. Quality control (QC) samples correspond to equivolume mixtures of the 9 hydrolysates (three control, three EDBP-AEM and three EDBM-MCP) injected at the beginning, the middle and the end of the HPLC-MS/MS analysis session. (**B**) Heat maps showing amino acid occurrence in the identified peptides in the primary sequences of the bovine hemoglobin α and β chains after its hydrolysis in the three conditions. Underlined regions highlight peptide pattern differences. Heat maps were generated after combination of three LC-MS/MS analysis for each replicate.

For each amino acid from the protein sequence, a score was calculated according to its occurrence in each peptide sequence identified. The higher the occurrence, the more the peptide zone will tend towards red. The red areas represent the areas where amino acids are identified very often, the lighter areas where amino acids are poorly identified and the white areas where there has been no identification. This makes it easy to see the areas which are resistant to hydrolysis (red areas). For the three conditions, some color similarities were

observed in certain parts of the map as well as differences (underlined regions in **Figure IV-13**).

The number of identified peptides in the alpha chain of hemoglobin between the control and EDBM-AEM was roughly the same, with 112 and 110 peptides, respectively, while 123 peptides were identified in EDBM-MCP. The numbers of identified peptides for bovine hemoglobin β chain were 106, 110 and 106 for the control, EDBM-MCP and EDBM-AEM, respectively. A slightly higher number of identified peptides was identified in EDBM-MCP, for bovine hemoglobin α and β chains, in comparison with control and EDBM-AEM. This was probably due to the presence of precursors in EDBM-MCP which were not hydrolyzed in other shorter peptides because of a slow-down in the kinetics of enzymes. This finding confirmed the results observed for degree of hydrolysis. Moreover, this difference can also be explained by the fact that the three conditions were tested with different acidification times, different types of membranes and different mineral contents.

IV.5.4.4. Quantification of α137-141 derived from the new process

Since α137-141 was identified in the three conditions, its quantification was done by RP-HPLC. This measurement was made for C_{BH} = 1% (w/v), with ratio enzyme/substrate=1/11 (mole/mole) after three hours of hydrolysis for control, EDBM-MCP and EDBM-AEM. For control, $C_{\alpha 137\text{-}141}$ was 1.11 ± 0.16 µg/mL, while it was 1.13 ± 0.05 µg/mL in EDBM-MCP and 1.09 ± 0.09 µg/mL in EDBM-AEM. Statistical analysis demonstrated that $C_{\alpha 137\text{-}141}$ was the same ($p > 0.05$) for the three conditions. Therefore, the bipolar membranes made it possible to produce the same amount of α137-141 peptide as in the control, without the use of chemical agents.

IV.6. Conclusion

In this study, hydrolysis by EDBM was investigated to obtain the α137-141 peptide, a peptide of great interest for the food industry since it is considered as a natural antimicrobial and antioxydant peptide. It appeared from these results that EDBM was efficient to denature

hemoglobin by reducing the pH to 3 and then to regulate the pH of the solution during hydrolysis. Furthermore, it was demonstrated that hydrolysis in EDBM has the same enzymatic mechanism as the conventional hydrolysis, the « Zipper » mechanism. This mechanism made it possible to obtain a large peptide population that was approximately the same as in the control and without the use of chemical solvents. This new successful concept, based on the generation of protons from water dissociation to control hydrolysis, also allowed the production of α137-141 at the same concentration as conventional hydrolysis. Hence, EDBM-MCP, allowed simultaneous demineralization of the hemoglobin solution compared to conventional hydrolysis and EDBM-AEM and production of α137-141 peptide with a low mineral content. In comparison, EDBM-AEM allowed the production of α137-141 peptide more rapidly without membrane fouling and involving minimal global resistance and consequently a lower energy consumption.

In this study, electrodialysis with bipolar membrane (EDBM) was demonstrated for the first time as an eco-efficient and innovative technology to produce peptide hydrolysates from enzymatic hydrolysis of bovine hemoglobin with low mineral salt concentration. This was due to the specific characteristics of the bipolar membranes which allow us to generate *in situ* H^+ and OH^- ions from water dissociation under an electrical field and consequently do not need the use of any chemical agents. Further experiments will focus on minimizing peptide fouling on the monovalent perm-selective membrane, which can be an effective means of improving bovine hemoglobin demineralization, by applying a pulsed electric field or an over-limiting current regime, by changing the configuration of EDBM or by modification of ion-exchange membranes (Bazinet and Geoffroy, 2020).

This new green application of EDBM to the production of α137-141 from a by-product of slaughterhouses fits perfectly with the concept of circular economy. Indeed, hemoglobin from blood, once hydrolyzed, allows the bioproduction of active peptides that can then be used in meat or meat products to increase their preservation and innocuity.

IV.7. Acknowledgements

The Natural Sciences and Engineering Research Council of Canada (NSERC) financial support is acknowledged. This work was supported by the NSERC Industrial Research Chair METABIOLAC (Grant IRCPJ 499946-15 to Ismail Fliss), the NSERC strategic partnership grants for projects Program (Grant SPGP 5212082 to Laurent Bazinet) and the NSERC Discovery Grants Program (Grant SD 210829409 to Laurent Bazinet). This work was realized within the French-Canadian International Associated Laboratory on «Bioproduction of natural antimicrobials» (LIAAN). The authors thank Véronique Perreault and Diane Gagnon, research professionals at Laval University and Rémi Przybylski, doctor at Charles Violette Institute for their technical assistance and for their help at the lab. The authors are grateful to the Advanced High Throughput Technologies Platform for Biorefineries Catalysts Design « REALCAT » funded by a French governmental subsidy managed by the French National Research Agency (ANR) within the frame of the « Future Investments program (ANR-11- EQPX-0037) ». The Hauts-de-France region and the FEDER, the Ecole Centrale de Lille and the Centrale Initiatives Foundation are also warmly acknowledged for their financial contributions to the acquisition of REALCAT platform equipment.

IV. *Deuxième Article*

IV.1. Résumé

L'hémoglobine est une protéine riche en peptides antibactériens et antioxydants après son hydrolyse. Dans le contexte actuel de sécurité alimentaire, de tels peptides bioactifs issus de l'hydrolyse enzymatique de l'hémoglobine représentent des conservateurs potentiels prometteurs pour le secteur alimentaire. Dans cette étude, pour produire des peptides bioactifs, l'hydrolyse de l'hémoglobine a été réalisée dans un milieu à pH régulé sans utilisation de solvants chimiques par un procédé éco-efficient : l'électrodialyse avec membranes bipolaires (EDMB). La configuration bipolaire/monopolaires utilisant les ions H^+ et OH^- générés par les membranes bipolaires a été étudiée. Le but de cette étude était

d'identifier les peptides bioactifs produits par EDMB en comparaison avec l'hydrolyse conventionnelle et d'identifier leur activité biologique. L'utilisation de l'EDMB pour l'hydrolyse enzymatique de l'hémoglobine a permis la production de 17 peptides bioactifs. Les hydrolysats obtenus ont montré une excellente activité antibactérienne, une activité antioxydante et, pour la première fois, une activité antifongique.

Mots clés : Hémoglobine bovine, Électrodialyse avec membranes bipolaires, Peptides bioactifs, Activité antibactérienne, Activité antifongique, Activité antioxydante.

IV.2. Abstract

Bovine cruor, a slaughterhouse waste, is mainly composed of hemoglobin, a protein rich in antibacterial and antioxidant peptides after its hydrolysis. In the current context of food safety, such bioactive peptides derived from enzymatic hydrolysis of hemoglobin represent potential promising preservatives for the food sector. In this work, the hemoglobin hydrolysis to produce bioactive peptides was performed in a regulated pH medium without the use of chemical agents and by an eco-efficient process: electrodialysis with bipolar membranes (EDBM). Bipolar/monopolar (anionic or cationic) configuration using the H^+ and OH^- ions generated by the bipolar membranes to regulate the pH was investigated. The aim of this study was to present and identify the bioactive peptides produced by EDBM in comparison with conventional hydrolysis and to identify their biological activity. The use of the EDBM for the enzymatic hydrolysis of hemoglobin has allowed for the production and identification of 17 bioactive peptides. Hydrolysates obtained by EDBM showed an excellent antimicrobial activity against six strains, antioxidant activity measured by four different tests and, for the first time, anti-fungal activities against five yeasts and mold strains. Consequently, this enzymatic hydrolysis carried out in regulated pH medium with bipolar membranes could provide bioactive peptides presenting antibacterial, antifungal and antioxidant interest.

Keywords: bovine hemoglobin; electrodialysis with bipolar membrane; bioactive peptides; antibacterial activity; antifungal activity; antioxidant activity.

IV.3. Introduction

According to a report published in 2018 by The World Bank, by 2050, the world is expected to generate 3.40 billion tons of waste annually, increasing drastically from today's 2.01 billion tons (Kaza *et al*., 2020). Hence, the industrial processes used in the food industries participate to this growing production of waste driven by rapid urbanization and growing populations. To move towards a circular or sustainable economy, the valorization of the many bio food industry co-products, underestimated and underused, by producing bioproducts with high added value, becomes a key issue. The circular economy is an economy operating in a loop and thus avoiding waste. Its objective is to produce goods but at the same time to strongly limit the consumption of non-renewable energy sources and raw materials.

Amongst these agri-food wastes, bovine blood from slaughterhouses, is a natural bioresource produced in all countries around the world. Billions of tons of blood are collected every year, and they are processed into blood meal and sold as low-value animal food and fertilizer or discarded as effluent (Aderson and Yu, 2008; Yu *et al*., 2010). This large quantity of animal blood generated is a problematic by-product for the meat industry since it has a very high pollutant load when discarded into the environment with minimal treatments (Del Hoyo and al., 2008; Bah *et al*., 2013). The Cruor represents 40% of the whole blood and contains mainly hemoglobin (90%), a protein broadly described as a rich source of bioactive peptides after its enzymatic hydrolysis (Adje *et al*., 2011; Gomes *et al*., 2010; Nedjar *et al*., 2008). Various biological activities, including opioid activity (Lignot *et al*., 1999; Zhao and Piot, 1997a; 1997b), hematopoietic (Vercaigne-Marko *et al*., 2000) and antihypertensive (Adje *et al*., 2011) have been reported for hemoglobin protein hydrolysate. The most described activities are antimicrobial (Daoud *et al*., 2011; Nedjar *et al*., 2006; Choisnard *et al*., 2002) and antioxidant activities (Przybylski *et al*., 2016). Many studies have shown that bioactive peptides derived from conventional enzymatic hydrolysis of hemoglobin can represent a natural alternative to synthetic preservatives that are suspected to induce pathological and toxic effects (Bauer *et al*., 2011; Imaida *et al*., 1983; Lanigan and Yamarik, 2002). These peptides have been used recently to protect meat, reducing lipid oxidation and microbial growth during food storage and distribution (Przybylski *et al*., 2016). Hence, in link with the

concept of the circular economy, blood from slaughterhouses can thus be recycled by the meat industry for the production of biopeptides with improved functions. Furthermore, this is also in direct link with the current context of food safety and food protection by means of natural products, adding value to proteins from wastes (Bah *et al*., 2013; Goot *et al*., 2016; Lafarga and Hayes, 2014).

However, the conventional process of hydrolysis includes several steps consisting of hemoglobin denaturation by chemical acidification, hydrolysis with pepsin enzyme and pH adjustment during hydrolysis. Due to the use of a chemical basis and acids, the final hydrolysates contain high levels of mineral salts. According to recent life cycle assessments, an electro-membrane process recognized as green process (Mikhaylin *et al*., 2018; Chaudron *et al*., 2019; Faucher *et al*., 2020) electrodialysis with a bipolar membrane (EDBM) was tested with two different membrane configurations (cationic (MCP) and anionic (AEM) membranes) as described by Abou-Diab *et al*. (2020a). It was reported that hydrolysis in EDBM showed the same enzymatic mechanism « Zipper » as observed in conventional hydrolysis. Moreover, EDBM allowed the generation of α137-141 in the same concentration as for the conventional hydrolysis. EDBM-MCP allowed the production of hydrolysates containing a low concentration of mineral salts while EDBM-AEM, allows the production of hydrolysates with a similar salt content as conventional hydrolysis. This was due to the specific characteristics of the bipolar membranes which allow to generate *in situ* H^+ ions from water dissociation under an electrical field and consequently no need to use any chemical agent during hydrolysis. However, this study focused on the production of α137-141 peptide and did not test the bioactivities of these hydrolysates produced by this new and eco-efficient technology.

The aim of the present study was to produce bioactive peptides derived from enzymatic hydrolysis of bovine hemoglobin using EDBM. The objectives of this study were to (1) identify and characterize the peptide population of the different conditions by UPLC-MS/MS, (2) identify the bioactive peptides resulting from this hydrolysis of hemoglobin and (3) study the biological activities of the hydrolysates obtained from EDBM. First, the antibacterial potential was investigated using the agar diffusion method and minimum

inhibitory concentration against six bacteria. Next, the antifungal activity was evaluated using the agar diffusion method against five strains. At the end, the antioxidant potential was investigated using several measurements, including β-carotene bleaching inhibition activity, DPPH radical scavenging activity assay, ABTS radical scavenging activity assay and evaluation of total antioxidant capacity to provide a clear idea about their real antioxidant potential.

IV.4. Materials and Methods

IV.4.1. Materials

Reagents. KCl was obtained from EMD Chemicals Inc., USA, Na_2SO_4 was supplied by ACP Inc. (Montréal, QC, Canada), KOH was purchased from Fisher Scientific (New Jersey, NJ, USA) and HCl was purchased from Anachemia, VWR Company International (ON, Canada). Chemicals required for the assays including 1,1-diphenyl-2-picrylhydrazyl (DPPH), Butylated hydroxytoluene (BHT), 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), β-carotene, and linoleic acid were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Neokyotorphin (α137-141) was purchased from GeneCust (Boynes, France). The ultrapure water was prepared using a Milli-Q system.

Cultures. *Listeria monocytogenes* (ATCC 19112), *Staphylococcus aureus* (ATCC 13709), *Micrococcus luteus* (ATCC 9341), *Escherichia coli* (ATCC 8733) and *Salmonella Newport* (ATCC 6962) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). *Kocuria rhizophila* (CIP 53.45), was from Collection de l'Institut Pasteur (CIP, Paris, France). *Aspergillus Niger* (3071-13) and *Paecilomyces spp* (5332-9a) were isolated from dairy product (strain collection of Denis Roy, Laval University, Québec, QC, Canada), *Mucor racemosus* (LMA-722) was obtained from Laboratoire de mycologie alimentaire (Laval university, Québec, QC, Canada), *Penicillium crustosum* (27,159) and *Rhodotorula mucilaginosa* (27,173) were isolated from dairy product (strain collection of General Mills Yoplait, France).

Pepsin. The pepsin lyophilized powder from porcine gastric mucosa was purchased from Sigma-Aldrich (P6887, Oakville, ON, Canada). It was used for the production of the hydrolysate. Pepsin activity was determined according to a protocol established by the supplier Sigma-Aldrich (Saint-Quentin Fallavier, France). Pepsin was stored at -20 °C.

Hemoglobin. The purified hemoglobin powder from bovine blood, dark brown, was purchased from Sigma-Aldrich (H2625, Oakville, ON, Canada). Hemoglobin was stored at 4 °C before use.

IV.4.2. Preparation of bovine hemoglobin hydrolysates

As described by Abou-Diab *et al*. (2020a), three conditions have been set up: hydrolysis by electrodialysis with bipolar and cationic membranes « EDBM-MCP », hydrolysis by electrodialysis with bipolar and anionic membranes « EDBM-AEM » and conventional hydrolysis in beaker considered as « control ».

IV.4.2.1. Stock solution preparation

In total, 15 g of purified bovine hemoglobin (BH) was solubilized in 100 mL of ultrapure water. After that, the solution was centrifuged at 6000× *g* for 30 min (Eppendorf AG, 22331 Hamburg, Centrifuge 5804 R, Brinkmann Instruments, Westbury, NY, USA). Drabkin's method was later used on the recovered supernatant to determine the real BH concentration (C_{BH}) (Crosby *et al*., 1954). From the concentration C of the stock solution, a solution of purified BH was prepared by dilution to a 1% (*w/v*) precise concentrations of hemoglobin C_{BH}.

IV.4.2.2. Hydrolysis process

Briefly, and according to Abou-Diab *et al*. (2020a), hydrolysis was carried out at pH 3 to denature the hemoglobin solution, at a constant temperature of 30 °C. pH was adjusted to 3 with hydrochloric acid 2 M in conventional hydrolysis and adjusted to 3 by bipolar membrane

which generated the H^+ ions from water dissociation under an electrical field in EDBM-MCP and EDBM-AEM. The hydrolysis reaction was started by the addition of pepsin (EC 3.4.23.1, 3200-4500 units mg^{-1} protein) at a 1:11 (mole/mole) enzyme/protein ratio. Samples were collected at T_0, $T_{2.5}$, T_{30}, T_{60}, T_{120} and T_{180} min of hydrolysis, corresponding to different degrees of hydrolysis. In total, 0.5 M of potassium hydroxide was added to stop the peptic hydrolysis reaction by adjusting the pH to 9. The samples (triplicates) were stored at -20 °C, and then they were lyophilized, and the recovered powder was ready for testing. The current intensity, conductivities, pH and temperature, for both 1% HB and KCl solutions, were recorded every 30 min during EDBM treatments.

IV.4.3. Electrodialysis cell

The electrodialysis (ED) cell was a Microflow-type cell with 10 cm^2 of effective membrane surface, purchased from ElectroCell AB (Taby, Sweden). The anode used was a dimensionally stable electrode and the cathode was a 316 SS electrode. A BK Precision power supply (Model BK9110-ND, Vancouver, BC, Canada) was employed at a constant voltage of 15 V, corresponding at the beginning of the process to a current density of 5.3 mA/cm^2. The cell configurations are those presented previously by Abou-Diab *et al*. (2020a) and displayed in **Figure IV-14**. Two ED configurations were tested: the ED configuration named «EDBM-AEM» (**figure IV-14a**) formed by stacking three anion-exchange membranes (AEM, Astom, Tokyo, Japan) and two bipolar membrane (BM, Astom, Tokyo, Japan) and the ED configuration named «EDBM-MCP» (**Figure IV-14b**) stacking one anion-exchange membrane (AEM, Astom, Tokyo, Japan), two monovalent cation permselective membranes (MCP, Japan Food grade Neosepta, Astom, Tokyo, Japan) and two bipolar membranes (BM, Astom, Tokyo, Japan). In both cases, the ED configuration defined three closed loops containing 200 mL of hemoglobin solution (1% Hg *w/v*), 200 mL of salt ion recovery (KCl 1 g/L (EMD Chemicals Inc, Port Wentworth, GA, USA)), and 250 mL of electrode-rinsing solution (Na_2SO_4 20 g/L (ACP Inc., Montréal, QC, Canada)).

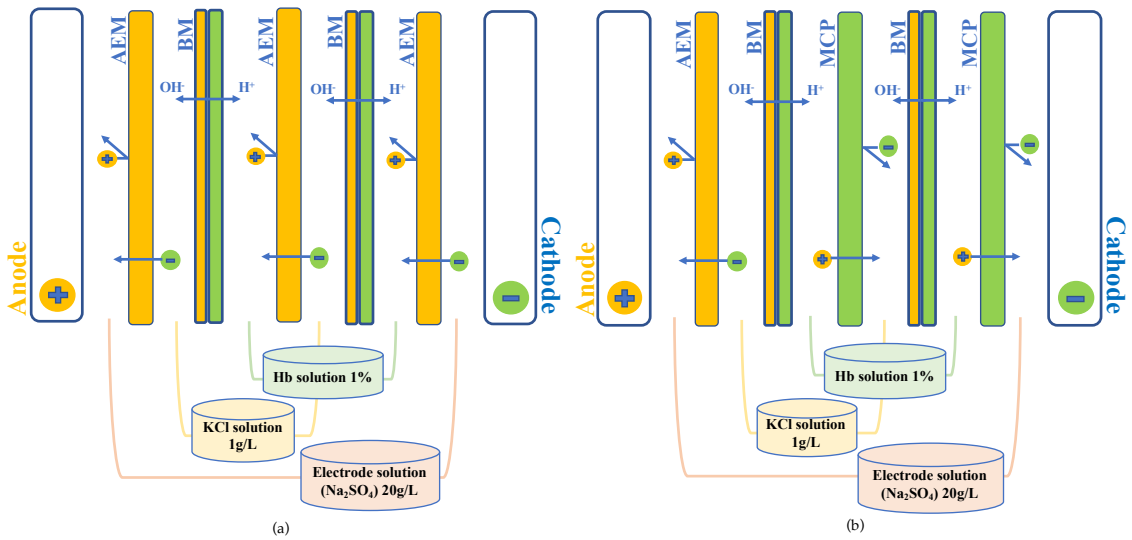

Figure IV-14. Schematic representation of electrodialysis cell configurations used for electro-acidification and hydrolysis of bovine hemoglobin in (a) electrodialysis with bipolar and anion exchange membranes (EDBM-AEM) and (b) electrodialysis with bipolar and monovalent cation permselective membranes (EDBM-MCP). MCP: monovalent cation permselective membrane; AEM: anion exchange membrane; BM: bipolar membrane.

From the results of the technological part of this study (Abou-Diab *et al.*, 2020a), EDBM, in batch mode by recirculation of the solution until pH 3 was obtained, was efficient to denature hemoglobin. The mean times required to electro-acidify the 1% HB solution were 390 ± 20 min and 60 ± 2 min in EDBM-MCP and EDBM-AEM, respectively, while the global process durations to produce peptides (electro-acidification + hydrolysis) were 570 ± 20 min and 240 ± 2 min in EDBM-MCP and EDBM-AEM, respectively. Conductivity values tend to decrease for cationic configuration because of partial demineralization (34%) caused by EDBM-MCP configuration and tend to increase for anionic configuration due to partial mineralization (88%) caused by migration of anions from KCl solution. Furthermore, EDBM-MCP, allowed the production of α137-141 peptide with a low mineral content but the MCPs exhibited a fouling, due to electrostatic interactions between peptides (positively charged) and the membranes (negatively charged). In comparison, EDBM-AEM, allowed for the production of the α137-141 peptide more rapidly without membrane fouling. The subsequent sections will focus on identifying the biological activity of the hydrolysates produced.

IV.4.4. Analysis

IV.4.4.1. RP-UPLC

Materials, Software and Elution Program Used: All samples were analyzed with two different UPLC to identify peptide population and bioactive peptides.

First method: RP-UPLC analyses performed on all samples used a 1290 Infinity II UPLC (Agilent Technologies, Santa Clara, CA, USA). The RP-UPLC apparatus was composed of a binary pump (G7120A), a multisampler (G7167B), an in-line degasser and a variable wavelength detector (VWD G7114B) adjusted to 214 nm. 0.22 µm PVDF filter were used to filter hydrolysis fractions into a glass vial. Samples were loaded (0.25 µL) into a Poroshell 120 EC-C18 column (2.1 × 100 mm i.d., 2.7 microns, Agilent, Santa Clara, CA, USA). A flow rate of 500 µL/min at 23° C was used to operate the column. A gradient was applied for the mixture of solvent A (LC-MS grade water with 0.1% formic acid) and solvent B (LC-MS grade ACN with 0.1% formic acid) with solvent B increasing from 1% to 13% in 6 min, to 35% until 25 min, and to 100% until 35 min and holding until 45 min, then back to initial conditions. Each sample analysis was performed in triplicate for ensuring technical reproducibility.

Second method: After sample drying, peptides were dissolved in H2O/0.1% TFA at 0.5 mg/mL and centrifuged for 5 min at 10,000× *g*. 10 µL were separated by chromatography at 30 °C on an ACQUITY UPLC system (Waters Corporation, Guyancourt, France) equipped with a Kinetex 2.6 µm C18 100 Å (150 × 4.6 mm) column (Phenomenex). Acetonitrile gradient was set at 0.5 mL/min (the mobile phases were composed of solvent A (0.1% (*v/v*) formic acid/99.9% (*v/v*) water) and solvent B (0.1% (*v/v*) formic acid/99.9% (*v/v*) acetonitrile): from 5% to 15% solvent B during the first 30 min, from 15% to 30% over 60 min, from 30% to 50% over 10 min and then maintained during 10 min at 95% solvent B. The eluate was then directed into the electrospray ionization source of the qTOF Synapt G2-Si™ (Waters Corporation). The analysis was performed in sensitivity, positive ion and data dependent analysis (DDA) modes. The source temperature was at 150 °C and the capillary

and cone voltages were fixed, respectively, at 3000 and 60 V. Data for m/z values were collected in the range of 100 to 2000 Da with a scan time of 0.2 sec. A maximum of 10 precursor ions were chosen for MS/MS analysis with an intensity threshold of 100,000 with A Collision-induced dissociation (CID) fragmentation mode and a scan time of 0.1 sec were chosen for MS/MS data collection.

Identification of Peptide Population: PEAKS Studio (version 8.5, Bioinformatics Solutions Inc., Waterloo, ON, Canada) software and its database search tools were used for peptide identification. The UniProt database restricted to Bos Taurus was used. A mass tolerance of 35 ppm, 3 missing cleavage sites and an MS/MS tolerance of 0.2 Da were allowed while a variable methionine oxidation was considered. Protein and peptide identity relevance was judged according to their score in the research software (p value of 0.05 ($p < 0.05$), False Discovery Rate < 1%). For the search of peptides from the pepsic hydrolysate by Peaks® software, the Bos taurus bovine hemoglobin alpha and beta chain sequences were taken as model proteins. The number of unique and common peptides identified in all samples were subsequently presented in Venn diagram format using R software (R Core Team (2013).

Identification of Bioactive Peptides by Mass Spectrometry Analyses: To identify and quantify the relative abundances of the peptides present in the samples, a hybrid ion mobility quadrupole time of flight mass spectrometer 6560, IM-Q-TOF, Agilent, Santa Clara, USA) was used. All LC-MS/MS experiments were acquired using Q-TOF. A positive mode at Extended Dynamic Range, 2 Ghz, 3200 m/z with a scan range between 100 and 3200 m/z was used to record the MS/MS signals. The drying gas was nitrogen (purity 99,999% UHP-T (Praxair, Québec, QC, Canada)) at 13.0 L/min and 150 °C and was also used as nebulizer gas at 30 psig. The capillary voltage was set at 3500 V while the nozzle voltage at 300 V and the one of the fragmentor at 400 V. The instrument calibration was carried out by using an ESI-L low concentration tuning mix (G1969-85000, Agilent Technologies, Santa Clara, CA, USA). Data acquisition and analyses were performed with the Agilent Mass Hunter Software package (LC/MS Data Acquisition, Version B.08.00 and Qualitative Analysis for IM-MS, Version B.07.00 Service Pack 2 with BioConfirm Software, Santa Clara, CA, USA).

UPLC-MS/MS coupling was used to analyze the peptides. This coupling, offering the advantage of a complementarity between UV profile from the UPLC and the masses of the corresponding compounds for each point of the chromatogram. This also gives the amino acid composition of each compound. Several parameters were considered to ensure a good identification of bioactive peptides: the retention time, the monoisotopic molecular mass and the second-order derivative spectral analysis of peptides testified of the presence (or absence) of aromatic amino acids (Zhao *et al*., 1995). The presence of tyrosine (Tyr), tryptophan (Trp) or phenylalanine (Phe) in the peptide sequence indicates the presence of bioactive peptide. The peptides have been identified by comparing them with the known hemoglobin alpha and beta sequences with the BioConfirm software. Then bioactive peptides were identified by comparing them to the literature (Nedjar *et al*., 2008; Lignot *et al*., 1999; Adje *et al*., 2011; Takagi *et al*., 1982; Chang *et al*., 1980; Piot *et al*., 1992).

IV.4.4.2. Biological activities

Antibacterial Activity

- Agar diffusion method

The antibacterial activity was determined by the method of Yaba Adje, (2010). Six bacterial species were tested for determination of the antibacterial activity: *Staphylococcus aureus* (ATCC 13709), *Listeria monocytogenes* (ATCC 19112), *Micrococcus luteus* (ATCC 9341), *Kocuria rhizophila* (CIP 53.45), *Escherichia coli* (ATCC 8733) and *Salmonella Newport* (ATCC 6962). These bacterial species are commonly responsible for food alteration (Borch and Arinder, 2002; Catiau *et al*., 2011). These bacterial species were stored at -20 °C in glycerol containing nutrient broth and were sub-cultured twice in Muller-Hinton broth (MH) at 37 °C under agitation (60 rpm). After 24 h of incubation at 37 °C in nutrient broth, absorbance of the pre-cultures was determined at 620 nm using the medium MH as a blank. A dilution series of 1/100 and of 1/1000 were carried out in tryptone salt (TS) for gram (+) and gram (-) bacteria, respectively, in order to adjust inoculum concentration to $\times 10^6$ CFU/mL. Seeding was carried out by the spot-on-lawn method on petri dishes containing 15

mL of MH agar previously casted and dried. A volume of 10 µL of the samples filtered at 0.25 µm, as well as the positive control, were then deposited on the surface of the agar in the form of a spot and plates were incubated 24 h at 37 °C. The antibacterial activity was measured as the diameter of the clear zone of growth inhibition compared with a positive control, Ampicillin (0.1 mg/mL) for all bacterial species and Colistin (0.1 mg/mL) for *Escherichia coli*. This antimicrobial test was performed in triplicate.

- Minimal inhibitory concentration (MIC) determination

The MIC (Minimum Inhibitory Concentration) corresponds to the lowest concentration of the peptide hydrolysates allowing the inhibition of the bacterial strain growth. It is a liquid growth inhibition assay performed in a sterile 96-well microplate, of which the response was read after 24 h incubation (Froidevaux *et al*., 2001). This test makes it possible to quantify the antimicrobial activity of hydrolysates, isolated peptides or any other antimicrobial agents. Each well contained 100 µL of Mueller-Hinton medium and was supplemented with 100 µL of peptide fraction (80 mg/mL) serially diluted in half. In total, 100 µL of the tested strain pre-diluted in Mueller-Hinton to a final bacterial load of 10^5 CFU/mL was added last in each well. The inhibition of bacterial growth was monitored by measuring absorbance at 600 nm on a microplate reader (Safas, model MP96 UV-Vis Spectrophotometer, Agilent Technologies, Santa Clara, CA, USA) after 24 h of incubation at 37 °C. All measurements of MIC values were repeated in triplicate. The absorbance values of wells containing peptide hydrolysates were compared to those of negative controls consisting of a Mueller-Hinton medium and control culture. All the data are expressed as mean ± SD and are the mean of three replicates.

Antifungal Activity

Fungal strains used in this study were chosen because they represent some of the most abundant fungal species causing significant food contamination; they were isolated from the environment, food or dairy products (Pitt and Hocking, 2009). Five fungal strains were used for determination of antifungal activity: *Aspergillus Niger* (3071-13)*, Mucor racemosus*

(LMA-722), *Penicillium crustosum* (27,159), *Rhodotorula mucilaginosa* (27,173) and *Paecilomyces spp* (5332-9a). These microorganisms were maintained in glycerol stocks at -80 °C. Then they were recovered on Potato Dextrose Agar (PDA, BD-Difco, Sparks, MD, USA) plates and incubated at 25 °C for a week for the molds and for 72 h for *Rhodotorula mucilaginosa*. For *Rhodotorula mucilaginosa,* cells were then recovered with a swab and incubated on PDA plates for 48 h at 25 °C. For the four molds, conidia were recovered with a swab soaked with peptone water containing 10% Tween 80 and were inoculated on PDA plates for another week at 25 °C. Conidia and vegetative cells of *Rhodotorula mucilaginosa* were then harvested using the same method and transferred in peptone water. Cell concentration was measured by an automated cell counter (Invitrogen™ AMQAF1000, Oakville, ON, Canada) and adjusted to $\times 10^6$ CFU/mL after dilutions in peptone water to obtain the same inoculum concentration for all the strains. The antifungal activity of extracts was assessed in agar diffusion assays. Briefly, a volume of 250 µL of each antifungal culture was then incorporated into falcon tubes containing 25 mL of PDA medium at 45 °C to obtain a final inoculum concentration of $\times 10^4$ CFU/mL. Inoculated media were then poured into sterile Petri dishes of 100 mm in diameter and solidified. Eight wells were uniformly dug on each inoculated PDA plate using a 5 mL serological pipette. In each well, 80 µL of hemoglobin solution at 10 and 20 mg/mL were spilled. The agar plates were incubated at 25 °C for 48 h, then the diameter of the inhibition zones indicating antifungal activity was measured (in millimeter), as indicated by the Clinical and Laboratory Standards Institute (CLSI 2016, CLSI 2017). A solution of 16.7 µg/mL natamycin (Sigma-Aldrich, Saint-Quentin Fallavier, France) was used as a positive control and sterile distilled water as negative control. All the data are expressed as mean ± SD and are the mean of three replicates.

Antioxidant Bioactivities

Four chemical *in vitro* assays, based on different antioxidant mechanisms, were used in this study to evaluate the antioxidant activity of our generated peptides.

- Antioxidant assay using the β-carotene bleaching method

The ability of the hydrolysate to prevent the bleaching of β-carotene was determined as described by Koleva *et al*. (2002). Briefly, a stock solution of β-carotene (4 mg), linoleic acid (100 µL) and Tween-40 (800 µL) was dissolved in 4 mL of chloroform. The latter was completely evaporated under vacuum (rotary evaporator, Heidolph, Schwabach, Germany) at 45 °C, then 400 mL of distilled water was added, and the resulting mixture was vigorously stirred. The β-carotene/linoleic acid emulsion obtained was freshly prepared before each experiment. An aliquot (5 mL) of the emulsion was transferred to tubes containing 500 µL of each sample prepared in ultrapure water at different concentrations. The absorbance at 470 nm (Shimadzu UV-1650 PC Spectrophotometer, shimadzu corporation, Kyoto, Japan) of each sample was measured before and after incubation at 50 °C for 2 h. Butylated hydroxytoluene (BHT) at a concentration of 0.5 mg/mL was used as positive control. Relative antioxidant activity was calculated according to following equation:

$$\mathrm{RAA}(\%) = \frac{\text{A sample}}{\text{A control}} \times 100$$

where A blank consisted of 500 µL of distilled water instead of the sample solution, A sample is the absorbance of samples (with the emulsion) and A control is the absorbance of BHT (with the emulsion). All the data are expressed as mean ± SD and are the mean of three replicates.

- DPPH radical scavenging capacity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging capacity of samples was determined as described by Bersuder *et al*. (1998). A volume of 500 µL of each sample prepared in ultrapure water at different concentrations was added to 250 µL of 99% ethanol and 375 µL of DPPH solution (0.02% in ethanol) as free radical source. The mixtures were shaken and then incubated for 60 min in a dark room at 30 °C. Scavenging capacity was measured spectrophotometrically (Shimadzu UV-1650 PC Spectrophotometer, shimadzu corporation, Kyoto, Japan) by monitoring the decrease in absorbance at 517 nm. Lower

absorbance of the reaction mixture indicated higher DPPH free radical scavenging activity. BHT was used as positive control. DPPH radical scavenging capacity was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A\ control - A\ sample}{A\ control} \times 100$$

where $A_{control}$ represents the absorbance of all the reagents without the samples and A_{sample} is the absorbance of the sample (with the DPPH solution). $DPPH^+$ radical scavenging activity was expressed as the half-maximal inhibition concentrations (IC_{50}) and Trolox Equivalent Antioxidant Capacity (TEAC) values. The IC_{50} was calculated graphically by linear regressions of the percentage curve inhibition according to different concentrations of the hydrolysates tested. It represents the value of the sample concentration required to inhibit 50% of DPPH radicals. The lower this value, the more the compound was anti-free radical (Gulcin, 2010). The TEAC value was defined as the concentration of standard Trolox, a water-soluble vitamin E analogue that exhibited the same antioxidant capacity as a 1 mg/mL solution concentration of the antioxidant compound under investigation (Maisuthisakul *et al*., 2007). It is used as a reference antioxidant in biology for evaluation of resistance to oxidative stress. All analyses were carried out in triplicate and results represented the mean values with standard deviations.

- Antioxidant properties products by ABTS assay

The ABTS (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activities were determined according to the method described by Re *et al*. (1999). The cationic radical $ABTS^+$ was obtained by aqueous reaction between 7 mM ABTS and 4.95 mM potassium persulfate. This was prepared 12 to 16 h before use. The solution was stable when stored at room temperature and protected from light. A part of this solution was diluted with ethanol at 30 °C, in order to obtain an absorbance at 734 nm of less than 1. The hydrolysates were previously dissolved in ultrapure water at different concentrations and then, 10 µL of each sample were mixed with 1 mL of the diluted $ABTS^+$ solution. The absorbance of $ABTS^+$ was measured at 734 nm (Shimadzu UV-1650 PC Spectrophotometer,

shimadzu corporation, Kyoto, Japan) and at 30 °C exactly 6 min after the initial mixing. Appropriate solvent blanks were run in each assay. The results are expressed as the percentage inhibition according to the following equation:

$$\text{ABTS}^{+} \text{ radical scavenging activity (\%)} = (1 - \frac{A\ sample}{A\ Blank}) \times 100$$

where A blank is the absorbance of the control reaction (containing all reagents except the sample) and A sample is the absorbance of samples (with the ABTS solution). $ABTS^{+}$ radical scavenging activity was expressed as the half-maximal inhibition concentration (IC_{50}) and TEAC values. All the data are expressed as mean ± SD and are the mean of three replicates.

- Evaluation of total antioxidant capacity

Total antioxidant capacity of the hydrolysates was determined according to the method described by Prieto *et al*. (1999). It is a quick and simple method for determining antioxidant activity, based on an oxidation-reduction reaction. This technique is based on the reduction of molybdenum Mo present as molybdate MoO_4^{2-} ions to molybdenum MoO^{2+} in the presence of the hydrolysate to form a phosphate/molybdenum ($Mo_3O_{16}P_4$) green complex at acidic pH. This test allowed us to evaluate the antioxidant status of the peptides present in the hydrolysates as reducing agents in a colorimetric redox reaction. Peptide hydrolysates were previously dissolved in ultrapure water at different concentrations (2.5 mg/mL, 5 mg/mL, 10 mg/mL and 20 mg/mL) and then an aliquot of 300 µL of each sample was mixed with 3 mL reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 °C for 90 min. The absorbance of the aqueous solution of each sample was measured at 695 nm (Shimadzu UV-1650 PC Spectrophotometer, shimadzu corporation, Kyoto, Japan), after cooling at room temperature, against a blank. A typical blank solution contained 3 mL of reagent solution and the appropriate volume of the same solvent used for the sample, and it was incubated under the same conditions as the rest of the samples. BHT (0.5 mg/mL) was used as positive standard. A Trolox solution at different concentrations, ranging from 0 to 0.75 mg/mL, was

used for calibration. The antioxidant activity is expressed in Trolox Equivalent Antioxidant Capacity (TEAC) values (mg/mL) using the equation given by the calibration line:

$$A = a \times [C_{Trolox}] + b$$

where A the absorbance at 695 nm, C the equivalent antioxidant concentration (mg/mL). The concentration is determined according to the equation of the standard range curve of a reference antioxidant such as: Trolox, a and b, respectively, the origin and the slope of the Trolox calibration line. All the data are expressed as mean ± SD and are the mean of three replicates.

IV.4.4.3. Statistical analyses

All analyses were performed in triplicate and three independent repetitions were done for each condition. Data were subjected to one-way or two-way analyses of variance (ANOVA). Tukey tests were also performed on data using SigmaPlot software (Version 14.0) to determine which treatment was statistically different from the others at a probability level *p* of 0.05.

IV.5. Results and Discussion

IV.5.1. Identification and characterization of the peptide population derived from EDBM-MCP and EDBM-AEM

In our study, « Zipper » mechanism allowed obtaining a large peptide population in the three conditions as described by Abou-Diab *et al*. (2020a). However, mass spectrometry is required to identify these peptide populations and to be able to compare them with control.

IV.5.1.1. Schematic representations

For readability, the results will be described in the form of a figure presenting the amino acid sequence of hemoglobin chains and all of the peptides identified for the comparison between the three pepsic hydrolysates derived from the conventional hydrolysis process, EDBM-MCP and EDBM-AEM, respectively. Peptide sequences from the alpha and beta-chains of hemoglobin, found by mass spectrometry analysis, in the protein hydrolysate of the conventional hydrolysis process, EDBM-MCP and EDBM-AEM are shown, in **Figures IV-15, IV-16, IV-17, IV-18, IV-19** and **IV-20.** The recovery rates for the alpha chain were 98.5% (112 peptides), 98,5% (123 peptides) and 91.5% (110 peptides) for conventional hydrolysis process, EDBM-MCP and EDBM-AEM, respectively, while for the beta chain, the respective recovery rates were 100% (106 peptides), 100% (110 peptides) and 98% (106 peptides). The majority of the sequences of alpha chain were found for control and EDBM-MCP among the peptides identified by Peaks Studio, while for EDBM-AEM some short sequences were not found, but the numbers of sequences identified were still very high (more than 91%). Moreover, the majority of the sequences of beta chains were found for control, EDBM-MCP and EDBM-AEM. We noticed that in these three conditions more than 91% of α and β chain were recovered, this showed the great resemblance between the peptides produced even with the new process which did not use chemical solvents.

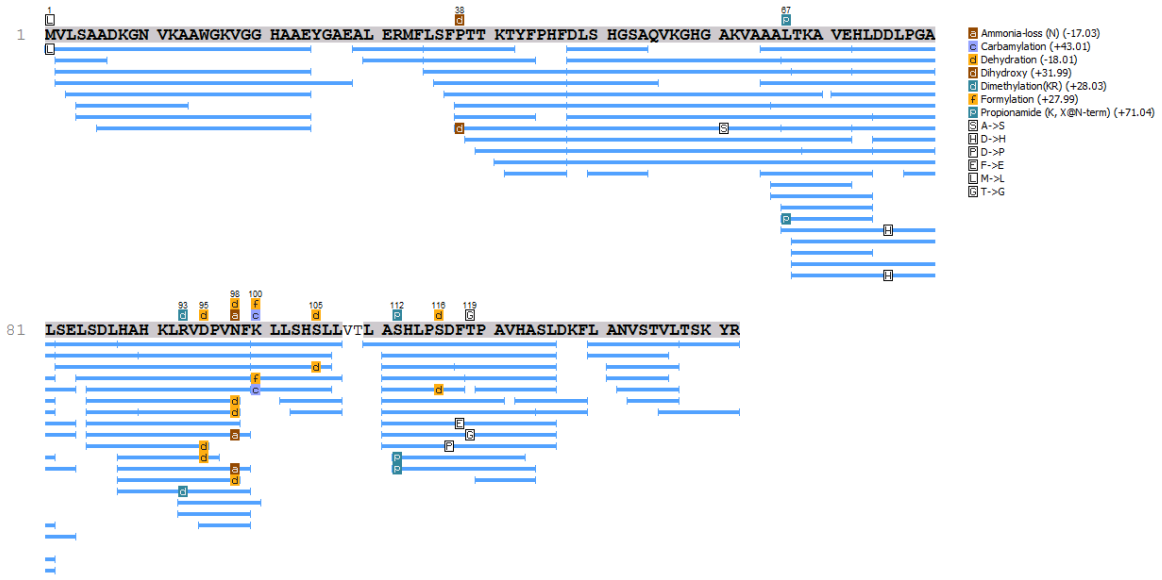

Figure IV-15. Schematic representation of the peptides resulting from the enzymatic hydrolysis of the α chain of bovine hemoglobin in control.

Figure IV-16. Schematic representation of the peptides resulting from the enzymatic hydrolysis of the β chain of bovine hemoglobin in control.

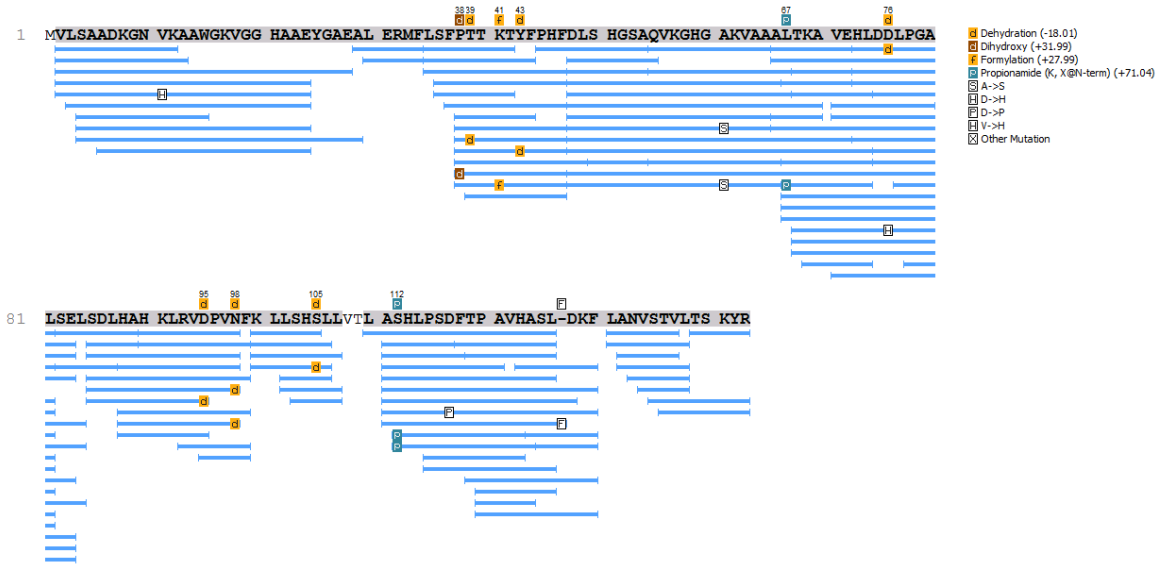

Figure IV-17. Schematic representation of the peptides resulting from the enzymatic hydrolysis of the α chain of bovine hemoglobin in EDBM-MCP.

Figure IV-18. Schematic representation of the peptides resulting from the enzymatic hydrolysis of the β chain of bovine hemoglobin in EDBM-MCP.

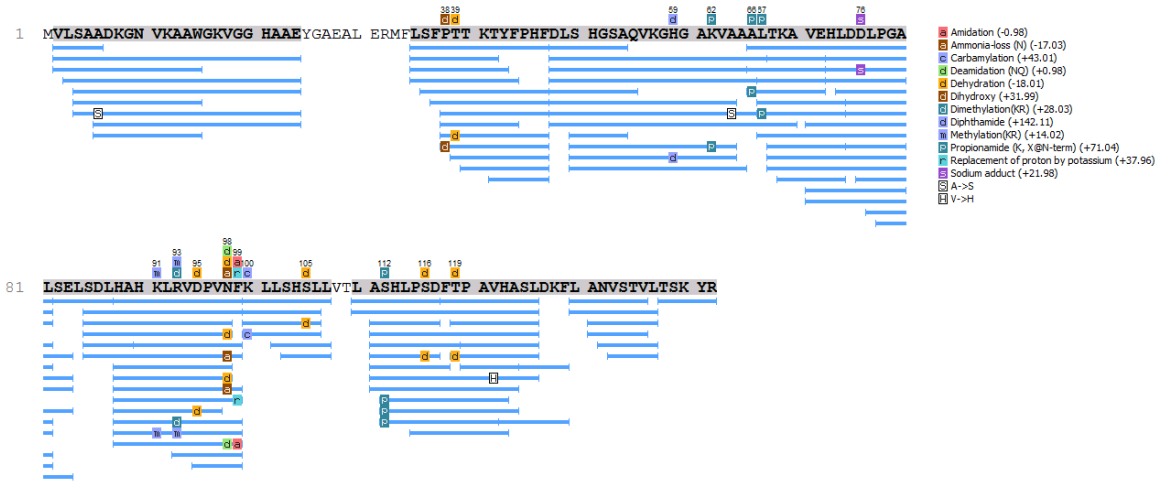

Figure IV-19. Schematic representation of the peptides resulting from the enzymatic hydrolysis of the α chain of bovine hemoglobin in EDBM-AEM.

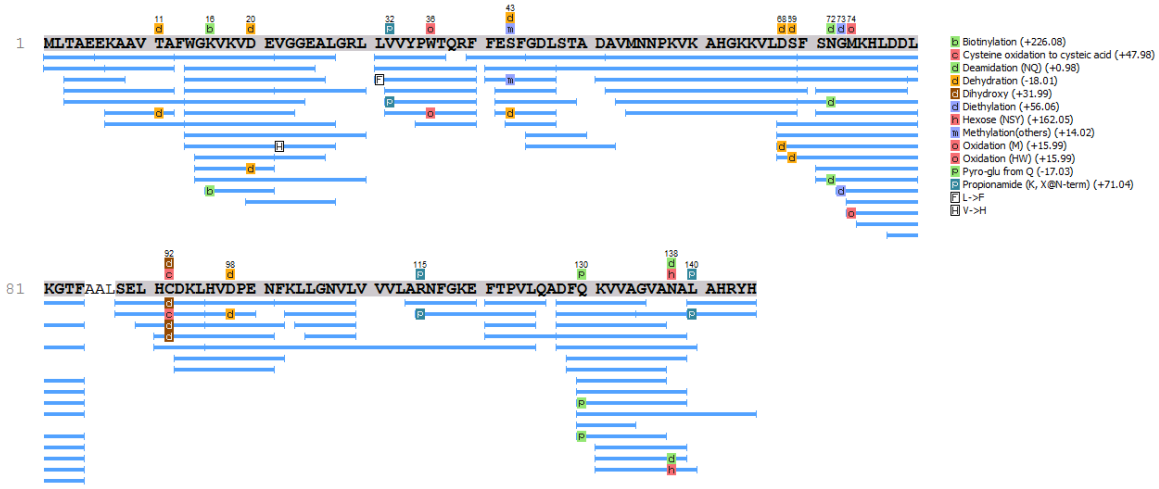

Figure IV-20. Schematic representation of the peptides resulting from the enzymatic hydrolysis of the β chain of bovine hemoglobin in EDBM-AEM.

IV.5.1.2. Venn diagrams

Venn diagrams showed all possible peptide population relations between control, EDBM-MCP and EDBM-AEM (**Figure 21A-C**). As can be seen, **Figure 21A** showed 171 peptides in common between control and EDBM-MCP. **Figure 21B** showed 162 peptides in common between control and EDBM-AEM. Moreover, the Venn diagram in **Figure 21C** showed 143 peptides in common between control, EDBM-MCP and EDBM-AEM. By using bipolar membrane electrodialysis with the ion-exchange membranes, whether cationic or anionic, we obtained roughly the same result. The bipolar membrane made it possible to reproduce the same peptide population as in the control under normal conditions with a slight difference because the three conditions do not follow the same parameters throughout the process as shown equally by Abou-Diab *et al*. (2020a).

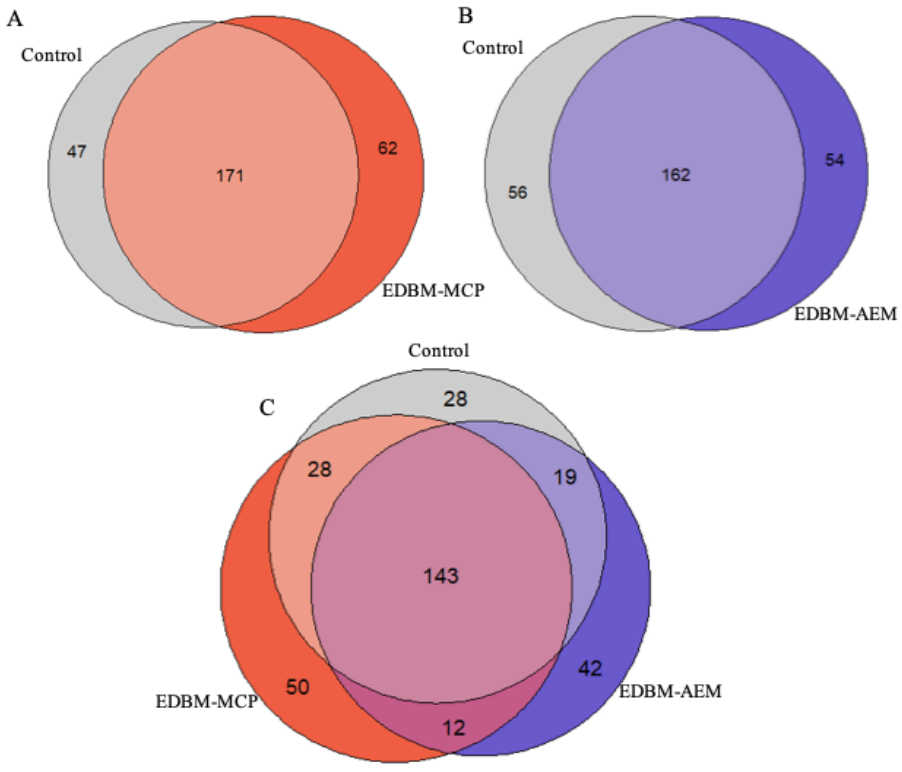

Figure IV-21. Venn diagrams showing the different peptide populations produced by control and electrodialysis with bipolar membrane (EDBM)-MCP (A), control and EDBM-AEM (B) and control, EDBM-MCP and EDBM-AEM (C).

IV.5.2. Identification of bioactive peptides resulting from bovine hemoglobin hydrolysis by EDBM-MCP and EDBM-AEM

One of the most important aim of this study was to identify the bioactive peptides obtained following the hydrolysis of bovine hemoglobin in EDBM and to compare them with the peptides obtained in control. Bioactive peptide sequences identified by UPLC-MS/MS are presented in **Tableau IV-4**.

Tableau IV-4. Bioactive peptide sequences identified by UPLC-MS/MS resulting from hydrolysis of bovine hemoglobin for the three different hydrolysis processes tested.

Biological Activity	Position	Sequence	Molecular Weight (Da)	Control	EDBM -MCP	EDBM -AEM
Antimicrobial	α1-29	VLSAADKGNVKAAWGKVGGHAAEYGAEAL	2839	+	+	+
	α1-27	VLSAADKGNVKAAWGKVGGHAAEYGAE	2655	+	+	+
	α1-23	VLSAADKGNVKAAWGKVGGHAAE	2235	+	+	+
	α34-46	LSFPTTKTYFPHF	1585	+	+	+
	α33-46	**FLSFPTTKTYFPHF**	**1732**	-	+	-
	α37-46	PTTKTYFPHF	1238	+	+	+
	α137-141	TSKYR	653	+	+	+
	β1-13	MLTAEEKAAVTAF	1381	+	+	+
	β126-145	QADFQKVVAGVANALAHRYH	2194	+	+	+
	β140-145	LAHRYH	795	+	+	+
	α99-105	KLLSHSL	796	+	+	+
	α99-106	KLLSHSLL	910	+	+	+
Hematopoietic	**α76-82**	**LPGALSE**	**685**	-	+	-
Opioid	α137-141	TSKYR	653	+	+	+
	β32-37	VVYPWT	763	+	+	+
	β31-37	LVVYPWT	876	+	+	+
	β32-40	VVYPWTQRF	1195	+	+	+
	β31-40	LVVYPWTQRF	1308	+	+	+
Potentiator of bradykinin	α129-134	LANVST	603	+	+	+
	α110-125	ASHLPSDFTPAVHASL	1649	+	+	+
Coronaro-constrictor	**β32-36**	**VVYPW**	**663**	-	+	-
Antihypertensive	α99-105	KLLSHSL	796	+	+	+
Antioxidant	α137-141	TSKYR	653	+	+	+

Among this large peptide population, numerous bioactive peptides were found in EDBM-MCP and EDBM-AEM, after three hours of hydrolysis, using bipolar membranes and without the use of chemical acids. The peptides identified by UPLC-MS/MS and compared with the literature show several bioactivities: antimicrobial (Adje *et al*., 2011; Nedjar *et al*., 2008; Dubois *et al*., 2005), Hematopoietic (Vercaigne-Marco *et al*., 2000), opioid (Lignot *et al*., 1999; Zhao and Piot, 1997a; 997b), potentiator of bradykinin (Lignot *et al*., 1999; Piot *et*

al., 1992), Coronaro-constrictor (Barkhudaryan *et al*., 1992), antihypertensive (Adje *et al*., 2011) and antioxidant (Przybylski *et al*., 2016). The majority of the identified bioactive peptides were the same under all three conditions. The three hydrolysis processes produced 11 peptides in common having antimicrobial activity, 5 opioid peptides, 2 potentiator of bradykinin, 1 antihypertensive and 1 antioxidant peptide in common. The α33-46 which have an antimicrobial activity appeared in the EDBM-MCP and did not appear in the other conditions. Furthermore, α76-82 and β32-36 peptides which have hematopoietic and Coronaro-constrictor activity, respectively, appeared in the EDBM-MCP and did not appear in the other conditions. The bioactive peptides (α33-46, α76-82 and β32-36) presented in the EDBM-MCP and not present in the conventional and EDBM-AEM hydrolysis were precursors. Being an intermediate peptide, α33-46, α76-82 and β32-36, were rapidly hydrolyzed and no longer present after 3 h of hydrolysis in control and EDBM-AEM which agreed with the work of Dubois *et al*. (2005), for bovine hemoglobin. Their presence in the EDBM-MCP and their absence in the conventional and EDBM-AEM conditions means that there was a slow-down in enzymatic kinetics during the hydrolysis of hemoglobin in EDBM-MCP as proposed by Abou-Diab *et al*. (2020a) which confirms the result found above in Venn diagrams.

IV.5.3. Biological activities of the hydrolysates obtained from EDBM-MCP and EDBM-AEM

IV.5.3.1. Antimicrobial Test

Antibacterial Activity

The results presented in **Tableau IV-5** showed that, at 20 mg/mL, all hemoglobin hydrolysates derived from the three hydrolysis processes displayed antibacterial activity against the six tested bacterial species, with a clear growth inhibition zone. There is no significant difference in antibacterial activity between the hydrolysates derived from different conditions against a tested strain. Among the six bacterial strains, *S. aureus* and *K. rhizophila* presented the highest sensitivity to the hydrolysates. The hydrolysates of all

conditions were noted to have strong protective effect against pathogenic and non-pathogenic bacteria.

Tableau IV-5. Antibacterial activity of bovine hemoglobin hydrolysates obtained with treatment by pepsin.

Bacteria Strains	Bovine Hemoglobin Hydrolysates		
	Control	EDBM-MCP	EDBM-AEM
Staphylococcus aureus	+++	+++	+++
Listeria monocytogenes	++	++	++
Micrococcus luteus	+	+	+
Kocuria rhizophila	+++	+++	+++
Escherichia coli	+	+	+
Salmonella Newport	+	+	+

Inhibition zones: +++: > 1.5 cm; ++ 0.5–1.5 cm; +: < 0.5 cm, and -: no activity.

MIC Determination of Antibacterial Peptide Hydrolysates

The hydrolysates were assessed for their MIC values in mg/mL because they contained multiple peptides. All samples showed activity against Gram-positive and Gram-negative bacteria. The results of MIC confirmed the findings previously found with the Agar Diffusion Method. However, the MIC values recorded for the active peptides against *S. aureus, L. monocytogenes* and *K. rhizophila* (between 0.31 to 2.5 mg/mL) were considerably lower than the MIC values registered against *M. luteus, E. coli and S. Newport* (between 5 to 10 mg/mL).

The antimicrobial effect of control and EDBM-AEM hydrolysate corresponded to MIC values of 0.31 mg/mL for *S. aureus* and 0.62 mg/mL for EDBM-MCP hydrolysates, which were statistically different ($p < 0.05$). Control hydrolysate displayed important antibacterial activity against *L. monocytogenes*, with MIC value of 1.25 mg/mL lower than the MIC value of EDBM-MCP and EDBM-AEM hydrolysates which have a value of 2.5 mg/mL. The hydrolysate from all conditions also showed antibacterial activity against *K. rhizophila* with identical MIC values of 0.31 mg/mL. The MIC value of 5 mg/mL for *M. luteus* obtained for control hydrolysate was statistically different ($p < 0.05$) from MIC values of 10 mg/mL for EDBM-MCP and EDBM-AEM hydrolysates. Identical antibacterial activities were also

observed for all hydrolysates against *E. coli* and *S. Newport* with a MIC value of 10 mg/mL (**Tableau IV-6**).

Tableau IV-6. MIC values of the antibacterial peptide hydrolysates.

Bacteria Strains	Bovine Hemoglobin Hydrolysates		
	MIC		
	Control	EDBM-MCP	EDBM-AEM
	mg/mL	mg/mL	mg/mL
Staphylococcus aureus	0.31 ± 0.0 [a]	0.62 ± 0.0 [b]	0.31 ± 0.0 [a]
Listeria monocytogenes	1.25 ± 0.0 [a]	2.5 ± 0.0 [b]	2.5 ± 0.0 [b]
Micrococcus luteus	5 ± 0.0 [a]	10 ± 0.0 [b]	10 ± 0.0 [b]
Kocuria rhizophila	0.31 ± 0.0 [a]	0.31 ± 0.0 [a]	0.31 ± 0.0 [a]
Escherichia coli	10 ± 0.0 [a]	10 ± 0.0 [a]	10 ± 0.0 [a]
Salmonella Newport	10 ± 0.0 [a]	10 ± 0.0 [a]	10 ± 0.0 [a]

The minimum inhibitory concentration (MIC) of the peptide hydrolysates was determined in a microtiter plate assay system after 24 h of incubation at 30 or 37 °C. **[a-b]:** Population means for each bacteria within each row with different letters are significantly different, similar letter means no significant difference $p < 0.05$ (Anova, Tukey).

In this study, we demonstrated that bovine hemoglobin hydrolysates, derived from conventional hydrolysis or from hydrolysis in EDBM, possessed antimicrobial activities against microorganisms that are of interest in the food industry. Our findings are in line with previous work reported by Zouari *et al*. (2020) who showed the inhibitory effect of bovine or porcine hemoglobin hydrolysates against *M. luteus, L. Innocua, E. coli* and *S. Newport*. The hemoglobin peptides could be candidate for food preservation. In poultry feed, these results are promising for the development of an alternative or supplement for reducing the use of antibiotics (Jean, 2015).

IV.5.3.2. Antifungal Test

Antifungal activities of bovine hemoglobin hydrolysates (10 and 20 mg/mL) obtained with treatment by pepsin are shown in **Figure IV-22**. Protein hydrolysates from hydrolysis control, EDBM-MCP and EDBM-AEM at a concentration of 20 mg/mL were strongly active against *Paecilomyces spp*, inducing zones of inhibition of 12.70 mm while hydrolysates at a concentration of 10 mg/mL induced a zone of inhibition of 11.90 mm, statistical analysis

demonstrated that there is significant difference ($p < 0.05$) between these two values. The control hydrolysate at a concentration of 20 mg/mL was more active against *Penicillium crustosum* (11.90 mm inhibition zone) than with EDBM-MCP and EDBM-AEM hydrolysates at the same concentration (9.52 mm inhibition zone). All hydrolysates at a concentration of 10 mg/mL and 20 mg/mL were active against *Aspergillus Niger* producing a 4.76 mm inhibition zone in comparison with negative control, which has no inhibition zone. Statistical analysis demonstrated that there is significant difference ($p < 0.05$) in the inhibition zones formed by the hydrolysates against *Rhodotorula mucilaginosa*. At 20 mg/mL, the control hydrolysate presented an inhibition zone (11.11 mm) larger than the one of EDBM-AEM hydrolysate (9.52 mm) and even larger than by EDBM-MCP (4.76 mm). Always at 20 mg/mL, the control hydrolysate presented a clear activity against *Mucor racemosus* (inhibition zone of 11.90 mm) statistically similar ($p > 0.05$) to EDBM-AEM (11.11 mm) but statistically higher ($p < 0.05$) than the one of EDBM-MCP (4.76 mm). At a concentration of 10 mg/mL, all hydrolysates were active against *Mucor racemosus* inducing similar inhibition zone of 4.36 mm ($p > 0.05$). These tests showed that the most inhibited strains (based on inhibition zone diameter) belonged to *Paecilomyces spp, Penicillium crustosum* and *Mucor racemosus*. It should be noted that hydrolysates with a higher peptide concentration show higher antifungal activity. The hydrolysates derived from EDBM have an inhibitory effect on the fungal growth of these strains despite the small differences observed with the control. By way of comparison, the antifungal activity of the goat milk whey hydrolysates at a final concentration of 250 mg/mL induced inhibition zone of 8 mm against *Penicillium camemberti* and <8 mm against *Penicillium roqueforti* (Luz *et al*., 2020), while here the antifungal activity of bovine hemoglobin hydrolysates (20 mg/mL) derived from control, EDBM-MCP and EDBM-AEM induced inhibition zone >8 mm against *Penicillium crustosum*. Thus, EDBM is an appealing technology allowing to produce hydrolysates with an interesting antifungal activity. These results are promising for the development of an alternative or supplement for reducing the use of antifungal chemical additives and drugs.

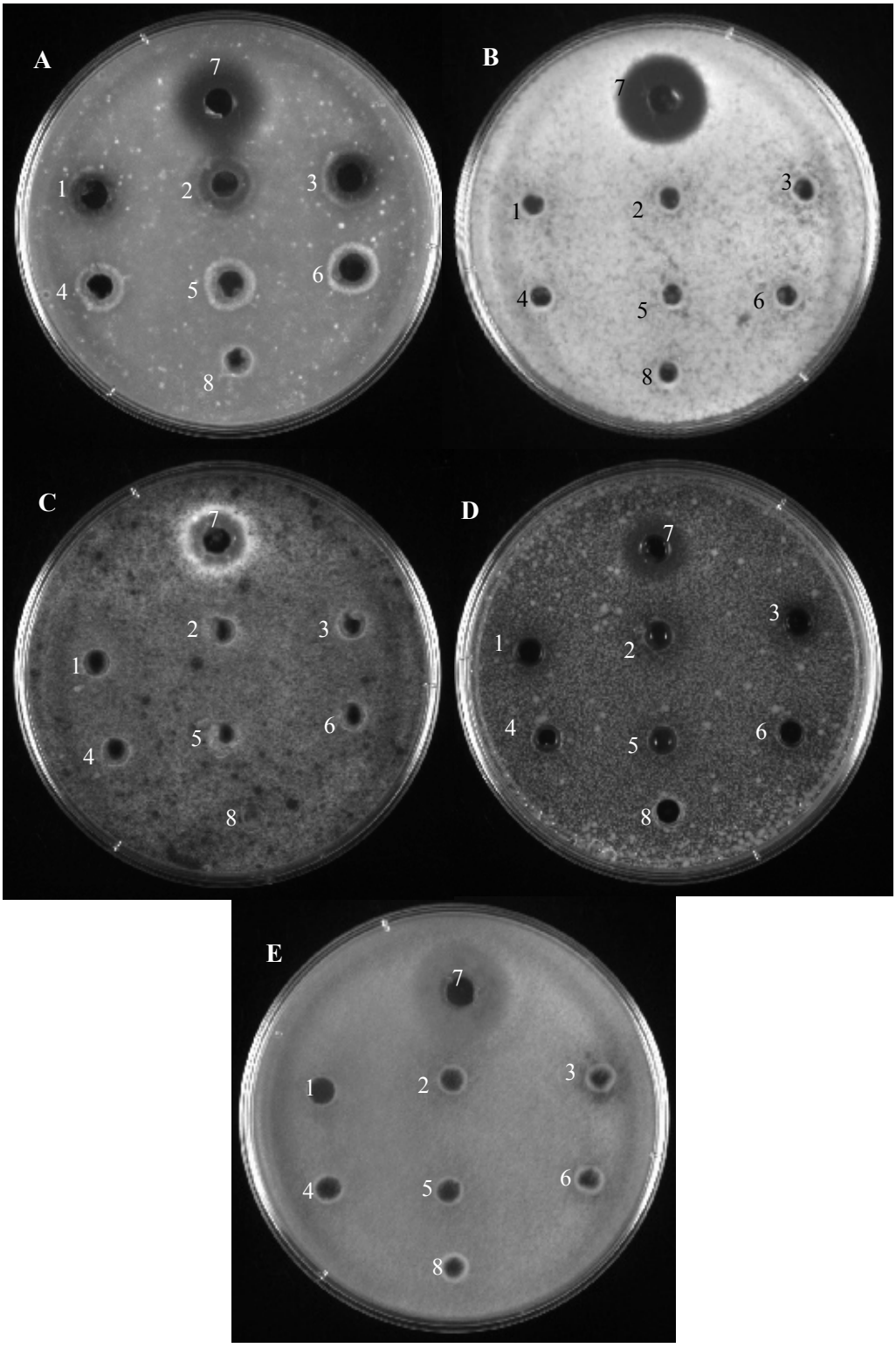

Figure IV-22. Antifungal activity of bovine hemoglobin hydrolysates produced by three different hydrolysis processes on Potato Dextrose Agar (PDA) overlaid with a suspension (104 spores per plate) of *Paecilomyces spp* (**A**), *Penicillium crustosum* (**B**), *Aspergillus Niger* (**C**), *Rhodotorula mucilaginosa* (**D**) and *Mucor racemosus* (**E**), 1-8 refer to EDBM-MCP (20 mg/mL), EDBM-AEM (20 mg/mL),

Hydrolysis Control (20 mg/mL), EDBM-MCP (10 mg/mL), EDBM-AEM (10 mg/mL), Hydrolysis Control (10 mg/mL), control +, control -, respectively.

To the best of our knowledge, this was the first study demonstrating the ability of bioactive peptides derived from the hydrolysis of bovine hemoglobin to inhibit fungal growth.

IV.5.3.3. Antioxidant activities of bovine hemoglobin hydrolysates

- β-carotene bleaching inhibition activity

Lipid oxidation products react with proteins causing their oxidation. Carbohydrates are also susceptible to oxidation, but they are less sensitive than lipids and proteins (Zhuang *et al.*, 2013). In this study, lipid peroxidation inhibition activity of conventional hydrolysis (control), EDBM-MCP and EDBM-AEM hydrolysates was determined by assessing their ability to inhibit oxidation of linoleic acid in an emulsified model system.

The antioxidative activity of control, EDBM-MCP and EDBM-AEM hydrolysates measured by the β-carotene bleaching assay are represented in **Figure IV-23**. Different concentrations of hydrolysates were tested and compared to the standard antioxidant, BHT (0.5 mg/mL) and synthetic bioactive peptide derived from hemoglobin hydrolysis, neokyotorphin (0.5 mg/mL). All hydrolysates inhibited the oxidation of β-carotene at different degrees according to the concentration. The more the value is close to 100%, the more the antioxidant activity of the tested product corresponds to that of the BHT reference antioxidant (99.56 ± 0.36%). The relative antioxidant activity (RAA) of NKT is 93.26 ± 1.4%, statistically lower ($p < 0.05$) but close to the one of BHT. This peptide, therefore, has a lipid protection function close to the one found for BHT. At a concentration of 10 mg/mL and 20 mg/mL, there was no significant difference ($p > 0.05$) in antioxidant activity between control, EDBM-MCP and EDBM-AEM. However, at a concentration of 2.5 mg/mL and 5 mg/mL, there was a significant difference ($p < 0.05$) in antioxidant activity between EDBM-MCP and EDBM-AEM. Furthermore, a concentration of 20 mg/mL showed a significantly ($p < 0.05$) higher antioxidant activity (92.15 ± 1.29%; 89.1 ± 0.78%; 90.38 ± 1.46%, respectively) than the

other concentrations. Relative antioxidant activity (%) increased with increasing protein hydrolysates concentrations. By way of comparison, the relative antioxidant activity of phenolic compounds of olive oil extracted in a traditional way is 67.40 ± 1.02% compared to BHT at 0.5 mg/mL (Merouane *et al.*, 2015). This equals to our hydrolysates dissolved at 10 mg/mL.

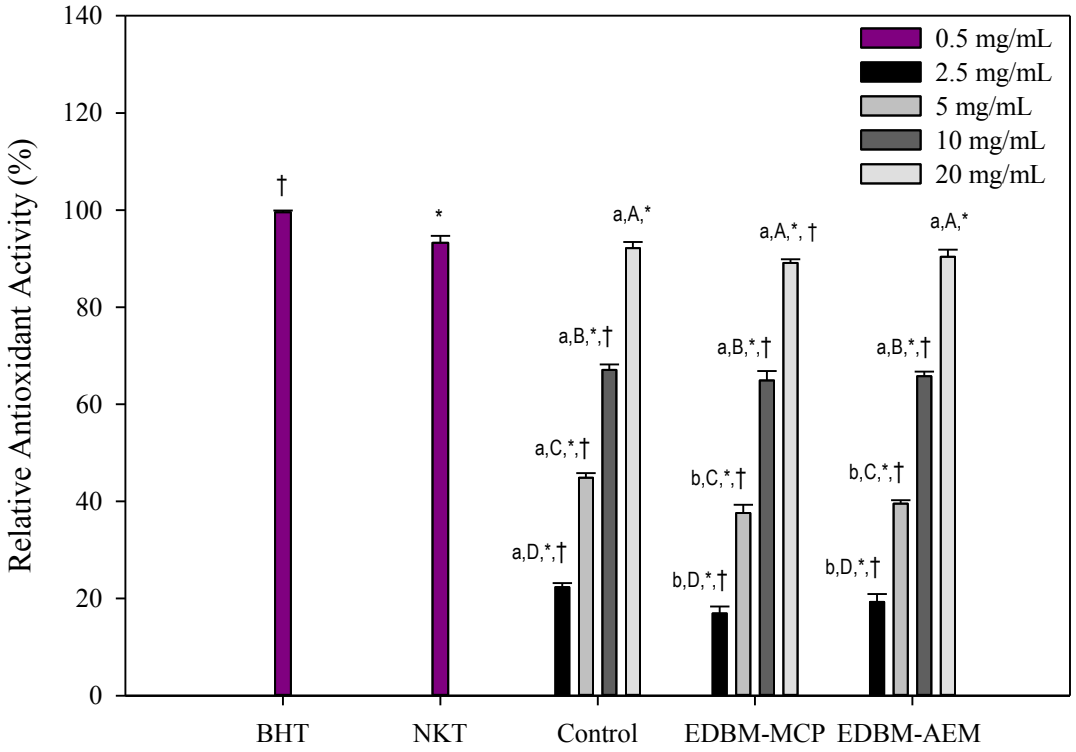

Figure IV-23. β-carotene bleaching inhibition activity of hemoglobin hydrolysates at different concentrations in Control, EDBM-MCP and EDBM-AEM. Values with (*) symbol are significantly different from BHT; Values with (†) symbol are significantly different from neokyotorphin; values with different lowercase letter (a-b) within the same concentration are significantly different; values with different capital letter (A-B-C-D) within the same condition are significantly different ($p < 0.05$, Tukey).

The concentration of 20 mg/mL of the hydrolysates and 0.5 mg/mL of NKT have antiradical activity close to that of BHT at 0.5 mg/mL. Bleaching of β-carotene has been slowed down significantly in both cases. These products can, therefore, be described as primary antioxidant scavengers of free radicals in emulsion, so they would allow lipid protection.

- DPPH free radical scavenging capacity

The DPPH radical scavenging activity method is based on the ability of trapping the DPPH radicals. The drop-in absorption followed by spectrophotometry makes it possible to measure the antioxidant activity.

The results (**Figure IV-24**) clearly indicated that hydrolysates obtained by treatment with pepsin exhibited a strong DPPH free radical scavenging activity. At 5 mg/mL, there was a significant difference ($p < 0.05$) in the antioxidant activity between control and EDBM-MCP while there was not significant difference ($p > 0.05$) between control and EDBM-AEM and between EDBM-AEM and EDBM-AEM. A concentration of 5 mg/mL for control, EDBM-MCP and EDBM-AEM showed significantly ($p < 0.05$) higher antioxidant activity (95.7 ± 2.8%; 87.3 ± 2.3%; 89.38 ± 2.19%, respectively) than the other concentrations. The antioxidant activity of bovine hemoglobin hydrolysates increased with increasing peptide concentrations. Our findings are in line with previous works reported by He Tang *et al*. (2009) who reported that the DPPH-scavenging activity increased with increasing buckwheat protein hydrolysates concentrations (0-1.0 mg/mL) using alcalase.

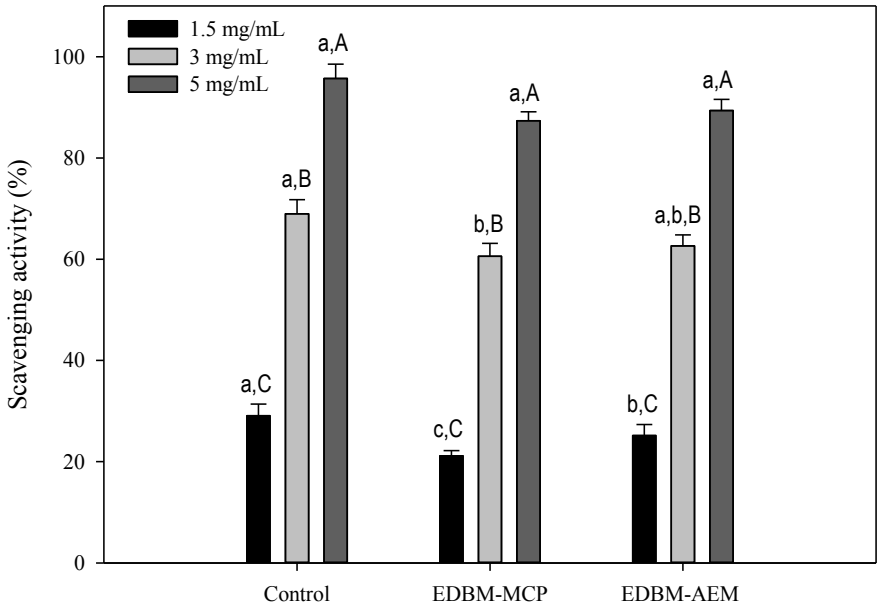

Figure IV-24. DPPH scavenging activities of hemoglobin hydrolysates in control, EDBM-MCP and EDBM-AEM at different concentrations. Values with different lowercase letter (a-b-c) within the same concentration are significantly different; values with different capital letter (A-B-C) within the same condition are significantly different ($p < 0.05$, Tukey).

The DPPH radical scavenging activities of the peptide hydrolysates were also expressed as the half-maximal inhibition concentrations (IC_{50}) and Trolox Equivalent Antioxidant Capacity (TEAC). The IC_{50} value is applied as an indication to evaluate the scavenging activity. The lower the IC_{50} value, the higher the free radical scavenging ability. The comparative IC_{50} and TEAC values of the tested hemoglobin hydrolysates were shown in **Tableau IV-7**. The DPPH radical scavenging activity of control showed different ($p < 0.05$) IC_{50} (2.55 ± 0.02 mg/mL) compared to EDBM-MCP (2.86 ± 0.03 mg/mL) and EDBM-AEM (2.72 ± 0.04 mg/mL). However, NKT showed an IC_{50} (0.61 ± 0.05 mg/mL) close to that of Trolox (0.38 ± 0.03 mg/mL) but both statistically lower than the ones of control, EDBM-MCP and EDBM-AEM. The TEAC of control showed different ($p < 0.05$) values compared to EDBM-MCP and EDBM-AEM. In comparison with Trolox, the DPPH radical scavenging activity of control, EDBM-MCP and EDBM-AEM showed lower radical scavenging activity (TEAC value lower than 1). Additionally, the DPPH radical scavenging activity of NKT showed higher radical scavenging activity (0.62 ± 0.09) than the peptide hydrolysates.

Tableau IV-7. IC_{50} and Trolox Equivalent Antioxidant Capacity (TEAC) coefficients of hemoglobin hydrolysates for the DPPH method.

DPPH	Control	EDBM-MCP	EDBM-AEM	NKT	Trolox
IC_{50} (mg/mL)	2.55 ± 0.02 [a]	2.86 ± 0.03 [b]	2.72 ± 0.04 [c]	0.61 ± 0.05 [d]	0.38 ± 0.03 [e]
TEAC	0.15 ± 0.03 [a]	0.13 ± 0.02 [b]	0.14 ± 0.01 [c]	0.62 ± 0.09 [d]	1 [e]

[a-e]: Means values within each row with different letters are significantly different, similar letter means no significant difference at a probability level of 0.05 (Anova, Tukey).

- Antioxidant properties products by ABTS assay

The ABTS radical scavenging activity method is based on the ability of molecules to quench the ABTS radical cation, in comparison with Trolox, which was used as a reference molecule. The comparative IC_{50} and TEAC values of the tested hydrolysates are shown in **Tableau IV-8**.

To inhibit 50% of ABTS radicals, concentrations of hemoglobin hydrolysates between 3.42 and 3.66 mg/mL were needed. Statistical analysis showed that there was significant difference ($p < 0.05$) of IC_{50} for hemoglobin hydrolysates between control, EDBM-MCP and EDBM-AEM, but the values were very close. A concentration of 0.53 mg/mL of NKT inhibits 50% of ABTS radicals; this value is very close to Trolox but is statistically different ($p < 0.05$). Concerning TEAC, the ABTS radical scavenging activity of hemoglobin hydrolysates derived from control, EDBM-MCP and EDBM-AEM exhibited lower radical scavenging activity than Trolox (TEAC value lower than 1) while NKT showed a very close radical scavenging activity to Trolox (0.92 ± 0.01).

Tableau IV-8. IC_{50} and TEAC coefficients of hemoglobin hydrolysates for ABTS method.

ABTS	Control	EDBM-MCP	EDBM-AEM	NKT	Trolox
IC_{50} (mg/mL)	3.42 ± 0.01 [a]	3.66 ± 0.03 [b]	3.55 ± 0.04 [c]	0.53 ± 0.05 [d]	0.49 ± 0.09 [e]
TEAC	0.14 ± 0.03 [a]	0.13 ± 0.02 [b]	0.14 ± 0.01 [a]	0.92 ± 0.01 [c]	1 [d]

[a-e]: Means values within each row with different letters are significantly different, similar letter means no significant difference $p < 0.05$ (Anova, Tukey).

This result showed that hemoglobin hydrolysates have the ability to act as an antioxidative compound but due to the large number of bioactive peptides and the competitiveness between

them they showed lower capacity compared to the reference antioxidant Trolox. In comparison, NKT which is the reference bioactive peptide showed an antioxidant activity close to Trolox.

- Evaluation of total antioxidant capacity

The total antioxidant capacity of the hydrolysates was evaluated at four different concentrations (2.5 mg/mL, 5 mg/mL, 10 mg/mL and 20 mg/mL), and Trolox was used as a reference antioxidant. The total antioxidant capacity of the hydrolysates was expressed as TEAC (mg/mL).

The results shown in **Figure IV-25** clearly indicate a change in antioxidant capacity as a function of hydrolysate concentration. As expected, a concentration of 20 mg/mL for control, EDBM-MCP and EDBM-AEM showed significantly higher antioxidant activity than the other concentrations. EDBM-AEM (20 mg/mL) showed the highest TEAC compared to other conditions. There is significantly different antioxidant capacity between the different conditions for the same concentration; the TEAC increased from 0.146 ± 0.007, 0.126 ± 0.000 and 0.163 ± 0.001 mg/mL at a concentration of 2.5 mg/mL to 0.338 ± 0.021, 0.258 ± 0.038 and 0.371 ± 0.000 mg/mL at 20 mg/mL for conventional hydrolysis, EDBM-MCP and EDBM-AEM, respectively. BHT (0.5 mg/mL) has an antioxidant activity of 0.699 ± 0.007 mg/mL determined under the same conditions. TEAC of our 20 mg/mL hydrolysates is approximately two times lower than for 0.5 mg/mL BHT. The antioxidant activity of 20 mg/mL of hydrolysate is, therefore, equivalent to 0.25 mg/mL of BHT by the phosphor-molybdenum method.

Bovine hemoglobin hydrolysates of control, EDBM-MCP and EDBM-AEM have demonstrated important antioxidant properties and can be considered as promising antioxidants. The antioxidant properties of peptides are influenced by molecular size, composition, hydrophobicity and electron transferring ability of amino acid residues in the sequence (Qian *et al*., 2008). Hydrolysis is required to generate smaller bioactive peptides that possess biological activity. Antioxidant activity can be due to the total denatured

hemoglobin favoring the «Zipper» mechanism which made it possible to produce low molecular weight hydrolysates having important radical scavenging activities.

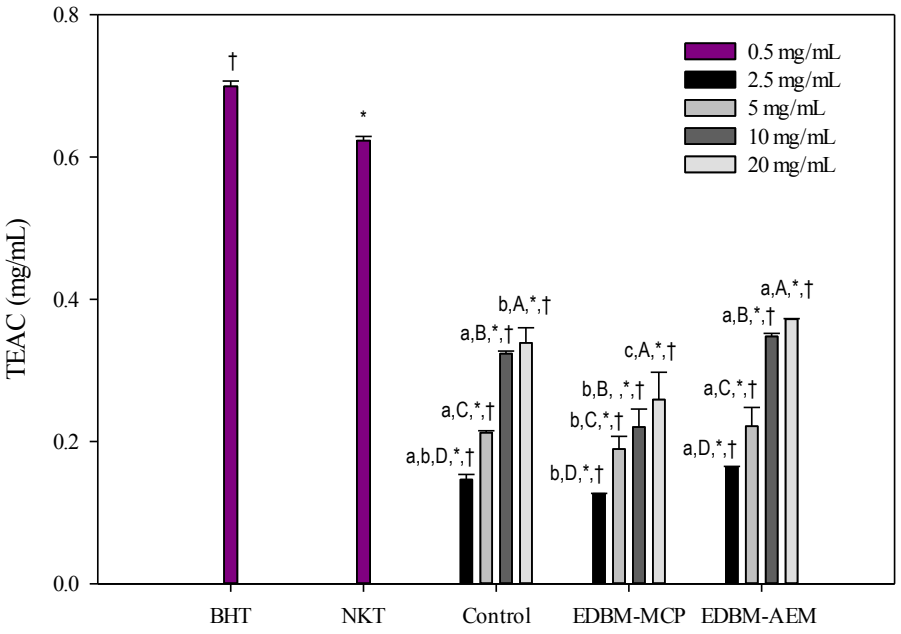

Figure IV-25. Total antioxidant capacity of hemoglobin hydrolysates in control, EDBM-MCP and EDBM-AEM at different concentrations. Values with (*) symbol are significantly different from BHT; Values with (†) symbol are significantly different from neokyotorphin; values with different lowercase letter (a-b) within the same concentration are significantly different; values with different capital letter (A-B-C-D) within the same condition are significantly different ($p < 0.05$, Tukey).

IV.6. Conclusion

The electrodialysis process using bipolar membranes combined with other ion-exchange membranes has been successful, and it has made it possible to adjust the pH and make the hydrolysis under the right conditions using the H^+ and OH^- generated by the bipolar membranes and without the use of chemical solvents. By the «Zipper» mechanism, EDBM, whatever the configuration of MCP or AEM, allowed for a recovery rate of the alpha and beta chain of bovine hemoglobin greater than 91% and similar to the conventional hydrolysis. Furthermore, following the identification of peptide sequences by mass spectrometry, EDBM was demonstrated to produce 17 bioactive peptides that are already known for their activities.

More specifically the production of neokyotorphin (α137-141) which has shown several biological activities, which has a great importance in the food industry.

The results of the present work showed an excellent microbial growth inhibition of bovine hemoglobin peptide hydrolysates derived from conventional hydrolysis or from hydrolysis by EDBM against *S. aureus*, *L. monocytogenes, K. rhizophila*, *M. luteus, E. coli* and *S. Newport.*

This study reports, for the first time, the capacity of these hydrolysates to inhibit the growth of *Aspergillus Niger*, *Mucor racemosus, Penicillium crustosum, Rhodotorula mucilaginosa* and *Paecilomyces spp*. Moreover, these results confirmed that bovine hemoglobin hydrolysates, whatever their mode of production, had antioxidant capacities. Hence, some peptides in the hydrolysates are new potent free radical scavengers that could be used as natural antioxidants in food matrices or products.

This new green application of EDBM and the production of antimicrobial, antifungal and antioxidant peptide, from waste, fits perfectly with the concept of circular economy. However, the issue of fouling highlighted and explained in the technological part of this study (Abou-Diab *et al*., 2020a) has to be fixed before further steps. Different approaches are already being considered and are under way, such as applying pulsed electric field, changing the EDBM configuration or modifying the ion-exchange membranes (Abou-Diab *et al*., 2020a; Bazinet and Geoffroy, 2020).

IV.7. Acknowledgements

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IV. Atteinte des objectifs et avancement des connaissances

Les objectifs qui étaient de déterminer les paramètres optimaux de dénaturation et d'hydrolyse enzymatique par électro-acidification pour produire le peptide α137-141 ont été atteints. L'optimisation du procédé a été effectuée tout d'abord en déterminant un nouveau niveau de conductivité optimal, 1,5 mS/cm, suite à plusieurs essais permettant une hydrolyse efficace sans ajout d'une grande quantité de sel en tout début. De plus, la valeur du pH privilégiant un mécanisme « Zipper » a été identifiée. Ce mécanisme est apparu identique dans toutes les conditions lorsque le pH était maintenu à 3 permettant une dénaturation complète de l'hémoglobine. Par ce mécanisme, une grande population peptidique similaire au contrôle et sans utilisation de produits chimiques a été identifiée. Ce nouveau concept, basé sur la génération de protons à partir de la dissociation de l'eau, a également permis la production de l'α137-141 à la même concentration que l'hydrolyse conventionnelle.

Par ailleurs, ces travaux ont permis d'atteindre l'objectif visant à déterminer le type de membrane monopolaire à utiliser en association avec les membranes bipolaires permettant de réduire le colmatage des membranes par l'hémoglobine. Ce travail a montré que l'EDMB-MCP permettait la déminéralisation simultanée de la solution d'hémoglobine par rapport à l'hydrolyse conventionnelle et à l'EDMB-AEM, la production du peptide α137-141 avec une faible teneur en minéraux mais avec apparition de colmatage sur les MCP. En comparaison,

l'EDMB-AEM a permis la production du peptide α137-141 plus rapidement sans colmatage des membranes et en impliquant une résistance globale minimale et par conséquent une consommation moindre d'énergie. En effet, l'utilisation de membranes anioniques a permis d'empêcher le passage des protons dans le compartiment adjacent, ce qui explique l'accélération de l'acidification par rapport aux membranes cationiques. De plus, les groupes fonctionnels chargés positivement des membranes échangeuses d'anions ont probablement exercé une répulsion électrique sur les peptides chargés eux aussi positivement à pH acide, réduisant ainsi les interactions potentielles avec la membrane monopolaire et par le fait même, les phénomènes de colmatage des membranes monopolaires par des dépôts de peptides ou d'associations peptides-hème. Cependant, le colmatage n'a pu être évité complètement.

Dans cette étude, l'électrodialyse avec membranes bipolaires (EDMB) a été démontrée en tant que technologie innovante pour contrôler l'avancement d'une réaction enzymatique et pour produire des hydrolysats peptidiques à partir de l'hydrolyse de l'hémoglobine bovine sans ajout de produits chimiques. Cela était dû aux caractéristiques des membranes bipolaires qui permettent de générer *in situ* des ions H^+ et OH^- à partir de la dissociation de l'eau sous un champ électrique et nécessitent par conséquent l'utilisation d'aucun agent chimique. L'EDMB peut être considérée comme un procédé alternatif à la méthode conventionnelle pour la dénaturation et l'hydrolyse enzymatique de l'hémoglobine bovine.

Les résultats du présent travail ont aussi concouru à répondre à l'objectif concernant l'étude des activités biologiques et ont montré une excellente inhibition de la croissance bactérienne des hydrolysats d'hémoglobine bovine issus de l'hydrolyse conventionnelle ou de l'hydrolyse par EDMB contre *S. aureus, L. monocytogenes, K. rhizophila, M. luteus, E. coli et S. Newport.* De plus, ces résultats ont confirmé que les hydrolysats d'hémoglobine bovine, quel que soit leur mode de production, avaient des capacités antioxydantes. Par conséquent, certains peptides dans les hydrolysats sont de nouveaux capteurs de radicaux libres puissants qui pourraient être utilisés comme antioxydants naturels dans des matrices ou des produits alimentaires.

Ces résultats ont contribué à un avancement des connaissances sur la compréhension des interactions peptides et peptides-hème avec les membranes cationiques et anioniques, l'impact du pH sur l'hémoglobine, les différents mécanismes enzymatiques qui en résultent et leur conséquence sur la population peptidique, et enfin les différents mécanismes d'actions appliqués par les peptides générés nécessaire pour avoir des activités antibactérienne, antifongique et antioxydante. Cette étude rapporte aussi, pour la première fois, la capacité de ces hydrolysats à inhiber la croissance d'*Aspergillus niger, Mucor racemosus, Penicillium crustosum, Rhodotorula mucilaginosa* et *Paecilomyces spp*. En se basant sur les résultats de cette étude, d'autres expériences se concentreront sur l'élimination complète du colmatage peptidique en améliorant la déminéralisation de l'hémoglobine bovine tout en utilisant une nouvelle configuration.

Chapitre V

PRODUCTION ÉCO-CIRCULAIRE DE PEPTIDES BIOACTIFS DÉMINÉRALISÉS À PARTIR D'HÉMOGLOBINE BOVINE EN EFFECTUANT SIMULTANÉMENT, LES MULTIPLES ÉTAPES NÉCESSAIRES, EN UTILISANT L'ÉLECTRODIALYSE AVEC MEMBRANES BIPOLAIRES.

V. Production éco-circulaire de peptides bioactifs déminéralisés à partir d'hémoglobine bovine en effectuant simultanément, les multiples étapes nécessaires, en utilisant l'électrodialyse avec membranes bipolaires.

V. Transition contextuelle

Dans le chapitre précédent, l'optimisation a porté sur le type de membranes monopolaires associées aux membranes bipolaires, le niveau de conductivité, la valeur du pH et le mécanisme enzymatique. L'EDMB-MCP a permis la production d'hydrolysats contenant une faible concentration en sels minéraux mais avec formation de colmatage sur la MCP, tandis que l'EDMB-AEM a permis la production d'hydrolysats sans colmatage mais avec une concentration en sel similaire à l'hydrolyse conventionnelle. L'hydrolyse en EDMB a montré le même mécanisme enzymatique, « Zipper », identique à celui obtenu en hydrolyse conventionnelle (contrôle). Après avoir dénaturé la solution et maintenu le pH de la solution d'hémoglobine durant l'hydrolyse enzymatique par électroacidification suivant des conditions optimisées, l'étape suivante du projet consistait à arrêter l'action de l'enzyme par électrobasification et déminéraliser les hydrolysats d'hémoglobine. En se basant sur les travaux des chapitres précédents qui ont utilisé une configuration à 2 compartiments afin d'évaluer la faisabilité du procédé et d'optimiser les conditions, le protocole de cette dernière partie sera basé sur une configuration à 3 compartiments. L'objectif principal de cette étude sera d'étudier le procédé de préparation d'hydrolysats d'hémoglobine bovine déminéralisé par EDMB en testant pour la première fois et en simultané les différentes étapes de fabrication de peptides bioactifs au sein d'un système d'EDMB, à savoir la dénaturation et l'hydrolyse enzymatique de l'hémoglobine (compartiment acidifié), l'inactivation de la pepsine (compartiment basifié) et déminéralisation des hydrolysats (compartiment central de diluat).

Les objectifs spécifiques de cette étude seront de : 1) étudier l'évolution du pH et de la conductivité dans les trois compartiments, 2) caractériser le colmatage des membranes, 3)

quantifier la composition minérale, 4) identifier le mécanisme enzymatique impliqué dans l'hydrolyse de l'EDMB, 5) caractériser la population peptidique obtenu après hydrolyse et enfin 6) étudier les activités biologiques des hydrolysats obtenus à partir de l'EDMB.

Ce chapitre a fait l'objet de deux articles: Le premier intitulé : « **Eco-circular production of demineralized bioactive peptides from bovine hemoglobin by performing the necessary steps simultaneously using Bipolar Membrane Electrodialysis** », accepté dans *ACS Sustainable Chemistry & Engineering,* le 24 novembre 2021. Les auteurs sont: Mira Abou-Diab (Candidate au Ph. D.: planification et réalisation des expériences, analyse des résultats et rédaction de l'article), Jacinthe Thibodeau (Professionnelle de recherche côté canadien : supervision de l'étudiant pour les analyses d'électrodialyse et LC-MS, correction du manuscrit), Ismail Fliss (Co-directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit), Pascal Dhulster (Co-directeur de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit), Naima Nedjar (Directrice de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit) et Laurent Bazinet (Directeur de thèse côté canadien: supervision scientifique de l'étudiant, correction et révision du manuscrit).

Le second ayant pour titre: « **Production of demineralized Antibacterial, Antifungal and Antioxidant peptides from bovine hemoglobin using an optimized « multiple-steps » system: Electrodialysis with Bipolar Membrane** », soumis pour publication dans *Membranes,* le 20 août 2021. Les auteurs sont: Mira Abou-Diab (Candidate au Ph. D.: planification et réalisation des expériences, analyse des résultats et rédaction de l'article), Jacinthe Thibodeau (Professionnelle de recherche côté canadien : supervision de l'étudiant pour les analyses d'électrodialyse et LC-MS, correction du manuscrit), Ismail Fliss (Co-directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit), Pascal Dhulster (Co-directeur de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit), Laurent Bazinet (Directeur de thèse côté canadien : supervision scientifique de l'étudiant, correction et révision du manuscrit) et Naima Nedjar (Directrice de thèse côté Français : supervision scientifique de l'étudiant, correction et révision du manuscrit).

V. *Premier article*

V.1. Résumé

Il existe un grand intérêt pour les peptides dérivés de l'hémoglobine bovine. Le but de cette étude était de tester une nouvelle configuration originale d'EDBM, permettant en simultané la dénaturation de l'hémoglobine, l'inactivation de la pepsine et la déminéralisation des hydrolysats, pour produire des peptides bioactifs sans ajout de produits chimiques et sans colmatage membranaire. Jusqu'à présent, ce procédé vert n'a jamais été réalisé sans colmatage. Un procédé « 3-en-1 » utilisant une configuration d'EDBM à trois compartiments a été mise en place. L'EDMB s'est avérée efficace pour dénaturer l'hémoglobine, inactiver la réaction enzymatique et déminéraliser à 85% les hydrolysats finaux. Cependant, un colmatage a encore été observé sur la membrane anionique dû à la précipitation de l'hème. Pour cette raison, une étape supplémentaire de décoloration a été proposée avant la déminéralisation. Ainsi, un procédé global « 4-en-1 » d'EDMB a été développé dans la présente étude produisant des bio-peptides à faible teneur en minéraux pouvant être utilisés comme conservateurs naturels de viande.

Mots clés : Électrodialyse avec membranes bipolaires, Hémoglobine bovine, Hydrolyse enzymatique, Électro-acidification, Électro-basification, Déminéralisation, Décoloration, Économie circulaire.

V.2. Abstract

There is a great interest in peptides derived from bovine hemoglobin, a protein from slaughterhouse waste, as they have been demonstrated as a potential novel class of natural conservative agents. The aim of the present study was to test a new original electrodialysis with bipolar membrane (EDBM) configuration, allowing the simultaneous denaturation of hemoglobin, inactivation of pepsin and demineralization of the hydrolysates, to produce bioactive peptides without adding chemicals and avoiding membrane fouling. Indeed, membrane fouling is the main concern of this process since it decreases the process

performances and the lifespan of membranes. Up to now, this ecofriendly process has never been achieved without fouling. A complete « 3-in-1 » process using a three-compartment EDBM configuration was carried-out and compared to a conventional process using chemical acid and base. The EDBM method was found to be as effective as the conventional method for denaturing the hemoglobin and for inactivating the enzymatic reaction. Furthermore, the use of EDBM allowed a demineralization rate of 85% of the hemoglobin hydrolysate in the diluate compartment. However, fouling was still observed on the anionic membrane due to heme precipitation. For this reason, an additional step of discoloration was carried-out before demineralization to avoid fouling. Hence, an innovative global « 4-in-1 » process operating from the hemoglobin hydrolysis to the recovery of demineralized bioactive peptides using an EDBM system with no fouling was proposed. Moving towards a circular economy, this sustainable technology has a great potential for industrial hydrolysis of blood, a slaughterhouse by-product produced in large quantities all around the world, since it produces biopeptides with a lower mineral content that can be used as natural preservatives on meat.

Keywords: Electrodialysis with bipolar membrane, Bovine hemoglobin, Enzymatic hydrolysis, Electro-acidification, Electro-basification, Demineralization, Discoloration, Circular economy.

V.3. Introduction

Hemoglobin is a protein that constitutes 90% of the total cruor volume, a bovine blood by-product from slaughterhouses, considered as agricultural waste. Bovine hemoglobin has a globular structure in its native form. This protein is formed of four polypeptide chains, two α chains and two β chains, and an overall molecular mass of 64.5 kDa. Each of the chains has a protein part, called globin, and a non-protein part. The latter represents the hemoglobin heme, a tetrapyrrolic complex of ferrous iron, which is positioned within the compact globular structure formed by each protomer. Hem is responsible for the red color of the blood. The enzymatic hydrolysis of hemoglobin is a source of many peptides with biological activities (Adje *et al*., 2011a; Adje *et al*., 2011b; Gomes *et al.,* 2010; Nedjar *et al*., 2008).

The conventional method for producing these bioactive peptides consists of native hemoglobin denaturation and pH adjustment during enzymatic hydrolysis by chemical acidification, inactivation of enzymatic reaction by chemical basification and a potential additional step of hemoglobin hydrolysate discoloration by chemical acidification (Adje *et al*., 2011a; Adje *et al*., 2011b; Gomes *et al.,* 2010; Nedjar *et al*., 2008; Nedjar *et al*., 2006; Zouari *et al*., 2020). Indeed, the presence of hem, responsible for the red color of the hydrolysates, makes it difficult to valorize the products in some applications. Therefore, discoloration is carried-out at industrial scale to avoid organoleptic changes following the incorporation of hemoglobin bioactive peptides into the formulation of feed intended for human or animal consumption due to iron oxidation. However, the addition of salts during the acidification and hydrolysis phases of hemoglobin, using chemical acid, could impact the structure of hemoglobin or the activity of pepsin as demonstrated by Abou-Diab *et al*. (2021). Furthermore, the hydrolysates produced contain high levels of mineral salts which may decrease the purity of the final product as described by Lin Teng Shee *et al*. (2008) for chitosan oligomers and whey protein fractions Lin Teng Shee *et al*. (2005). High salt concentrations in the final hydrolysates could also impact the antimicrobial activity of peptides. Experimental studies have shown that antimicrobial activity of the antimicrobial peptides (AMPs) is dependent on the concentration of salt and demonstrated that antimicrobial activity is typically reduced at higher salt concentrations (Matsuzaki *et al*., 1991; Lee *et al*., 1997; Kandasamy and Larson, 2006). Moreover, the peptides produced will be used as natural preservatives within the food matrix, such as minced meat type or its derivatives (Przybylski *et al*., 2016). However, high salt content in preservatives is a root cause of health risks such as heart and kidney diseases, high blood pressure, etc. (Frisoli *et al*., 2012; Heerspink *et al*., 2012; Cappuccio, 2013) and does not meet the consumer needs, who ask for healthy foods. Therefore, salt content needs to be reduced.

Recently, electrodialysis with bipolar membrane (EDBM) was carried out for bovine hemoglobin denaturation and enzymatic hydrolysis by electro-acidification to produce hydrolysates with demonstrated antimicrobial and antioxidant activities (Abou-Diab *et al*., 2020a; 2020b). EDBM is a proven green and eco-efficient technology (Mikhaylin *et al*.,

2018; Faucher *et al*., 2020) for acid and base generation using protons and hydroxide ions generated by water dissociation at the interface of the bipolar membrane under an electrical field obtained from renewable sources, without hazardous chemicals needed (Pourcelly, 2020). According to Abou-Diab *et al.* (2020a) denaturation and hydrolysis of bovine hemoglobin was most effective using a bipolar/anionic membrane configuration, allowing more rapid production of bioactive peptides. Moreover, this configuration did not show any membrane fouling and consequently the global resistance and the energy consumption were low. However, the final hydrolysates contained high mineral concentrations. In contrast, demineralization was successfully achieved with a bipolar/cationic membrane configuration, leading to the production of bioactive peptides fractions having a low mineral content, but with the presence of membrane fouling (Abou-Diab *et al*. 2020a). Nevertheless, a further demineralization step would be necessary to reduce minerals of the final hemoglobin hydrolysate which increased during the electro-acidification and electro-basification steps.

In this context and according to the circular economy philosophy, the general objective of this study was to propose a complete and new optimal process, using a three-compartment EDBM configuration, to recycle and valorize bovine blood by-product from slaughterhouse to produce a hemoglobin hydrolysate having antimicrobial and antioxidant activities suitable for applications in the meat industry. Each compartment will be used to perform, simultaneously hemoglobin denaturation, enzymatic hydrolysis, pepsin inactivation, and hemoglobin hydrolysate demineralization so that the process produces no waste. The specific objectives of this study were to (1) study the evolution of pH and conductivity in the three compartments, (2) characterize membrane fouling, (3) quantify the mineral composition, (4) identify the enzymatic mechanism taking place in EDBM hydrolysis, and (5) characterize the peptide population obtained after hydrolysis. The EDBM process was compared to conventional hydrolysis using chemical acid and base.

V.4. Materials and Methods

V.4.1. Materials

Hemoglobin and pepsin. Hemoglobin (Hb) and pepsin were purchased from Sigma-Aldrich (Oakville, ON, Canada). The purified hemoglobin powder from bovine blood, dark brown (H2625) was composed of ≃ 98% of proteins, ≤ 2% of ashes, ≤ 8% of water and 0.25-0.35% of iron (Fe). Hemoglobin was stored at 4 °C before use. The pepsin (P6887) was a lyophilized powder from porcine gastric mucosa (EC 3.4.23.1, 3200-4500 units/ mg protein). The activity of pepsin was measured at 3250 AU/mg of protein according to a protocol established by the supplier, Sigma-Aldrich. Pepsin was stored at -20 °C.

V.4.2. Protocols

V.4.2.1. Preparation of bovine hemoglobin hydrolysates using EDBM

V.4.2.1.1. EDBM configuration

The EDBM system consisted of a three-compartment electrodialysis cell (**Figure V-1**). The module was a MP type cell (100 cm^2 of effective surface) equipped with a dimensionally stable anode (DSA) and a 316 SS cathode from ElectroCell AB (Taby, Sweden). The ionic membranes were two bipolar membranes (BM, Astom, Tokyo, Japan), one anion-exchange membrane (AEM, Astom, Tokyo, Japan) and one monovalent cation perm-selective membranes (MCP, Astom, Tokyo, Japan). The electrode rinsing solution was a 20 g/L Na_2SO_4 solution (600 mL). The other reservoirs during the « 3-in-1 » process were filled with 600 mL of the following solutions: (1) a 1% hemoglobin solution in the acidified compartment, (2) a 3 h hemoglobin hydrolysate solution with active pepsin in the basified compartment, and (3) a 3 h hemoglobin hydrolysate solution with inactive pepsin in the diluate compartment (**Figure V-1**). A constant voltage of 15 V, corresponding at the beginning of the process to a current density of 6.63 mA/cm^2 was applied during the EDBM experiments using a power supply (Model HPD 30-10, Xantrex, Burnaby, BC, Canada). The

flow rates were maintained constant at 800 mL/min for the electrode-rinsing solution and at 450 mL/min for the other three solutions. This configuration was the same as the one used by Lin Teng Shee *et al*. [8] and reported to be effective for chitosan demineralization.

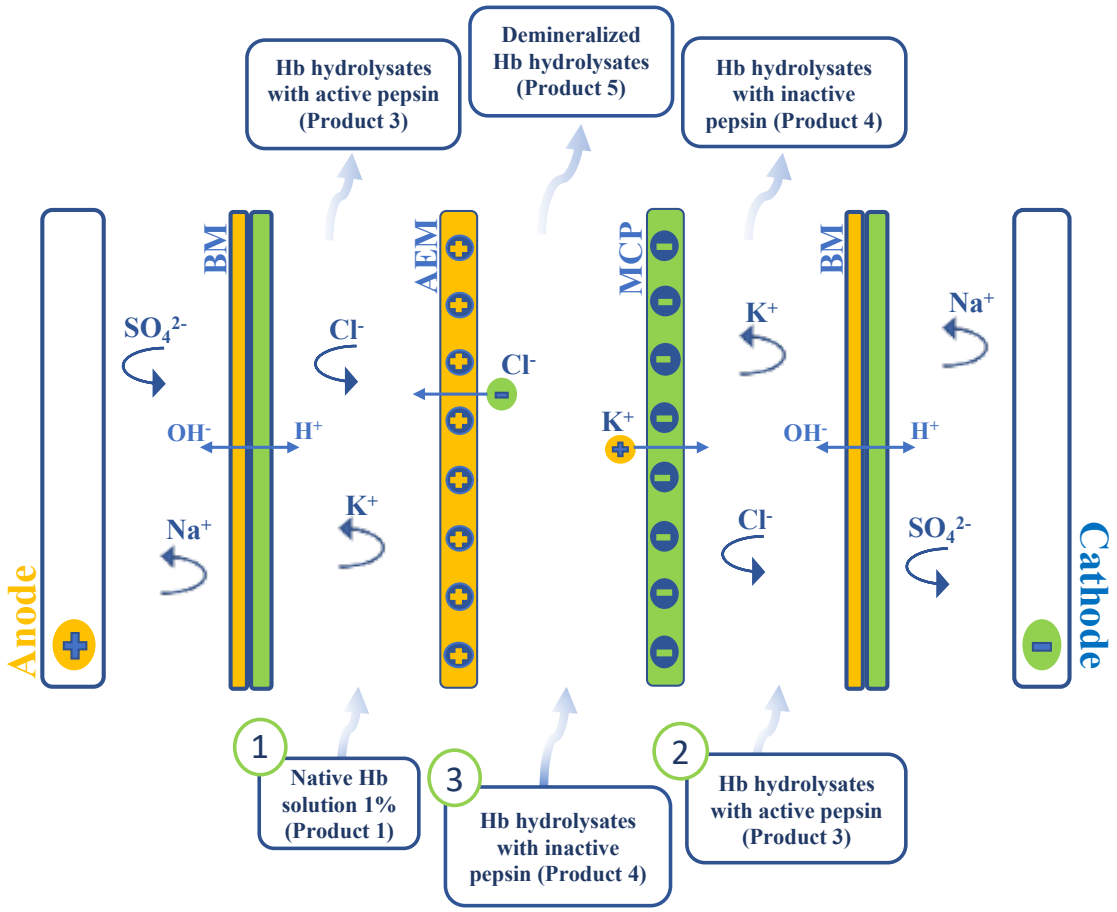

Figure V-1. Schematic representation of the three-compartment bipolar membrane electrodialysis cell configuration for production of demineralized hemoglobin hydrolysates: (1) electro-acidified compartment; (2) electro-basified compartment; (3) demineralized compartment. BM, bipolar membrane; MCP, monovalent cation perm-selective membrane and AEM, anion exchange membrane.

V.4.2.1.2. EDBM protocol

The production of bovine hemoglobin demineralized hydrolysates starts with hemoglobin solution denaturation and subsequently its enzymatic hydrolysis by pepsin, follows by pepsin inactivation, and ends with hemoglobin hydrolysates demineralization. However, before all three EDBM steps were performed in a simultaneous way, three preliminary steps were

carried-out in EDBM to obtain the solutions for all « 3-in-1 » compartments as shown in **Figure V-2.**

In the first step, the acidification compartment was used to denature the 1% native hemoglobin solution (product 1) by electro-acidification according to the previous method by Abou-Diab *et al*. (2020a). Briefly, a stock solution was prepared by adding 15 g of purified bovine hemoglobin (BH) into 100 mL of ultrapure water. From determination of the accurate hemoglobin concentration of the stock solution with the Drabkin's method (Crosby *et al*., 1954), a 1% (w/v) solution of BH was prepared. The 1% BH solution conductivity was adjusted to 1.5 mS/cm with KCl as explained by Abou-Diab *et al*. (2020a). Because a certain conductivity was required during ED experiments, for ion migration and to avoid « water-splitting ». The hemoglobin solution had a pH of 6.94 ± 0.02 and was adjusted at pH 3 by applying an electric current leading to the electrochemical production of protons (H^+) at the cationic interface of the bipolar membrane in contact with the BH solution. At the end of the acidification process, when pH 3 was reached, we obtained a denatured hemoglobin solution (product 2). Hemoglobin is completely denatured at pH 3. Afterward, porcine pepsin was solubilized in ultrapure water, with an enzyme/substrate ratio equal to 1/11 (mole/mole) and was added to product 2 to start the three-hour hydrolysis. While maintaining pH at 3 by turning « on » the current as needed, enzymatic hydrolysis was realized in the acidified compartment. At the end of the hydrolysis process, the hemoglobin hydrolysates with active pepsin led to product 3. Simultaneously, KOH (0.1M) was generated in the basified compartment and KCl solution (2 g/L) demineralized in the diluate compartment due to the migration of anions through the anionic membrane to the acidified compartment and the migration of the cations through the cationic membrane to the basified compartment.

In the second step, after a 3 hour-hydrolysis, the enzymatic reaction of hemoglobin solution with active pepsin was terminated by circulating the hemoglobin hydrolysate, recovered from the acidified compartment (product 3), in the basified compartment. Hemoglobin hydrolysate at pH 3, was adjusted at pH 9, by electro-basification, using the hydroxide ions (OH^-) produced at the anionic interface of the bipolar membrane and led to product 4.

Simultaneously, HCl (0.01 M) was being generated in the acidified compartment and KCl solution (2 g/L) was being demineralized in the diluate compartment.

In the third step, the denaturation and the enzymatic hydrolysis of a new hemoglobin solution (new product 1) was carried out in the acidification compartment as described previously in step 1 and led to a new product 3. Simultaneously, KOH was being generated in the compartment of basification and a KCl solution was being demineralized in the diluate compartment. After a 3 hours-enzymatic hydrolysis, the reaction was ready to be stopped in the basified compartment.

In the fourth step (3-in-1), the three compartments were running all together. The acidification compartment was used for a new hemoglobin solution denaturation and enzymatic hydrolysis. The basification compartment was used for pepsin inactivation of product 3 recovered from the third step. The diluate compartment located between the acidification and the basification compartments was used for alkaline hemoglobin hydrolysate solution (product 4) demineralization recovered from the second step, leading to product 5: demineralized Raw Hydrolysates (RH) produced by EDBM (EDBM-RH), as shown in **Figure V-1**.

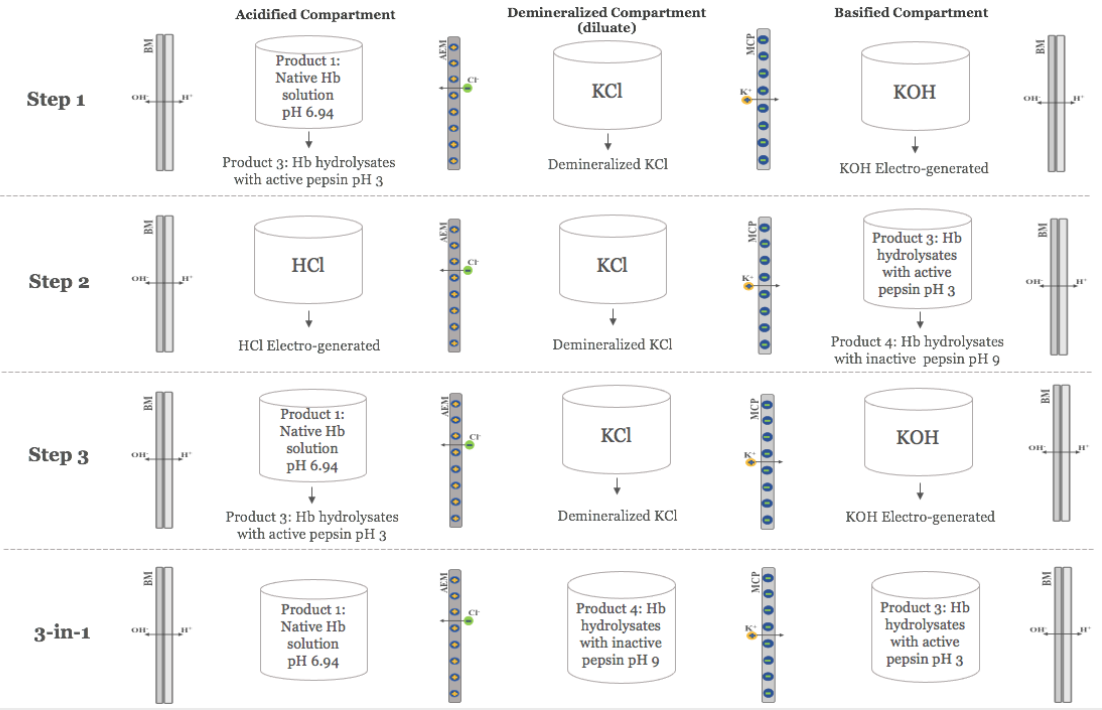

Figure V-2. Scheme of preliminary process steps for bovine hemoglobin transformation using electrodialysis with bipolar membrane (EDBM).

V.4.2.2. Preparation of bovine hemoglobin hydrolysates using conventional hydrolysis

Hemoglobin hydrolysates were also prepared using the conventional hydrolysis (CH) method and was considered as control. The conventional method was carried-out by adding 15 g of purified bovine hemoglobin (BH) into 100 mL of ultrapure water. From the real concentration C of the stock solution determined according to the Drabkin's method, a solution of BH was prepared by dilution to a 1 % (w/v). The pH of the 1 % hemoglobin solution was 6.87 ± 0.09 and its conductivity value 0.0597 ± 0.0039 mS/cm. To denature the hemoglobin solution (600 mL), the pH was adjusted at 3 with 2 M HCl (Anachemia, VWR Company International, ON, Canada). Then, the hydrolysis was performed at pH 3 in the presence of porcine pepsin solubilized in ultrapure water, with an enzyme/substrate ratio equal to 1/11 (mole/mole). Throughout the 3 hours of hydrolysis, the pH was maintained at 3 by adding HCl if needed. After 3 hours of enzymatic hydrolysis, the reaction was ended by chemical basification of hemoglobin with addition of 0.5 M KOH (Fisher Scientific, New

Jersey, USA) to the 600 mL raw hydrolysates produced by conventional hydrolysis (CH-RH).

Furthermore, hem is responsible for the red color of hemoglobin and contains iron. The discoloration, which consists of separating the hem (non-protein part) from the globin (protein part) of hemoglobin is important to limit color change in food formulation due to iron oxidation. Hence, a discoloration step was carried-out on hemoglobin hydrolysate, in control, where reaction was stopped by the pH precipitation method (Adje *et al*., 2011a; Adje *et al*., 2011b; Nedjar *et al*., 2008; Hedhili, 2014). The method consisted of lowering the pH of the hemoglobin hydrolysate using HCl, thus making heme insoluble. It was followed by a centrifugation step, which allowed heme precipitation, therefore causing the discoloration of the hydrolysate. For this, a pH range was produced for each of the different samples obtained from the peptic hydrolysate after inactivation of the enzyme. Briefly, to determine the optimum discoloration pH, 1 mL of hydrolysate at pH 9 was taken and adjusted to a pH range of 3.5 to 6.5 with HCl (2 M). After 3 min centrifugation at 10 000 x g, the absorbance of the supernatant was measured at 392 nm. The sample with the lowest absorbance at 392 nm was chosen as the optimum discoloration pH. After determining the optimal value for discoloration, which was 4.5, the method was applied to the total volume of the final hydrolysate. Under agitation (60 min), the pH of the hydrolysate was slowly lowered to a value of 4.5 by addition of HCl (2 M). After 24 hours, the hydrolysate was centrifuged during 30 min at 6000 x g and discolored hydrolysates produced by conventional hydrolysis were recovered (CH-DH).

Samples were collected from EDBM and conventional hydrolysis for chemical analysis at 0 min before adding the pepsin and then after pepsin addition at 2.5, 30, 60, 120 and 180 min during the hydrolysis step corresponding to different degrees of hydrolysis. The reaction in each sample was stopped by addition of KOH (0.5 M) (Fisher Scientific, New Jersey, USA) to bring the sample to pH 9, allowing to irreversibly inactivate the enzyme. Other samples were collected at the end of the basification phase and after demineralization in EDBM from the diluate compartment. The samples were stored at -20 °C. Physicochemical parameters were followed all along the EDBM process by measuring the pH, conductivity, and

temperature of the solutions in the three compartments. Voltage and current intensity data were recorded. Moreover, measurements of pH, conductivity, ash levels, mineral concentration, degree of hydrolysis, hydrolysis mechanism and identification of the peptide population by UPLC-QTOF were performed on the raw hydrolysates (RH) and discolored hydrolysates (DH) produced by conventional hydrolysis for comparison with EDBM hydrolysates.

V.4.3. Analyses

V.4.3.1. Determination of hemoglobin concentration: Drabkin's method

Drabkin's reagent is a spectrophotometric method used for the quantitative determination of hemoglobin concentration in whole blood. Briefly, 40 µL of the sample were added to 10 mL of the Drabkin's reagent (D5941, Sigma-Aldrich, Oakville, ON, Canada) for 15 min at room temperature. Then, the absorbance was read at a wavelength of 540 nm by a UV spectrophotometer (ChemStation 8453A UV-Vis Spectrophotometer, Agilent Technologies, Santa Clara, CA, USA) and reported into the calibration curve. From the concentration C of the stock solution, solutions of purified bovine hemoglobin were prepared by dilution to precise concentrations of hemoglobin; C_{BH}: 1% (w/v).

V.4.3.2. pH

The pH in the three compartments was measured using a pH meter equipped with a pH electrode containing an automatic temperature compensation (ATC) probe (model SP70, symphony, VWR international, Montreal, Canada). To stop the hydrolysis reaction in the hemoglobin hydrolysate samples, a fine tip pH electrode (Mettler Toledo, LE422, micro, 3 mm, United Kingdom) was used to measure the pH.

V.4.3.3. Conductivity

Electrical conductivity of the solutions in the three compartments was measured with an YSI conductivity meter (Model 3100, Yellow Springs Instrument, Yellow Springs, OH, USA) combined with an YSI 3252 electrode with a cell constant of 1 cm^{-1}. The demineralization rate was calculated using the change in conductivity between the start and the end of the electrodialysis treatment, according to:

$$\text{Demineralization Rate} = (1 - \text{Conductivity}_{\text{End}}/\text{Conductivity}_{\text{Start}}) \times 100$$

V.4.3.4. Global electrical resistance and relative energy consumption

Electrical resistance was calculated according to Ohm's law: R = U/I, where R is the global system resistance (Ω), I the current intensity (A) and U the voltage applied between the electrodes of the ED cell (V). The voltage and the current intensity were directly read on the power supply. Relative energy consumption was calculated to measure the efficiency of EDBM process using the following equation:

$$E_c = \frac{E}{m} = \int_0^t \frac{I \times U}{3600 \times m}\, dt$$

with E_c, the relative energy consumption (Wh/g peptide); E, the energy consumption of the process (Wh); I, the current intensity (A); U, the voltage (V); m, the mass of peptide recovered (g) and t, the experiment duration (s). The estimation of global electrical resistance and relative energy consumption were based on the very last 3-in-1 step in **Figure V-2**.

V.4.3.5. Membrane thickness and electrical conductivity

Membrane thickness was measured with an electronic digital micrometer (Model Marathon Watch Company LTD., Richmond Hill, ON, Canada). Membrane electrical conductance (G) was measured with a specially designed cell (Laboratoire des Matériaux Échangeurs d'Ions,

Créteil, France) following the procedure described by Mikhaylin *et al*. (2016). The NaCl reference solution was 0.5 M. The membrane conductivity was calculated according to Lteif *et al*. (1999) and Lebrun *et al*. (2003):

$$R_m = R_{m+s} - R_s$$

where R_m is the electrical resistance of the membrane (in Ω), R_{m+s} the electrical resistance of the membrane and the reference solution measured together (in Ω), and R_s the electrical resistance of the reference solution (in Ω). The resistance R is equal to 1/G. The membrane electrical conductivity (κ) was calculated as follow:

$$\kappa = L/R_m A$$

where L is the membrane thickness (in cm) and A is the electrode area (1 cm^2), these measurements were recorded before and after each replicate.

V.4.3.6. Total solid and ash contents

The total solid content was determined by drying hemoglobin solution samples at 95 °C overnight. The ash content was measured by heating the dried samples at 550 °C for 20 h according to the AOAC international method 945.46 (AOAC, 1990).

V.4.3.7. Mineral concentration measurement

The samples collected after 180 min of hydrolysis were thawed at 4 °C before their dilution 1/20 in ultrapure water. Potassium, sodium and sulfur concentrations were determined by optical emission spectrometry with inductively coupled plasma as atomisation and excitation source (ICP-OES Agilent 5110 SVDV Agilent Technologies, Victoria, Australia), using the following wavelengths (in nm): 766.491 (K), 588.995; 589.592 (Na), 181.972; 180.669 (S). The analyses for all ions were performed in axial and/or radial view. For chlorides, the samples were analyzed on a Flow Injection Analysis (FIA) (Quikchem 8500 series 2,

Zellweger Analytic, inc., Lachat Instruments Division, Milwaukee, WI, USA) with Quikchem method 10-117-07-1-C: Determination of chloride by Flow injection analysis colorimetry.

V.4.3.8. Determination of the degree of hydrolysis

The degree of hydrolysis was quantified by the ortho-phthaldialdehyde (OPA) technique as described by Abou-Diab *et al*. (2020a), adapted from Church *et al*. (1983) and Spellman *et al*. (2003).

V.4.3.9. Identification of hydrolysis mechanism

All samples were analyzed by UPLC-QTOF to identify the hydrolysis mechanism and the peptide sequences. A 1290 Infinity II UPLC (Agilent Technologies, Santa Clara, CA, USA) system was used for the RP-UPLC analyses. The equipment consisted of a binary pump (G7120A), a multisampler (G7167B), an in-line degasser and a variable wavelength detector (VWD G7114B) adjusted to 214 nm. Hydrolysis fractions were filtered through 0.22µm PVDF filter into a glass vial. Samples were loaded (0.25µL) into a Poroshell 120 EC-C18 column (2.1 × 100mm i.d., 2.7 micron, Agilent, Santa Clara, USA). The column was operated at a flow rate of 500 µL/min at 23 °C. A gradient consisting of solvent A (LC-MS grade water with 0.1 % formic acid) and solvent B (LC-MS grade ACN with 0.1 % formic acid) was applied with solvent B going from 1 % to 13 % in 6 min, ramping to 35 % until 25 min, ramping again to 100 % until 35 min and holding until 45 min, then back to initial conditions. Each sample was run in triplicate for statistical evaluation of technical reproducibility.

V.4.3.10. Identification and characterization of peptide population

A hybrid ion mobility quadrupole time of flight mass spectrometer (6560 high-definition mass spectrometry (IM-Q-TOF), Agilent, Santa Clara, USA) was used to identify and quantify the relative abundances of the peptides present in the hydrolysates. All LC-MS/MS experiments were acquired using Q-TOF. Signals were recorded in positive mode at

Extended Dynamic Range, 2Ghz, 3200m/z with a scan range between 100-3200 m/z. Nitrogen was used as the drying gas (13.0 L/min and 150 °C) and as nebulizer gas (30 psig). The capillary voltage was set at 3500 V, the nozzle voltage at 300 V and the fragmentor at 400 V. ESI-L low concentration tuning mix (Agilent Technologies, Santa Clara, CA, USA) was used to calibrate the instrument. Data acquisition and analysis were performed using the Agilent Mass Hunter Software package (LC/MS Data Acquisition, Version B.08.00 and Qualitative Analysis for IM-MS, Version B.07.00 Service Pack 2 with BioConfirm Software). UPLC-QTOF combination was used to analyze the peptides by comparing them with the known hemoglobin alpha and beta sequences with the BioConfirm software as recently described by Abou-Diab *et al*. (2020a). Then bioactive peptides were identified by comparing them to the literature (Nedjar *et al*., 2008; Lignot *et al*., 1999; Zhao *et al*., 1995; Vercaigne-Marko *et al*., 2000; Takagi *et al*., 1982; Chang *et al*., 1980; Piot *et al.,* 1992).

V.4.3.11. Statistical analyses

All analyses were performed in triplicate and three independent repetitions were carried-out for each condition. Data were subjected to one-way or two-way analyses of variance (ANOVA). Tukey tests were also conducted on data using SigmaPlot software (Version 14.0) to determine which treatment was statistically different from the others at a probability level p of 0.05.

V.5. Results and discussion

V.5.1. Efficiency of the « 3-in-1 » process

V.5.1.1. Evolution of pH and conductivity

The global EDBM process duration for simultaneous phases of electro-acidification, electro-basification, and demineralization was 90 ± 2 min. The global process duration has been improved in comparison with previous study by Abou-Diab *et al*. (2020a), who reported a global process duration of 570 ± 20 min and 240 ± 2 min in EDBM-MCP and EDBM-AEM,

respectively. The evolution of pH and conductivity for acidification, basification and demineralization phases are shown in **Figure V-3.**

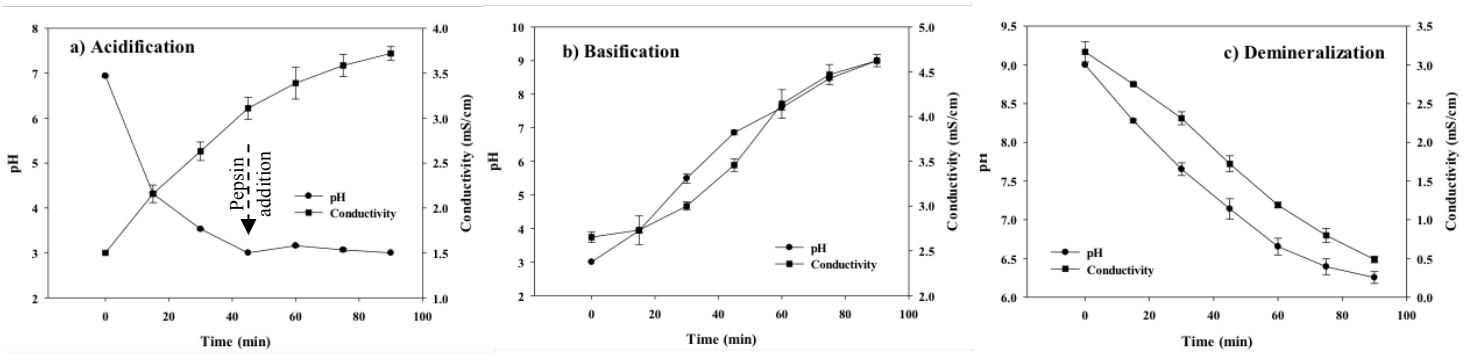

Figure V-3. Evolution of pH and conductivity as a function of time during a) acidification, b) basification, and c) demineralization running simultaneously in EDBM.

(a) Hemoglobin acidification. Bovine hemoglobin acidification was carried-out using an EDBM system or HCl for control experiments. This EDBM phase consisted of 2 steps: the denaturation of hemoglobin (first 45 min) and the enzymatic hydrolysis (last 45 min) as shown in **Figure V-3** (**a**). During the first step of denaturation, pH decreased of about 3.94 units, from pH 6.94 ± 0.02 to 3.00 ± 0.00, due to H^+ generated by the bipolar membranes. During the second step, after adjusting the pH to 3, pepsin was added for a three-hour hydrolysis. After adding the solubilized pepsin, in the acidified compartment, pH increased normally, as shown in **Figure V-3** (**a**). Throughout, the 45 min of hydrolysis, the pH was maintained at 3 in EDBM by applying current if needed. After 45 min of hydrolysis, at the end of the process (after 90 minutes), the pH was remained stable at 3. For this reason, hemoglobin solution was collected in a beaker and the hydrolysis was left to continue for another 135 minutes (total of 180 min of hydrolysis) outside the system because it was no longer needed to adjust the pH with current. Therefore, electrical energy was saved. In comparison, the pH was decreased and maintained at 3 in control by adding HCl if needed.

In parallel, during the denaturation, conductivity increased from 1.5 ± 0.0 to 3.11 ± 0.12 mS/cm in EDBM and from 0.059 ± 0.0039 to 1.15 ± 0.04 mS/cm in control. It continued to increase during the enzymatic reaction, to reach 3.72 ± 0.07 mS/cm (2.48 times increase) and

1.82 ± 0.08 mS/cm (30.84 times increase) in EDBM and control, respectively. Conductivity increased in EDBM due to the partial mineralization caused by the migration of Cl^- anions through the AEM from the demineralized compartment. While the conductivity increased in control due to the addition of HCl to maintain pH at 3.

(b) Hemoglobin hydrolysate basification. Enzymatic reaction of bovine hemoglobin with pepsin was terminated by basifying the solution using EDBM (**Figure V-3-b**) or chemical method. The increase in pH from 3 to 9 was done by the OH^- generated at the anionic interface of the bipolar membranes inducing basification of the solution or by addition of KOH in control. In parallel, conductivity increased from 2.65 ± 0.05 to 4.62 ± 0.06 mS/cm (1.74 time) and from 1.82 ± 0.08 to 3.64 ± 0.14 mS/cm (2 times) in EDBM and control, respectively. Conductivity increased in EDBM due to the partial mineralization caused by the migration of K^+ ions through the MCP from the demineralized compartment. While, for the control, the increase in conductivity was due to addition of KOH.

(c) Demineralization of hemoglobin hydrolysate by EDBM. After basification of the hemoglobin hydrolysate, the solution was demineralized in compartment 3 of the EDBM system (**Figure V-1**). A demineralization of 85% was obtained with conductivity decreasing from 3.17 ± 0.12 to 0.49 ± 0.04 mS/cm. This decrease, in the diluate compartment, was due to the migration of anions and cations through the anionic and cationic membranes, respectively. The cations removed from the diluate were mainly K^+ ions from KCl salt initially added, whereas the anions removed were mainly Cl^- and OH^- from the ions added initially and consecutively from basification. In parallel, the pH value decreased from 9 to 6.25 ± 0.07 (**Figure V-3-c**). The decrease in pH was explained by partial dealkalinization of the solution, since part of the OH^- migrated from the diluate compartment to the adjacent acidified compartment by crossing the anionic membrane, as described by Lin Teng Shee *et al*. (2008) for chitosan hydrolysate solution demineralization. To study the effect of the decrease in pH on the hemoglobin hydrolysate in the diluate compartment, chromatographic profiles of different demineralization rates (5% (pH = 8.28±0.02), 30%, 50%, 60%, 70%, 80% et 85% (pH = 6.25±0.07)) were analyzed over time as shown in **Figure V-4**. Chromatographic profiles of hemoglobin hydrolysate showed roughly the same profiles for

the different demineralization rates. However, the hem absorbance peak, at 28 min of retention time, gradually decreased from a value between 350-400 AU to a value between 150-200 AU for 5% and 85% of demineralization, respectively. It appeared that the decrease in pH impacted the hemoglobin hydrolysate in the diluate compartment leading to its precipitation. Indeed, hem is maintained in solution by its hydrophobic interactions with peptides (Lebrun *et al.,* 1998). The pH value (6.25) reached at the end of the demineralization phase is in the pH range between 4.5 and 6.5 where the solubility of the hydrolysate decreases sharply as described by Lebrun *et al*. (1998). They demonstrated that this interval corresponds to the pHi of peptides supporting hem in solution causing precipitation of hem-peptide associations by the break of their hydrophobic interactions.

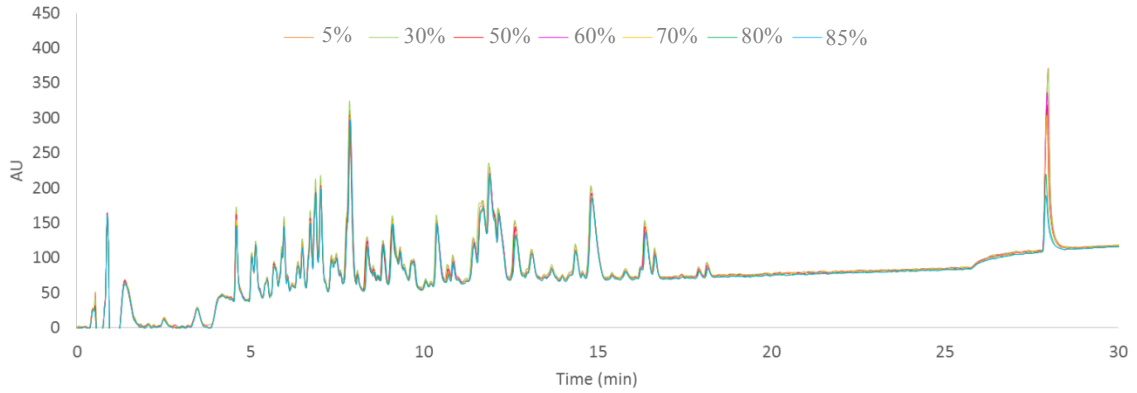

Figure V-4. Overlay of chromatographic profiles of different demineralization rates over time (5%, 30%, 50%, 60%, 70%, 80% et 85%) for bovine hemoglobin hydrolysate in EDBM at 214 nm by UPLC-QTOF, analyzed by C18 column.

V.5.1.2. Global resistance and relative energy consumption

The global resistance calculated from voltage and intensity, increased from 22.63 ± 0.29 Ω at the beginning of the process to reach 103 ± 2.14 Ω at the end of the process (**Figure V-5**). The increase in global resistance noticed was mainly due to the demineralization of the solution in the diluate compartment and to a possible change in the integrity of the membranes or fouling occured. However, the global resistance has been improved in comparison with the previous study by Abou-Diab *et al*. (2020a), who reported a 1086.3 ± 156.78 Ω and 198.29 ± 37.79 Ω at the end of the process in EDBM-MCP and EDBM-AEM,

respectively. The lower global resistance of the present study was caused by a higher current intensity due to the important ionic equilibrium since there was no impoverishment in ion in the compartments. The quantity of salt added at the beginning was sufficient and simultaneously reused in the three compartments. In addition, the electrical resistance was used for running three treatments simultaneously, allowing an efficient use of the electrical current. One electron was used for three operations instead of one operation in previous works (Abou-Diab *et al*., 2021; Abou-Diab *et al*., 2020a; Abou-Diab *et al*., 2020b).

Regarding the relative energy consumption, the average consumption at the end of the « 3-in-1 » process was 2.31 ± 0.18 Wh/g. By way of comparison, the relative energy consumption of casein isolates produced at a demineralization rate of 75%, by electrodialysis (ED) prior to electro-acidification with bipolar membrane (EDBM), was higher with a value of 3.08 (Wh/g) as reported by Bazinet *et al*. (2001). In the present study, « 3-in-1 » EDBM process is an interesting technology allowing to produce demineralized bovine hemoglobin hydrolysates with a low energy consumption.

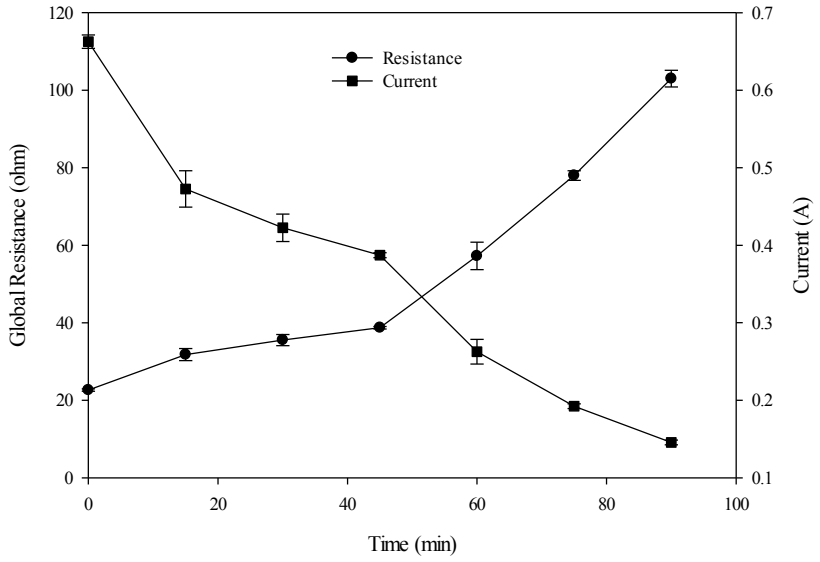

Figure V-5. Evolution of the system global resistance and current as a function of time during electrodialysis process.

V.5.1.3. Membrane characterization

In order to understand the cause of the variation in the global resistance, this section is dedicated to the characterization of the membranes after EDBM in terms of membrane thickness and electrical conductivity, as well as presenting photographs.

V.5.1.3.1. Membrane fouling

To evaluate whether fouling was present on membranes, at the end of EDBM process, photographs of each membrane were taken (**Figure V-6**). **Figure V-6** showed that, the MCP and BM did not present any fouling, while AEM facing the diluate compartment exhibited a thin brownish fouling.

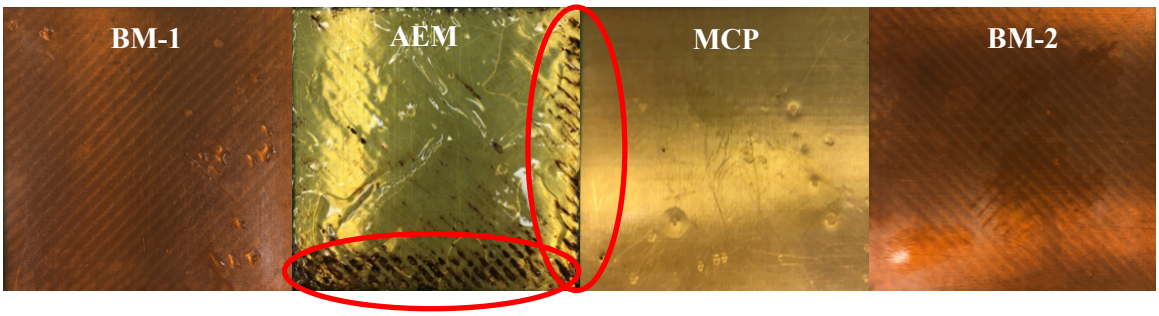

Figure V-6. Membrane photographs of BM (bipolar membrane), AEM (anion exchange membrane) and MCP (monovalent cation perm-selective membrane) after EDBM treatment.

V.1.1.1.1. Membrane thickness and conductivity

Membranes were also characterized by measuring their thickness (mm) and conductivity (mS/cm) before and after each treatment. Regarding membrane thickness, as shown in **Figure V-7**, the BM-1, BM-2 and MCP remained similar before and after EDBM treatment ($p > 0.05$), while a small but significant difference was observed ($p < 0.05$) for AEM before and after EDBM treatment.

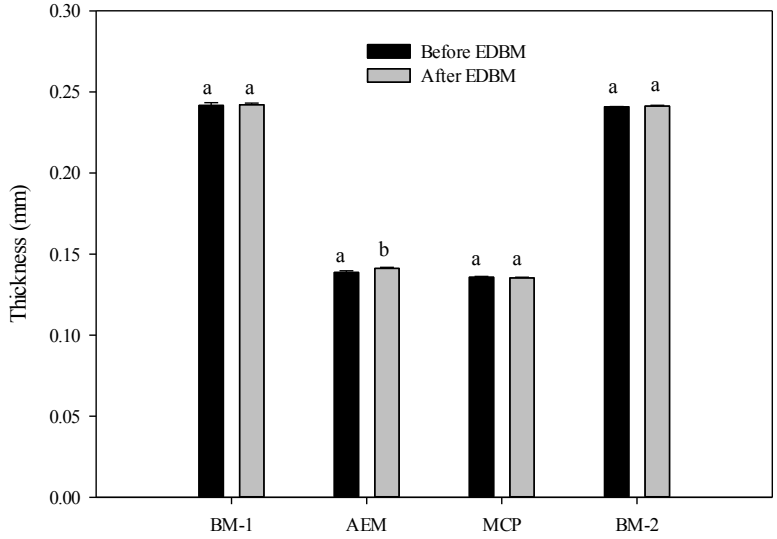

Figure V-7. Membrane thickness measurements for all membranes, before and after treatment for EDBM. Values with different lowercase letter (a-b) for the same membrane are significantly different ($P < 0.05$) before and after each EDBM treatment.

Statistical analysis demonstrated that electrical conductivity of BM-1 and BM-2 did not significantly ($p > 0.05$) change before and after the EDBM process. However, the conductivity decreased by 47% ($p < 0.05$) for AEM and by 41% ($p < 0.05$) for MCP (**Tableau V-1**). The variation in conductivity of AEM and MCP before and after the treatment did not affect the course of treatment in EDBM but explained the increase in global resistance.

Tableau V-1. Conductivity of BM, AEM and MCP before and after EDBM treatment.

Process	**EDBM**			
Conductivity (mS/cm)	**BM-1**	**AEM**	**MCP**	**BM-2**
Before	7.03 ± 0.06^{a}	5.63 ± 0.17^{a}	3.40 ± 0.29^{a}	7.06 ± 0.06^{a}
After	6.90 ± 0.18^{a}	3.00 ± 0.21^{b}	2.00 ± 0.21^{b}	6.90 ± 0.13^{a}

*$^{a-b}$: Values with different lowercase letter before and after the treatment for each type of membranes are significantly different ($p < 0.05$, Tukey).

As it can be seen, photographs taken at the end of the « 3-in-1 » EDBM process, showed a thin brownish layer on the anionic membrane on the diluate compartment side. In addition, the difference in membrane thickness and conductivity before and after the treatment on

AEM showed that there is some fouling. Therefore, the fouling on the anionic membrane (AEM) was due to hem or hem-peptides precipitation during hemoglobin hydrolysate demineralization and then to electrostatic interactions between precipitated hem and/or hem-peptides (negatively charged) and the AEM (positively charged; $N^+(CH_3)_3$), as described by Abou-Diab, *et al*. (2020a). During demineralization, as mentioned above, a decrease in hemoglobin solution pH caused hem and/or hem-peptides precipitation. The pH of the solution in the diluate compartment was close to 6.25, indeed at this pH value, hem is negatively charged because of its two carboxylic acid groups, which allowed electrostatic interactions, after precipitation, between hem and/or hem-peptides associations and the AEM, or between peptides (negatively charged) and AEM, hence the presence of fouling. On the other hand, the decrease in conductivity for MCP (negatively charged) before and after the treatment, may be due to positively charged peptides forming a non-visual fouling on MCP.

V.1.1.2. Ash content and mineral composition

V.1.1.2.1. Evolution of ash content

The ash content evolution, from the 1% initial HB solution to the end of the processes in control and EDBM is shown in **Tableau V-2**. For 1% HB solution, at the beginning of the treatment, as expected, the ash content was the same whatever the conditions ($p > 0.05$). In control, the ash content increased by 4.42 times after the acidification phase and by 1.5 time after the hydrolysis phase, due to the addition of HCl (2 M) solution. In the control, ash content continued to increase at the end of the basification, up to 3 times the initial value, due to the addition of KOH (0.5 M), to reach a final average value of 1820 ± 20 µg/mL. In comparison, after conductivity adjustment for EDBM, the ash content increased by 6.04 times and at the end of electro-acidification, the ash content increased by 1.62 times due to a partial mineralization caused by migration of Cl^- anions. At the end of the hydrolysis phase, ash content continued to increase by 1.49 times, and during the electro-basification phase, due to this partial mineralization caused by migration of K^+ ions. The final ash content between EDBM (180±40 µg/mL) and control hydrolysates (1820±20 µg/mL) was

significantly different ($p < 0.05$). As expected, for the demineralized hydrolysate the ash content was very low. The decrease in the ash content at the end of EDBM treatment was ≈ 92%, due to the migration of anions and cations through the anionic and cationic membranes.

Tableau V-2. Ash content evolution during different phases of hydrolysis in control and EDBM.

		Ash content (µg/mL)
Control	Hemoglobin solution	93.33±11.54
	End of chemical acidification	413.33±61.1
	End of hydrolysis	620±69.28
	End of chemical basification (CH-RH)	1820±20
	Discolored hydrolysate (CH-DH)	2100±34.64
EDBM	Hemoglobin solution	86±11.54
	Conductivity adjustment	520±52.91
	End of electro-acidification	846.67±61.1
	End of hydrolysis	1266.67±61.1
	End of electro-basification	2093.33±50.33
	Demineralized hydrolysate (EDBM-RH)	180±40
	Discolored-Demineralized hydrolysate (EDBM-DH)	169±20

V.1.1.2.2. Mineral composition

Determination of the mineral composition in the hemoglobin solution at the end of the EDBM process was important to confirm the efficiency of this new ED configuration to reduce the salt concentration in comparison with conventional hydrolysis. The minerals identified are chloride, potassium, sodium and sulfur. The concentrations of these minerals (mg/L) are shown in **Figure V-8.**

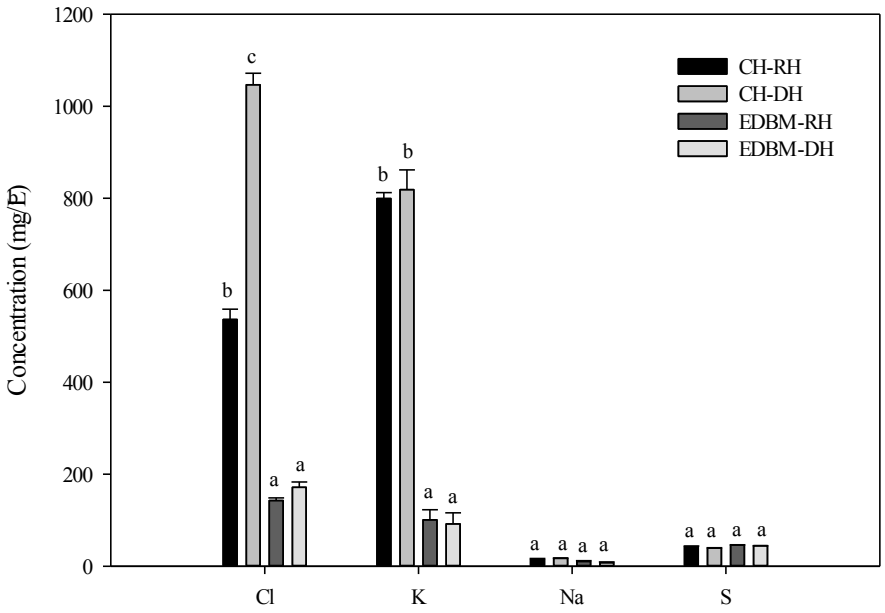

Figure V-8. Mineral concentration of hemoglobin solution at the end of the hydrolysis in raw hydrolysates produced by conventional hydrolysis (CH-RH), discolored hydrolysates produced by conventional hydrolysis (CH-DH), raw hydrolysates produced by EDBM (EDBM-RH) and discolored hydrolysates produced by EDBM (EDBM-DH). Values with different lowercase letter (a-b) within the same mineral element are significantly different between conditions.

Chloride concentration of hemoglobin solution at the end of the hydrolysis was significantly different ($p < 0.05$) between EDBM-RH and CH-RH. Lower chloride concentration observed in EDBM-RH in comparison with CH-RH was due to the migration of Cl^- anions through the anionic membrane from the diluate to the acidified compartment.

Potassium concentration of hemoglobin solution was significantly different ($p < 0.05$) between EDBM-RH and CH-RH. Lower potassium concentration observed in EDBM in comparison with control was due to the migration of K^+ ions through the cationic membrane from the diluate to the basified compartment. The higher K^+ concentration observed in control was due to the addition of KOH to stop the reaction.

Sodium and sulfur concentrations did not significantly change ($p > 0.05$) between EDBM-RH and CH-RH. The low concentration of sodium and sulfur observed is probably due to its

presence in the hemoglobin powder since none of these elements were added throughout the treatment.

V.1.1.3. Enzymatic kinetics and mechanism of action of the new process

V.1.1.3.1. Reaction mechanism of bovine hemoglobin hydrolysis by pepsin

The chromatographic profiles in EDBM were compared to the control and showed the same enzymatic kinetics throughout the hydrolysis at T0, T2.5, T30, T60, T120, and T180 min (**Figure V-9**). At T0, before adding the pepsin, three peaks are shown: hemoglobin α-chain and β-chain and hem. After a few minutes of hydrolysis, hemoglobin subunits were rapidly hydrolyzed into low molecular weight peptides. These were called intermediate peptides. They were themselves digested into shorter and more hydrophilic peptides to give final peptides that accumulated throughout the rest of the three hours of hydrolysis. The enzymatic hydrolysis of hemoglobin in EDBM displayed « zipper » mechanism identical to the one obtained in conventional hydrolysis (control). The « zipper » mechanism was described for the first time by Linderstrom-Lang, (1953). This mechanism occurs when the hemoglobin is completely denatured, allowing more numerous and accessible cutting sites for pepsin and therefore, yields a larger peptide population. The same mechanism was observed by Abou-Diab *et al*. (2020a) for enzymatic hydrolysis performed in EDBM with two different configurations (EDBM-MCP and EDBM-AEM).

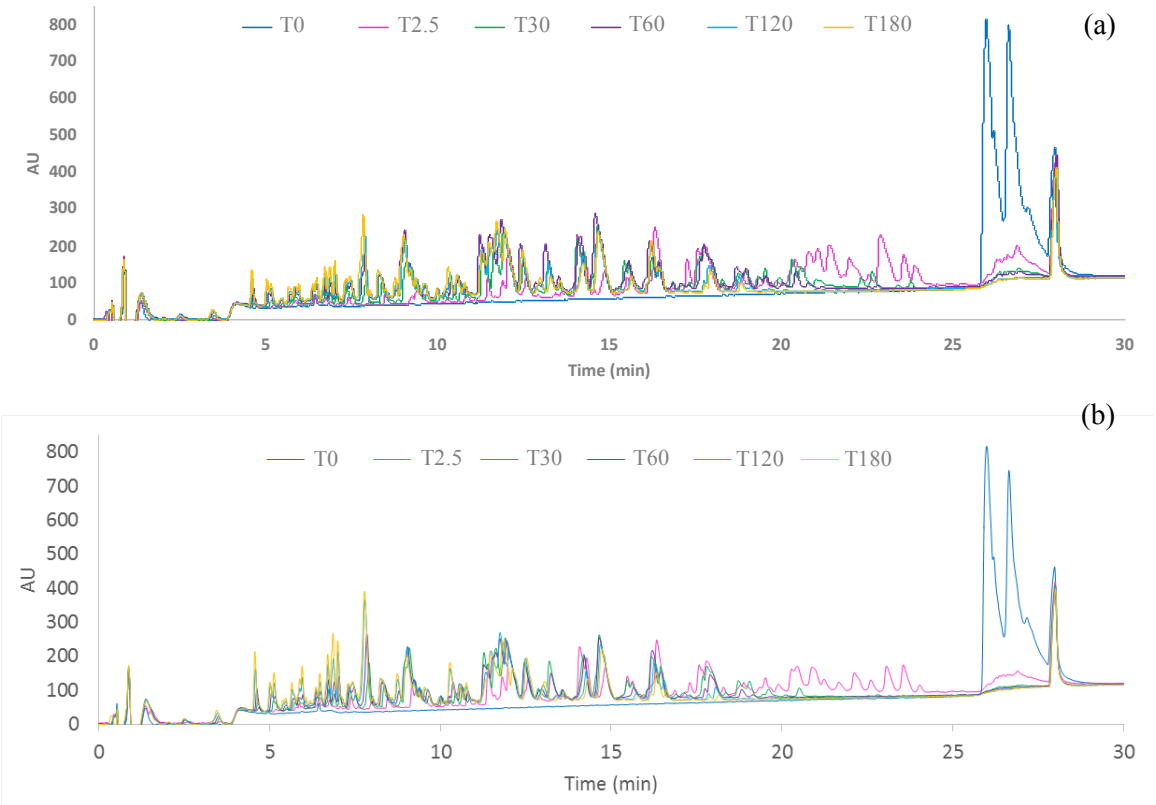

Figure V-9. Overlay of chromatographic profiles of bovine hemoglobin hydrolysis in control (a) and EDBM (b) at 214 nm by UPLC, analyzed on C18 column at different hydrolysis times for 3 hours (pH 3, 32°C, E/S = 1/11, CBH = 1%, w/v).

V.1.1.3.2. Determination of the degree of hydrolysis

Over the 180 minutes of hydrolysis, the DH has an identical tendency to increase in control and EDBM (**Figure V-10**). For control and EDBM, the DH evolution was identical, after 2.5, 30, 120 and 180 min of hydrolysis ($p > 0.05$). While statistical difference was observed ($p < 0.05$) after 60 min of hydrolysis. It is important to mention, that after 180 min of hydrolysis, hydrolysate in control was stopped using KOH solution while it was stopped in EDBM using the electrogenerated OH- by the BM. At T180 min, a similar DH was observed for EDBM (8.13 ± 0.06%) vs control (8.05 ± 0.06%), meaning that the electro-basification step was as effective as conventional basification.

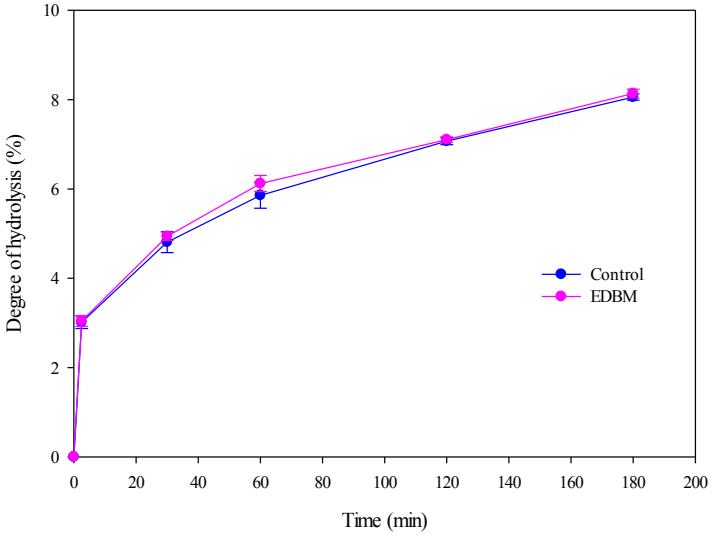

Figure V-10. Degree of hydrolysis as a function of hemoglobin hydrolysis duration in control and EDBM treated with pepsin during 180 min. Hydrolysis conditions: enzyme (U)/protein (mg) ratio of 1/11 (mole/mole), pH 3 and at 32°C.

V.1.1.4. Identification of bioactive peptides

The « zipper » mechanism allowed the production of a large peptide population composed of 455 and 473 peptides characterized by UPLC-QTOF for EDBM-RH and CH-RH, respectively. A lower number of peptides was observed for EDBM-RH process due to the precipitation during the demineralization. Among this large peptide population, it was important to identify, the bioactive peptides produced in EDBM and control, based on the literature (**Tableau V-3**). The bioactive peptides identified after the three-hour hydrolysis and the demineralization using EDBM and without the use of chemicals revealed several peptides with known bioactivities: antibacterial (Adje *et al*., 2011a; Nedjar *et al*., 2008; Dubois *et al*., 2005), opioid (Lignot *et al*., 1999; Zhao and Piot 1997a; 1997b), hematopoietic (Vercaigne-Marko *et al.,* 2000), potentiator of bradykinin (Lignot *et al*., 1999; Piot *et al*., 1992), hypolipidemic (Kagawa *et al*., 1996), bacterial growth stimulator (Zhao *et al*., 1996), antihypertensive (Adje *et al*., 2011b), dipeptidyl peptidase IV inhibitors (Caron *et al*., 2016) and antioxidant (Przybylski *et al*., 2016). However, a lower number of bioactive peptides were identified in EDBM-RH than in CH-RH. In EDBM-RH, 12 peptides in common with

CH-RH were found having antibacterial activity, 5 opioid, 3 hematopoietic, 3 potentiator of bradykinin, 1 hypolipidemic, 1 bacterial growth stimulator, 1 antihypertensive, 1 dipeptidyl peptidase IV inhibitor and 1 antioxidant. In comparison, some differences were observed. The α34-66, α33-45, α133-141 and α34-65 peptides appeared in CH-RH, but did not appear in EDBM-RH. This difference can be explained by the loss of peptides due to the precipitation. However, the β1-30, α137-140 and β32-39 peptides appeared in EDBM-RH, but not in CH-RH. This can be explained by the fact that the EDBM system and the control are two different conditions based on different methods and parameters and therefore could lead to roughly different peptide population. Overall, the EDBM process was efficient to produce many bioactive peptides and the demineralization was effective to remove salt with minimal loss of bioactive peptides due to the pH change in the diluate compartment and thus the precipitation.

Tableau V-3. Bioactive peptide sequences identified by UPLC-QTOF resulting from bovine hemoglobin hydrolysis in control and EDBM. (Peptides in bold are not present in all the conditions).

Biological activity	Position	Sequence	Molecular weight (Da)	m/z	CH-RH	CH-DH	EDBM-RH	EDBM-DH
Antibacterial	α1-29	VLSAADKGNVKAAWGKVGGHAAEYGAEAL	2839.4644	568.9018	+	+	+	+
	α1-28	VLSAADKGNVKAAWGKVGGHAAEYGAEA	2726.3813	546.2840	+	+	+	+
	α1-27	VLSAADKGNVKAAWGKVGGHAAEYGAE	2655.3476	532.0776	+	+	+	+
	α34-66	**LSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAAL**	**3482.8183**	**581.4765**	**+**	**+**	**-**	**+**
	α33-46	FLSFPTTKTYFPHF	1731.8675	578.2959	+	+	+	+
	α33-45	**FLSFPTTKTYFPH**	**1584.7971**	**793.4057**	**+**	**-**	**-**	**-**
	α34-46	LSFPTTKTYFPHF	1584.7973	529.2739	+	+	+	+
	α37-46	PTTKTYFPHF	1237.6136	413.5448	+	+	+	+
	α133-141	**STVLTSKYR**	**1053.5835**	**527.7971**	**+**	**+**	**-**	**+**
	α137-141	TSKYR	653.3506	327.6820	+	+	+	+
	β1-30	**MLTAEEKAAVTAFWGKVKVDEVGGEALGRL**	**3174.6777**	**635.9444**	**-**	**-**	**+**	**-**
	β1-13	MLTAEEKAAVTAF	1380.6955	691.3545	+	+	+	+
	β126-145	QADFQKVVAGVANALAHRYH	2194.142	549.5422	+	+	+	+
	β140-145	LAHRYH	795.4145	266.1456	+	+	+	+
	α34-65	**LSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAA**	**3369.7329**	**562.6297**	**+**	**+**	**-**	**+**
	α99-105	KLLSHSL	796.4818	399.2475	+	+	+	+
	α99-106	KLLSHSLL	909.5656	455.7901	+	+	+	+
Opioid	α137-141	TSKYR	653.3506	327.6820	+	+	+	+
	α137-140	**TSKY**	**497.2494**	**249.6318**	**-**	**-**	**+**	**-**
	β31-40	LVVYPWTQRF	1307.7023	654.8580	+	+	+	+
	β32-40	VVYPWTQRF	1194.6181	598.3160	+	+	+	+
	β32-39	**VVYPWTQR**	**1047.5502**	**524.7830**	**-**	**-**	**+**	**+**
	β31-37	LVVYPWT	876.4727	439.2449	+	+	+	+
	β32-37	VVYPWT	763.3909	382.7030	+	+	+	+
Hematopoietic	β2-12	LTAEEKAAVTA	1102.5864	552.3007	+	+	+	+
	β2-11	LTAEEKAAVT	1031.5501	516.7825	+	+	+	+
	β2-10	LTAEEKAAV	930.5028	466.2580	+	+	+	+
Potentiator of bradykinin	α110-125	ASHLPSDFTPAVHASL	1648.8213	550.6155	+	+	+	+
	α129-134	LANVST	603.3225	+1 only	+	+	+	+
	β129-134	FQKVVA	690.4082	346.2114	+	+	+	+
Hypolipidemic	β32-35	VVYP	476.2657	+1 only	+	+	+	+
Bacterial growth stimulator	**β48-52**	**STADA**	**463.1909**	**+1 only**	**+**	**-**	**+**	**-**
Antihypertensive	α99-105	KLLSHSL	796.4818	399.2475	+	+	+	+
Dipeptidyl peptidase IV inhibitor	α130-134	ANVST	490.2389	+1 only	+	+	+	+
Antioxidant	α137-141	TSKYR	653.3506	327.6820	+	+	+	+

This part showed that the 3-in-1 EDBM is a complete process. It can perform three operations in a single operating unit, including hemoglobin denaturation, pH adjustment during hydrolysis, pepsin inactivation and hemoglobin hydrolysate demineralization. Also, this process allows to generate acid and base necessary for electrodialysis cleaning. This innovative « 3-in-1 » process was also successful for producing bioactive peptides from bovine hemoglobin with a lower mineral salt concentration. However, a limitation was noticed due to membrane fouling and loss of some peptides after demineralization. Therefore, in order to optimize the process, an additional step was proposed.

V.1.2. Optimized multiple-step using EDBM process or « 4-in-1 »

According to the previous results, an additional step was tested with this integrated EDBM process in order to provide a complete optimized process and to avoid fouling. The new additional step was to discolor the hemoglobin hydrolysate using the characteristics of EDBM and without adding chemical agents. The discoloration consisted of separating the hem from the hydrolysate. It was achieved outside the system following the process steps scheme shown in **Figure V-2**, after electro-acidification, enzymatic hydrolysis and electro-basification, product 4 generated from step 2 was collected in a beaker. The discoloration was then realized on the hemoglobin hydrolysate with inactive pepsin (product 4) before the demineralization phase using the electro-generated HCl solution (0.045 M) from step 2 (**Figure V-2**). The complete EDBM system of this study permitted to electro-generate a HCl solution in the acidification compartment due to the specific characteristics of the bipolar membranes allowing the generation of the necessary ions without the use of chemicals. The discoloration was carried-out on product 4 by adjusting the pH at 4.5 after the steps described above. The electrogenerated acid was used instead of chemical HCl. After pH adjustment, the hem was separated from the peptides by centrifugation. The supernatant recovered after centrifugation consisted of the discolored hydrolysate after hem precipitation. This discolored solution was then demineralized in the diluate compartment to give a final discolored-demineralized hydrolysate (EDBM-DH). The « 4-in-1 » process provided the production of peptides with the same high rate of demineralization (85%) and with a

significantly similar ($p > 0.05$) relative energy consumption 2.29±0.16 Wh/g. The « 4-in-1 » EDBM method was compared to a conventional discoloration (CH-DH).

V.1.2.1. Membrane characterization

Photographs of the membranes were taken at the end of the « 4-in-1 » EDBM process (**Figure V-11**) and did not show any fouling.

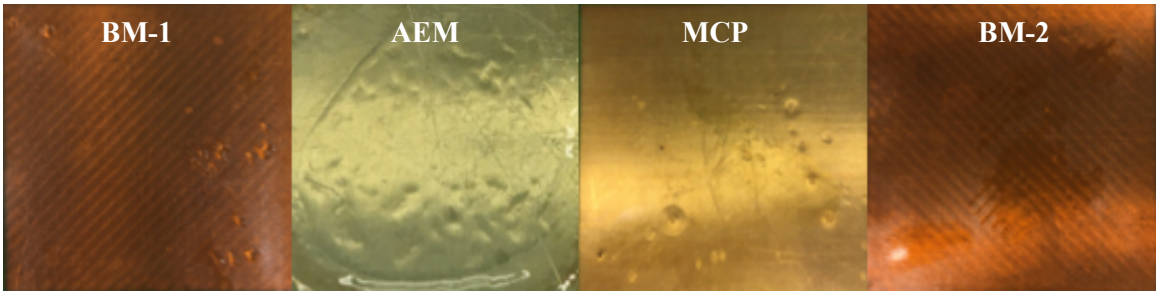

Figure V-11. Membrane photographs of BM (bipolar membrane), AEM (anion exchange membrane) and MCP (monovalent cation perm-selective membrane) after the « 4-in-1 » EDBM treatment.

In addition, membrane thickness was the same before and after the « 4-in-1 » treatment ($p > 0.05$). Concerning the electrical conductivity **(Tableau V-4)**, it decreased for AEM before and after the « 4-in-1 » EDBM treatment (25%) but was less compared to the « 3-in-1 » EDBM treatment (47%). This may be caused by a non-visual fouling produced by electrostatic interactions between peptides (negatively charged) and the AEM (positively charged).

Tableau V-4. Conductivity of BM, AEM and MCP before and after the « 4-in-1 » EDBM treatment.

Process	**EDBM**			
Conductivity (mS/cm)	**BM-1**	**AEM**	**MCP**	**BM-2**
Before	7.05±0.06^{a}	5.6±0.18^{a}	3.57±0.67^{a}	7.1±0.07^{a}
After	6.9±0.18^{a}	4.2±0.31^{b}	2.19±0.43^{b}	6.9±0.14^{a}

*$^{a-b}$: Values with different lowercase letter before and after the treatment for each type of membranes are significantly different ($p < 0.05$, Tukey).

V.1.2.2. Ash content and mineral composition

For EDBM-DH, the ash content was roughly the same and very low similarly to EDBM-RH, with an average value of 169 ± 20 µg/mL. In comparison, after discoloration, the ash content value reached 2100 ± 34.64 µg/mL in CH-DH. It increased, in CH-DH, by 1.07 times compared to CH-RH, due to the addition of chemical HCl (2 M) (**Tableau V-2**). Furthermore, as can be seen in **Figure V-8**, there is significant difference ($p < 0.05$) for chloride concentration between CH-RH and CH-DH. Higher chloride concentration observed after discoloration was due to the addition of HCl. In comparison, lower chloride and potassium concentrations were observed for EDBM-DH as a result of Cl^- anions and K^+ ions migrating through the anionic and cationic membranes, respectively.

V.1.2.3. Chromatographic profils and peptide population

The chromatographic profiles between CH-RH and CH-DH (**Figure V-12-a**), EDBM-RH and EDBM-DH (**Figure V-12-b**) were compared to ensure that the enzymatic kinetic remained the same. As it can be observed, the chromatographic profiles remained the same before and after the discoloration in conventional method and EDBM with elimination of the hem peak after discoloration. Chromatographic profiles of hydrolysis in EDBM and control consequently showed the same profiles at the end of the basification and the discoloration phase, meaning that the peptide population was similar, and that the acid electro-generated using for discoloration was as effective as the chemicals used in conventional discoloration. Moreover, based on the peptide population identified by UPLC-QTOF, 468 peptides were recovered after discoloration-demineralization in the « 4-in-1 » process instead of 455 peptides after demineralization in the « 3-in-1 » process since no precipitation was noticed in the « 4-in-1 ». Furthermore, **Tableau V-3** showed the same bioactive peptides after discoloration in CH-DH and EDBM-DH except for β32-39, it appeared in EDBM-DH but did not appear in CH-DH, this can be explained by the different parameters throughout the different conditions. The β48-52, which is a bacterial growth stimulator, appeared in CH-RH and EDBM-RH, but did not appear in CH-DH and EDBM-DH. This can be explained by the fact that the β48-52 has precipitated with hem during the discoloration. The « 4-in-1 » process

makes it possible to produce roughly the same peptide population containing a low concentration of mineral salt, without membrane fouling and with a low relative energy consumption.

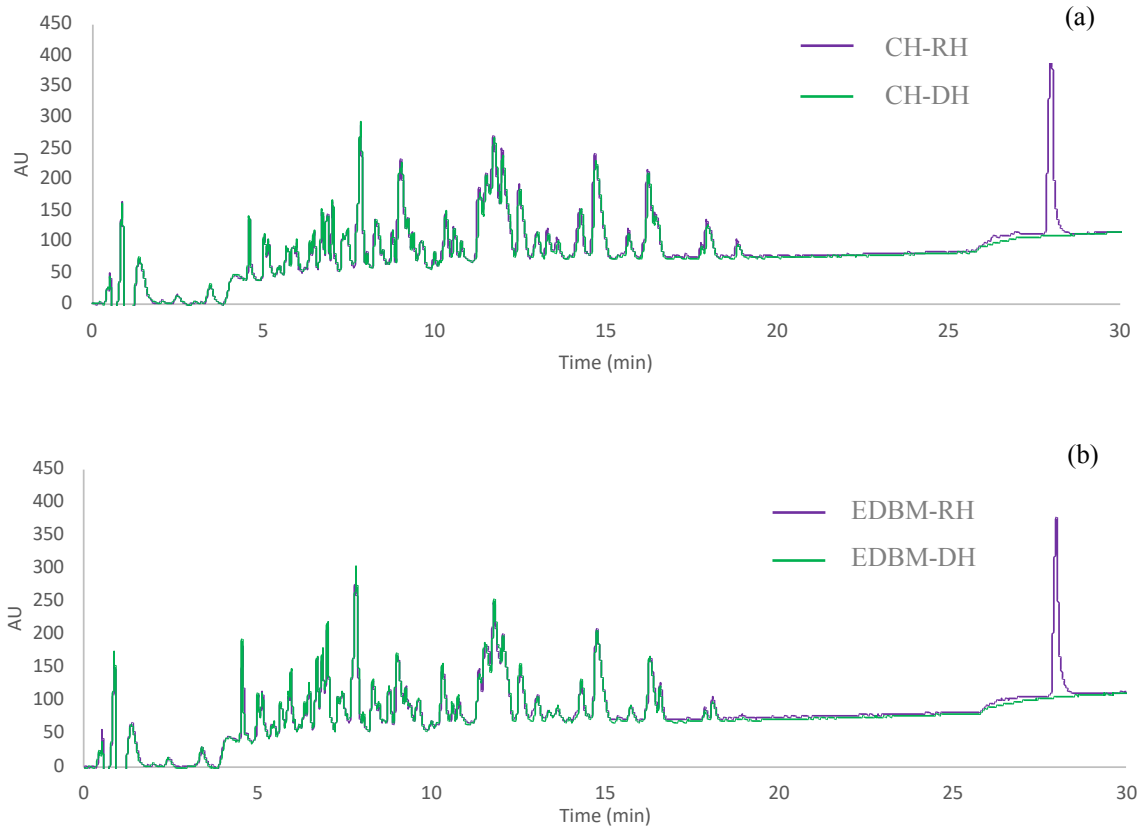

Figure V-12. Overlay of chromatographic profiles of bovine hemoglobin raw hydrolysate and discolored hydrolysate after 3 hours of hydrolysis in control (a) and EDBM (b) at 214 nm by UPLC-QTOF, analyzed by C18 column.

V.2. Conclusion

The global « 4-in-1 » electrodialysis process using bipolar membranes has revealed to be successful. This integrated process has made it possible to transform a by-product from slaughterhouses into a discolored and demineralized product containing bioactive peptides. It can perform 4 operations without adding chemicals, including hemoglobin denaturation, pH adjustment during hydrolysis, pepsin inactivation, hemoglobin hydrolysate discoloration and demineralization. This innovative « 4-in1 » process was also effective to maintain the

same « zipper » mechanism observed during conventional hydrolysis, to obtain a large peptide population without the loss of peptides and to generate bioactive peptides with low mineral salts and without membrane fouling. In addition, this process allowed the generation of acid and base necessary for electrodialysis cleaning or for the discoloration step. This was related to the physicochemical characteristics of bipolar membranes leading to the in situ electrogeneration of H+ and OH- ions from water splitting and hence, avoiding the use of chemical products. This method of electro-acidification and electro-basification is an alternative step to avoid using chemical acids and bases.

This new eco-efficient and sustainable EDBM system has a great importance at the industrial level since it perfectly fits within the concept of circular economy: hemoglobin from slaughterhouses blood, after hydrolysis, is then discolored and demineralized, favoring the bioproduction of natural active peptides with a low salt content that can be used as alternatives to chemical additives. Moreover, the final discolored-demineralized product obtained has a very low risk of deterioration due to iron oxidation which increases its potential commercial value. Meanwhile conventional methods produce bioactive peptides with high salt content, thus decreasing the purity of the peptides, causing undesirable effects on consumers health and impacting their biological activities. The specificities of the bipolar membranes make the EDBM process more eco-friendly. The electricity is the only energy necessary to operate EDBM system, which is considered as a clean energy source. Hence, no effluents are produced since all streams are utilized to generate the demineralized peptides. Currently, antibacterial, antifungal and antioxidant tests are in progress to assess the bioactive capacity of the hydrolysates generated. After that, a life cycle assessment (LCA) will be performed to evaluate the environmental profile associated with production of bioactive peptides and its contribution to the impact categories: climate change, ecosystem quality, human health and resources consumption, following our different scenarios: (1) conventional method (scenario 1) and (2) EDBM process (scenario 2).

V.3. Acknowledgements

The financial support of the Natural Sciences and Engineering Research Council of Canada (NSERC) is acknowledged. This study was supported by different NSERC grants: Grant IRCPJ 499946-15 to I. Fliss, Grant SPGP 5212082 to L. Bazinet and Grant SD 210829409 to L. Bazinet. This work was realized within the French-Canadian International Associated Laboratory on «Bioproduction of natural antimicrobials» (LIAAN). The authors thank V. Perreault and D. Gagnon, research professionals at Laval University for their technical assistance and for their help at the lab.

V. *Deuxième article*

V.1. Résumé

De nombreuses études ont montré que l'hémoglobine bovine, une protéine issue des déchets d'abattoirs, possède d'important potentiel biologique après hydrolyse enzymatique conventionnelle. Cependant, ces peptides actifs contiennent une forte proportion de sels minéraux. Ainsi, un procédé optimisé à multiple étapes d'EDMB a été réalisé pour produire des peptides décolorés et déminéralisés sans ajout d'agents chimiques. Le but de cette étude était de tester les activités antibactériennes, antifongiques et antioxydantes des hydrolysats d'hémoglobine décolorés et déminéralisés récupérés par l'EDMB et de les comparer avec des hydrolysats bruts et décolorés issus de l'hydrolyse conventionnelle. Les résultats obtenus démontrent que les hydrolysats décolorés-déminéralisés récupérés de l'EDMB avaient une activité antimicrobienne significative contre de nombreuses souches bactériennes et fongiques. Les hydrolysats ont également montré un puissant effet antioxydant. Par conséquent, ils peuvent être considérés comme des conservateurs naturels potentiels, à faible teneur en sel, prometteurs pour le secteur alimentaire.

Mots clés : Électrodialyse avec membranes bipolaires, Hémoglobine bovine, Hydrolysats déminéralisés, Hydrolysats décolorés-déminéralisés, Activité antibactérienne, Activité antifongique, Activité antioxydante.

V.2. Abstract

Numerous studies have shown that bovine hemoglobin, a protein from slaughterhouse waste, has important biological potential after conventional enzymatic hydrolysis. However, the active peptides could not be considered pure since they contained mineral salts. Therefore, an optimized « multi-step » process of electrodialysis with bipolar membranes (EDBM) was carried-out to produce discolored and demineralized peptides without the addition of chemical agents. The aim of this study was to test the antibacterial, antifungal and antioxidant activities of discolored and demineralized bovine hemoglobin hydrolysates recovered by EDBM and to compare them with raw and discolored hydrolysates derived from conventional hydrolysis. The results obtained demonstrate that discolored-demineralized hydrolysates recovered from EDBM had significant antimicrobial activity against many bacterial (Gram + and Gram -) and fungal (molds and yeast) strains. The hydrolysates also showed a potent antioxidant effect in four different antioxidant tests. Consequently, they can be considered as promising natural preservatives for the food sector with low salt content. To the best of our knowledge, no previous studies have identified the biological properties of discolored and demineralized bovine hemoglobin hydrolysates.

Keywords: Electrodialysis with bipolar membrane, bovine hemoglobin, demineralized hydrolysates, discolored-demineralized hydrolysates, antibacterial activity, antifungal activity, antioxidant activity.

V.3. Introduction

According to the World Health Organization (WHO), food contamination is a serious problem affecting many consumers across the world. Generally, food is considered contaminated when its flavor, texture, and appearance change, thus making it unhealthy for consumption. The major causes of deleterious quality changes in food may be due to microbial, chemical and physical contamination or oxidation (Holban, 2018).

Microbial contamination happens when a product has been contaminated by microorganisms, including bacteria, fungi, yeasts, protozoa, and even viruses (Melsungen, 2011). Microbial contamination of food has become the biggest issue that the industries are anxious about. The pathogenic microbes can cause several diseases, such as botulism, food poisoning, and certain other enteric infections by producing harmful toxic metabolites (Wirtanen and Salo, 2007). The most common food pathogenic bacteria are *Salmonella* spp.*, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Clostridium botulinum, Shigella* spp.*, Pseudomonas* spp.*,* and *Campylobacter jejuni* (Holban, 2018). Fungi are also major food contaminants, the most common fungal species, including molds and yeasts, found as food contaminant are *Aspergillus niger, Eurotium rubrum, Fusarium* sp.*, Paecilomyces* spp.*, Penicillium citrinum, Penicillium crustosum, Rhodotorula mucilaginosa, Candida intermedia, Saccharomyces boulardii* (Pitt and Hocking, 2009). In addition, oxidation of biomolecules has many undesirable impacts on organoleptic quality in food. Furthermore, oxidative stress can also modify proteins, DNA and small cellular molecules, and it is thought to have a significant role in the occurrence of diseases, such as cancer, arteriosclerosis, cardiovascular diseases, diabetes mellitus, neurological disorders and Alzheimer's disease (Halliwel, 2002; Stadtman, 2006).

To control microorganisms and oxidation reactions, preservatives are regularly added in food. The most commonly used antimicrobial and antioxidant compounds for the preservation of foods are: organic acids (acetic acid, benzoic acid, sorbic acid, etc.), nitrites and nitrates, sulfur dioxide and sulfites, ethylene oxide and propylene oxide, sugars and salts, alcohol, antibiotics, etc. (Holban, 2018). However, some of them are suspected to induce pathological and toxic effects. Moreover, microorganisms are becoming resistant to antibiotics and to preservatives (Brul and Coote, 1999). For this reason, and to respond to consumers growing demand for healthy and quality foods, the interest in producing natural preservatives has risen in the past decades. Bio-preservatives are produced from natural sources or formed in food and can prevent or delay spoilage related with chemical or biological deterioration that extend shelf life.

In this context, and in the framework of circular economy, bovine blood from slaughterhouse waste was demonstrated to be interesting by the production of natural antimicrobial peptides (Bah *et al*., 2013; Lafarga and Hayes, 2014). Moreover, the α137-141 peptide derived from bovine cruor waste has been demonstrated to be a promising natural preservative for the substitution of the synthetic additives widely used to protect meat during its storage and distribution through reducing the lipid oxidation by 60% and inhibiting growth of total viable colonies, yeasts and molds (Przybylski *et al*., 2016). Also, the biological properties (antibacterial, antifungal and antioxidant) of bovine hemoglobin hydrolysates from bovine blood slaughterhouse waste, produced using an innovative and green process: Electrodialysis with bipolar membrane (EDBM) have been proven (Abou-Diab *et al*., 2020b). However, further experiments were needed, to the latter, to: 1) settle the issue of membrane fouling which appeared during the production of hydrolysates as explained by Abou-Diab *et al*. (2020a), 2) remove, by discoloration, the hem contained in the hydrolysates responsible of the red color of blood, making difficult the valorization of the product due to iron oxidation and 3) reduce the final mineral salt concentration to be able to meet the consumer demands.

Therefore, a new optimized study performing a « multiple-step » EDBM process was carried out recently by Abou-Diab *et al*. (2021b) to produce demineralized and discolored-demineralized bovine hemoglobin hydrolysates without the addition of mineral salts. The optimized configuration of EDBM consisted of three-compartment combining homopolar membranes with bipolar membranes, whose membranes generate H^+ and OH^- ions from water dissociation under an electrical field and performed simultaneously: enzymatic hydrolysis, inactivation of reaction, discoloration and demineralization. However, this study focused on the technological part of producing bioactive peptides from bovine hemoglobin without membrane fouling and with a low salt concentration and did not test the biological activities of these hydrolysates.

Accordingly, the objective of the present study was to identify the antibacterial, antifungal and antioxidant activities of demineralized and discolored-demineralized bovine hemoglobin hydrolysates produced by EDBM. The results will be compared to raw and discolored hydrolysates produced using a conventional hydrolysis (CH) based on the use of many

chemical product as reported by Abou-Diab *et al*. (2021b). For this purpose, the antibacterial and antifungal potentials were investigated using the agar diffusion method and minimum inhibitory concentration against multiple pathogenic and non-pathogenic strains commonly known as food contaminants. Also, the antioxidant potential was investigated using four tests based on different mechanisms including β-carotene bleaching inhibition activity, DPPH radical scavenging activity assay, ABTS radical scavenging activity assay and evaluation of total antioxidant capacity to provide a clear idea about their real antioxidant potential. To the best of our knowledge, there is little information concerning the biological properties of a demineralized bovine hemoglobin hydrolysate.

V.4. Materials and Methods

V.4.1. Materials

Hemoglobin. The purified hemoglobin (Hb) powder from bovine blood, dark brown, was purchased from Sigma-Aldrich (H2625, Oakville, ON, Canada). Hemoglobin was stored at 4 °C before use.

Pepsin. The pepsin lyophilized powder from porcine gastric mucosa was purchased from Sigma-Aldrich (P6887, Oakville, ON, Canada). It was used to produce the hydrolysates. Pepsin activity was determined according to a protocol established by the supplier Sigma-Aldrich. Pepsin was stored at -20 °C.

Reagents. KCl was obtained from EMD Chemicals Inc., USA, Na_2SO_4 was supplied by ACP Inc. (Montréal, QC, Canada), KOH was purchased from Fisher Scientific (New Jersey, NJ, USA) and HCl was purchased from Anachemia, VWR Company International (ON, Canada). Chemicals required for the assays including 1,1-diphenyl-2-picrylhydrazyl (DPPH), Butylated hydroxytoluene (BHT), 2,20-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), β-carotene, and linoleic acid were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Neokyotorphin (TSKYR, α137-141) was purchased from GeneCust (Boynes, France).

Cultures. *Listeria monocytogenes* (ATCC 19112), *Staphylococcus aureus* (ATCC 13709), *Micrococcus luteus* (ATCC 9341), *Escherichia coli* (ATCC 8733) and *Salmonella newport* (ATCC 6962) were from the American Type Culture Collection (ATCC, Rockville, MD, USA). *Kocuria rhizophila* (CIP 53.45) was from Collection de l'Institut Pasteur (CIP, Paris, France). *Aspergillus niger* (3071-13) and *Paecilomyces* spp. (5332-9a) were isolated from dairy products (strain collection of Denis Roy, Laval University, Québec, QC, Canada), *Mucor racemosus* (LMA-722) was obtained from Laboratoire de mycologie alimentaire (Laval university, Québec, QC, Canada), *Penicillium crustosum* (27,159) and *Rhodotorula mucilaginosa* (27,173) were isolated from dairy products (strain collection of General Mills Yoplait, France).

V.4.2. Production of hemoglobin hydrolysates using EDBM

V.4.2.1. Electrodialysis cell

As described by Abou-Diab *et al*. (2021b), the electrodialysis (ED) experiments were performed using a MP type cell with an effective surface area of 100 cm^2. In summary, the ED configuration consisted of one anion-exchange membrane (AEM), one monovalent cation perm-selective membranes (MCP) and two bipolar membranes (BM) purchased from Astom, Ameridia (Tokyo, Japan). This arrangement defined 4 closed loops, each loop was connected to a separate external tank, allowing a continuous recirculation (**Figure V-13**).

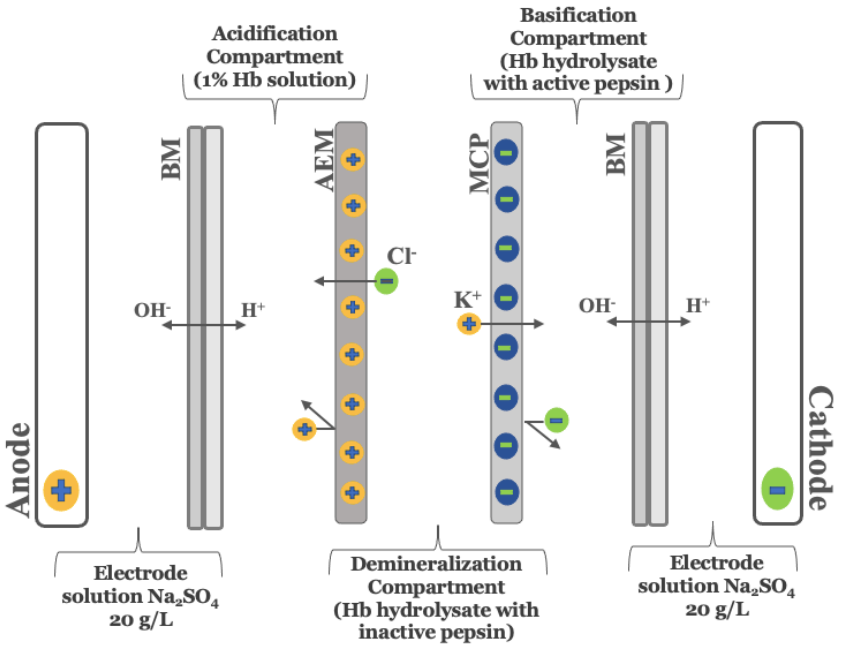

Figure V-13. Schematic representation of three-compartment bipolar membrane electrodialysis cell configuration for production of demineralized and discolored-demineralized hemoglobin hydrolysates running simultaneously used by Abou-Diab *et al*. (2021b). BM, bipolar membrane; MCP, monovalent cation perm-selective membrane and AEM, anion exchange membrane.

V.4.2.2. Preparation of bovine hemoglobin demineralized hydrolysates (EDBM-RH)

As described by Abou-Diab *et al*. (2021b), the production of a demineralized hemoglobin hydrolysates, requires many steps including denaturation, enzymatic hydrolysis, pepsin inactivation, and demineralization. However, before the steps were performed simultaneously, preliminary steps were carried out in EDBM without the addition of mineral salts to provide the solutions for all compartments.

To perform the hydrolysis, 15 g of purified bovine hemoglobin were solubilized in 100 mL of ultrapure water, followed by centrifugation at 6000 x g for 30 min (Eppendorf AG, 22331 Hamburg, Centrifuge 5804 R, Brinkmann Instruments, Westbury, NY, USA). Then, a 1% (w/v) hemoglobin solution was prepared from the recovered supernatant after determining the real concentration of Hb using Drabkin's method. After that, the 1% Hb solution was transferred into the EDBM acidification compartment for denaturation by lowering the pH to 3 using the protons (H^+) electro-generated at the cationic interface of the bipolar membrane

after water dissociation under an electrical field. The enzymatic hydrolysis reaction was started by the addition of the pepsin at an enzyme/protein ration of 1/11 (mole/mole) and performed at 32 °C and pH 3 for 3 h. Simultaneously, the reaction was stopped after 3 h in the basification compartment, by increasing the pH to 9 using the hydroxide ions (OH^-) produced at the anionic interface of the bipolar membrane. At the same time, the alkaline hydrolysate was demineralized in the diluate compartment due to the migration of anions through the anionic membrane towards the acidification compartment and the migration of the cations through the cationic membrane towards the basification compartment.

V.4.2.3. Preparation of bovine hemoglobin discolored-demineralized hydrolysate (EDBM-DH)

To perform discoloration, pH of hemoglobin hydrolysates with inactive pepsin (pH=9) recovered from the basification compartment was lowered at 4.5 using the electrogenerated HCl solution recovered from the acidification compartment as described by Abou-Diab *et al*. (2021b). This step was done outside the ED system and followed by centrifugation at 6000 x g for 30 min to separate the hem (non-protein part), responsible of the red color of the blood, from the globin (protein part) of the hemoglobin hydrolysates. Subsequently, the discolored hydrolysate was transferred into the diluate compartment for demineralization. In order to assess the repeatability, the experiments were performed in triplicates.

V.4.3. Production of hemoglobin hydrolysate using the conventional method

A hemoglobin hydrolysate was also prepared using the conventional method. Acidification of hemoglobin was carried out by addition of chemical HCl (2 M). After 3 h of hemoglobin hydrolysis with pepsin, chemical basification of hydrolysates was carried out by addition of KOH (0.5 M) and therefore a raw hydrolysate was produced (CH-RH). Afterwards, chemical discoloration was performed on the alkaline hydrolysate~~s~~ by addition of HCl (2M) followed by centrifugation and, therefore, a discolored hydrolysate was recovered (CH-DH). However, the mineral content of the final hydrolysate~~s~~ was high due to the use of ~~a~~ chemical acids and

bases, and without a subsequent demineralization step. Again, these experiments were performed in triplicates.

The biological properties were identified on raw and discolored hydrolysates recovered from EDBM and conventional method as shown in **Figure V-14**, to study the impact of demineralization and discoloration on biological activities.

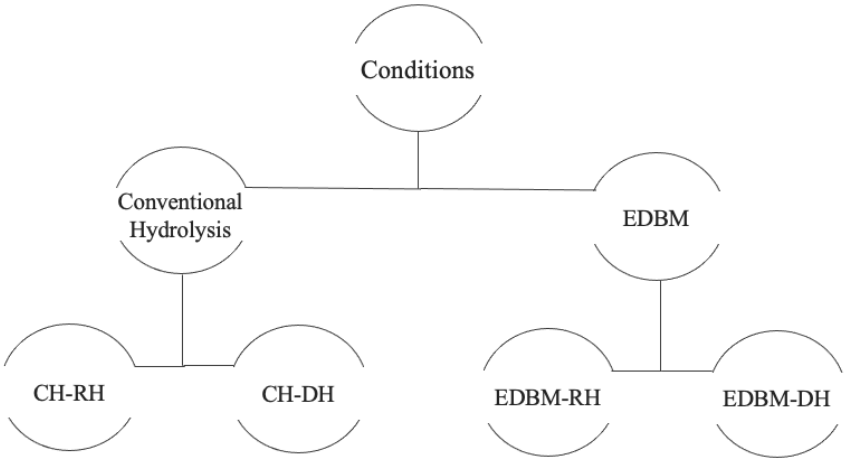

Figure V-14. Diagram summarizing the hydrolysates recovered in each condition.

V.4.4. Antibacterial Activity

V.4.4.1. Agar diffusion method

Bacterial species used in this study were chosen because they are commonly known to be responsible for food alteration (Borch and Arinder, 2002; Catiau *et al*., 2011). Five bacterial strains were used for the determination of antibacterial activity according to the method of Yaba Adje, (2010): *Listeria monocytogenes* (ATCC 19112), *Staphylococcus aureus* (ATCC 13709), *Kocuria rhizophila* (CIP 53.45), *Micrococcus luteus* (ATCC 9341), *Escherichia coli* (ATCC 8733) and *Salmonella newport* (ATCC 6962). Those bacterial species were kept at -20 °C in glycerol containing nutrient broth and were sub-cultured twice in Muller-Hinton broth (MH) at 30 °C under agitation (60 rpm) for *Micrococcus luteus* and at 37 °C for the other bacterial strains. After 24 h of incubation in nutrient broth, the absorbance of the pre-cultures was determined at 620 nm using the medium MH as a blank. After a dilution series,

seeding of the bacterial species at a concentration of $x10^6$ CFU/mL was carried out by the spot-on-lawn method on petri dishes containing 15 mL of MH agar previously casted and dried. A volume of 10 µL of the samples filtered on 0.25 µm, as well as the positive control, were then deposited on the surface of the agar in the form of a spot and plates were incubated 24 h at 37 °C. For *Micrococcus luteus,* petri dishes were incubated 24 h at 30 °C. The antibacterial activity was measured as the diameter of the clear zone of growth inhibition and recorded as diameter of inhibition in centimeter. Ampicillin (0.1 mg/mL) was used as positive control for all bacterial species and Colistin (0.1 mg/mL) as positive control for *Escherichia coli*. This antibacterial test was performed in triplicate.

V.4.4.2. Minimum inhibitory concentration (MIC)

The MIC assays were performed in sterile 96-well microplates (Costar 3799, Corning Incorporated, United Kingdom) for which the response was read after 24 h incubation. Each well contained 100 µL of MH and was supplemented with 100 µL of hemoglobin hydrolysates solutions (80 mg/mL) serially diluted in half. In total, 100 µL of the tested strain pre-diluted in MH to a final bacterial load of $x10^5$ CFU/mL was added last in each well. The inhibition of bacterial growth was monitored by measuring absorbance at 600 nm on a microplate reader (Safas, model MP96 UV-Vis Spectrophotometer, Agilent Technologies, Santa Clara, CA, USA) after 24 h of incubation at 37 °C. The absorbance of the wells corresponding to decreasing concentrations of peptides was compared to that of the well of a positive control consisting of MH containing the bacteria and to that of the well of a negative control consisting of only MH. The MIC was defined as the lowest concentration of peptides that caused no visible increase of absorbance at 600 nm after incubation at 37 °C for 24 h. All measurements of MIC values were performed in triplicate. All the data are expressed as mean ± SD and are the mean of three replicates.

V.4.5. Antifungal Activity

V.4.5.1. Agar diffusion method

The antifungal activity was evaluated against five strains. The fungal strains were: *Aspergillus niger* (3071-13), *Paecilomyces* spp. (5332-9a), *Mucor racemosus* (LMA-722), *Penicillium crustosum* (27,159) and *Rhodotorula mucilaginosa* (27,173). These strains were chosen since they represent some of the most abundant fungal species causing significant food contamination; they were isolated from the environment, food or dairy products products (Pitt and Hocking, 2009). These microorganisms were maintained in glycerol stocks at -80 °C. Then they were recovered on Potato Dextrose Agar (PDA, BD-Difco, Sparks, MD, USA) plates and incubated at 25 °C for a week for the molds and for 72 h for *Rhodotorula mucilaginosa*. For *Rhodotorula mucilaginosa*, cells were then recovered with a swab and incubated on PDA plates for 48 h at 25 °C. For the four molds, conidia were recovered with a swab soaked in a solution containing peptone water (10% w/v) and Tween 80 (0.1% w/v) and were inoculated on PDA plates for another week at 25 °C. Conidia and vegetative cells of *Rhodotorula mucilaginosa* were then harvested using the same method and transferred in peptone water. Cell concentration was measured by an automated cell counter (Invitrogen™ AMQAF1000, Oakville, ON, Canada) and adjusted to $x10^6$ CFU/mL after dilutions in peptone water to obtain the same inoculum concentration for all the strains. The antifungal activity of extracts was assessed in agar diffusion assays. Briefly, a volume of 250 µL of each fungal culture was then incorporated into Falcon tubes containing 25 mL of PDA medium at 45 °C to obtain a final inoculum concentration of $x10^4$ CFU/mL. Inoculated media were then poured into sterile petri dishes of 100 mm diameter and solidified. Eights wells were uniformly dug on each inoculated PDA plate using a 5 mL serological pipette. In each well, 80 µL of hemoglobin hydrolysates solutions at 10 and 20 mg/mL were spilled. The agar plates were incubated at 25 °C for 48 h, then the diameter of the inhibition zones indicating antifungal activity was measured (in millimeter), as indicated by the Clinical and Laboratory Standards Institute (CLSI 2016, CLSI 2017). A solution of 16.7 µg/mL natamycin (Sigma-Aldrich, Oakville, ON, Canada) was used as a positive control and sterile distilled water as negative control. All the data are expressed as mean ± SD and are the mean of three replicates.

V.4.5.2. Minimum inhibitory concentration (MIC)

The MIC assays were also applied to fungi and performed in sterile 96-well microplates. Each well contained 125 µL of peptone solution and was supplemented with 125 µL of hemoglobin hydrolysate solutions (80 mg/mL) serially diluted in half. Then, 50 µL of the molds and yeasts strains previously measured and adjusted at a concentration of $x10^5$ CFU/mL was added to each well. The inhibition capacity of the peptides was compared to that of the well of a positive control consisting of peptone solution containing the strain and to that of the well of a negative control consisting of peptone solution. MIC were determined as the lowest concentration of peptides that caused no visible increase after 48 h of incubation at 25 °C. All measurements of MIC values were done in triplicate. All the data are expressed as mean ± SD and are the mean of three replicates. Minimal fungicidal concentrations (MFC) were determined by spotting a 10µL of each well showing absence of growth onto Dichloran Rose-Bengal Chloramphenicol agar (DRBC, BD-Difco, Sparks, MD, USA) plates. After incubation at 25 °C for 48 h, the lowest dilution with no growth indicated the MFC. A compound was considered fungicidal when the MFC/MIC ratio was ≤ 4 and fungistatic when the MFC/MIC ratio was > 4 (Pfaller *et al*., 2004).

V.4.6. Antioxidant Activity

V.4.6.1. Antioxidant assay using the β-carotene bleaching method

The ability of the different hydrolysates produced by EDBM and control to prevent the bleaching of β-carotene was determined as described by Koleva *et al*. (2002). Briefly, 4 mg β-carotene in 4 mL chloroform was mixed with 800 µL of Tween-40 and 100 µL of linoleic acid. The chloroform was completely evaporated under vacuum (rotary evaporator, Heidolph, Schwabach, Germany) at 45 °C, then 400 mL of distilled water was added, and the resulting mixture was vigorously stirred. The emulsion obtained was freshly prepared before each experiment. An aliquot (5 mL) of the β-carotene-linoleic acid emulsion was transferred to tubes containing 500 µL of each sample at different concentrations. The tubes were placed in a water bath and incubated at 50 °C for 2 h. Thereafter, the absorbance of

each sample was measured at 470 nm (Shimadzu UV-1650 PC Spectrophotometer, shimadzu corporation, Kyoto, Japan). A blank consisted of 500 µL of distilled water instead of the sample solution. Butylated hydroxytoluene (BHT) at a concentration of 0.5 mg/mL was used as positive control. Relative antioxidant activity (RAA%) was calculated according to the following equation:

$$RAA(\%) = \frac{A\ sample}{A\ control} \times 100$$

where A_{sample} is the absorbance of samples (with the emulsion) and $A_{control}$ is the absorbance of BHT (with the emulsion). All the data are expressed as mean ± SD and are the mean of three replicates.

V.4.6.2. Antioxidant assay using DPPH radical scavenging capacity

The DPPH radical-scavenging capacity of samples was determined as described by Bersuder *et al*. (1998). A volume of 500 µL of each sample prepared in ultrapure water at different concentrations was added to 375 µL of 99% ethanol and 125 µL of DPPH solution (0.02% in ethanol) as free radical source. The mixtures were shaken and then incubated for 60 min in a dark room at 30 °C. Scavenging capacity was measured spectrophotometrically (UV mini 1240, UV/VIS spectrophotometer, SHIMDZU, Kyoto, Japan) by monitoring the decrease in absorbance at 517 nm. In its radical form, DPPH has an absorption band at 517 nm which disappears upon reduction by an antiradical compound. Lower absorbance of the reaction mixture indicated higher DPPH free radical-scavenging activity. DPPH radical-scavenging capacity was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A\ control - A\ sample}{A\ control} \times 100$$

where $A_{control}$ represents the absorbance of all the reagents without the samples and A_{sample} is the absorbance of the sample (with the DPPH solution). DPPH radical scavenging activity was expressed as the half-maximal inhibition concentrations (IC_{50}) and Trolox Equivalent Antioxidant Capacity (TEAC) values. The IC_{50} was calculated graphically by linear

regressions of the percentage curves inhibition according to different concentrations of the hydrolysates tested. It represents the value of the sample concentration required to inhibit 50% of DPPH radicals. The lower this value, the more the compound was anti-free radical (Gulcin, 2010). The TEAC value was defined as the concentration of standard Trolox, a water-soluble vitamin E analogue that exhibited the same antioxidant capacity as a 1 mg/mL solution concentration of the antioxidant compound under investigation (Maisuthisakul *et al*., 2007). It is used as a reference antioxidant in biology for evaluation of resistance to oxidative stress. All the data are expressed as mean ± SD and are the mean of three replicates.

V.4.6.3. Antioxidant assay using ABTS radical scavenging capacity

The ABTS radical scavenging activity was determined according to the method described by Re *et al*. (1999). The cationic radical $ABTS^+$ was obtained by aqueous reaction between 7 mM ABTS and 4.95 mM potassium persulfate. This solution was prepared 12 to 16 h before use and was stable when stored at room temperature and protected from light. Before use, this solution was diluted with ethanol at 30 °C, in order to obtain an absorbance at 734 nm of less than 1. The hydrolysates were previously dissolved in ultrapure water at different concentrations and then, 10 µL of each sample were mixed with 1 mL of the diluted $ABTS^+$ solution. The absorbance of $ABTS^+$ was measured at 734 nm (Shimadzu UV-1650 PC Spectrophotometer, Shimadzu corporation, Kyoto, Japan) and at 30 °C exactly 6 min after the initial mixing. Appropriate solvent blanks were run in each assay. The results are expressed as the percentage inhibition according to the following equation:

$$ABTS^+ \text{ radical scavenging activity (\%)} = \left(1 - \frac{A\ sample}{A\ control}\right) \times 100$$

where $A_{control}$ represents the absorbance of all the reagents without the samples and A_{sample} is the absorbance of the sample (with the ABTS solution). $ABTS^+$ radical scavenging activity was expressed as the half-maximal inhibition concentration (IC_{50}) and TEAC values. All the data are expressed as mean ± SD and are the mean of three replicates.

V.4.6.4. Evaluation of total antioxidant capacity

Total antioxidant capacity of the hydrolysates was determined according to the method described by Prieto *et al*. (1999). It is a quick and simple method for determining antioxidant activity, based on an oxidation-reduction reaction. This technique is based on the reduction of molybdenum Mo present as molybdate MoO_4^{2-} ions to molybdenum MoO^{2+} in the presence of the hydrolysate to form a phosphate/molybdenum ($Mo_3O_{16}P_4$) green complex at acidic pH. This test allowed to evaluate the antioxidant status of the peptides present in the hydrolysates as reducing agents in a colorimetric redox reaction. Peptide hydrolysates were previously dissolved in ultrapure water at different concentrations (2.5, 5, 10 and 20 mg/mL) and then an aliquot of 300 µL of each sample was mixed with 3 mL reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 °C for 90 min. The absorbance of the aqueous solution of each sample was measured at 695 nm (Shimadzu UV-1650 PC Spectrophotometer, Shimadzu corporation, Kyoto, Japan), after cooling at room temperature, against a blank. A typical blank solution contained 3 mL of reagent solution and the appropriate volume of the same solvent used for the sample, and it was incubated under the same conditions as the rest of the samples. A Trolox solution at different concentrations, ranging from 0 to 1 mg/mL, was used for calibration. The antioxidant activity is expressed in Trolox Equivalent Antioxidant Capacity (TEAC) values (mg/mL) using the equation given by the calibration line:

$$A = a \times [C_{Trolox}] + b$$

where A the absorbance at 695 nm, C the equivalent antioxidant concentration (mg/mL). The concentration is determined according to the equation of the standard range curve of a reference antioxidant such as: Trolox, a and b, respectively, the origin and the slope of the Trolox calibration line. All the data are expressed as mean ± SD and are the mean of three replicates.

V.4.7. Statistical Analyses

All analyses were performed in triplicate and three independent repetitions were done for each condition. Data were subjected to one-way or two-way analyses of variance (ANOVA). Tukey tests were also performed on data using SigmaPlot software (Version 14.0) to determine which treatment was statistically different from the others at a probability level p of 0.05.

V.5. Results and discussion

V.5.1. Antibacterial Activity

V.5.1.1. Agar diffusion method

Antibacterial activity investigated on CH-RH, CH-DH, EDBM-RH and EDBM-DH at 20 mg/mL are presented in **Tableau V-5**. All hemoglobin hydrolysates derived from EDBM and conventional hydrolysis displayed antibacterial activity against the six tested bacterial species, with a clear growth inhibition zone. There is no significant difference in antibacterial activity between CH-RH, CH-DH and EDBM-DH (20 mg/mL) against *Staphylococcus aureus* and *Kocuria rhizophila*. However, EDBM-RH presented a smaller and statistically lower ($p < 0.05$) inhibition zone than the other hydrolysates. Moreover, no significant difference between hydrolysates was observed against the other strains. *Staphylococcus aureus, Kocuria rhizophila* and *Listeria monocytogenes* presented the highest sensitivity to the hydrolysates.

Tableau V-5. Antibacterial activity of bovine hemoglobin hydrolysates.

Bacteria Strains	Bovine Hemoglobin Hydrolysates			
	CH-RH	**CH-DH**	**EDBM-RH**	**EDBM-DH**
Staphylococcus aureus	+++	+++	++	+++
Listeria monocytogenes	++	++	++	++
Micrococcus luteus	+	+	+	+
Kocuria rhizophila	+++	+++	++	+++
Escherichia coli	+	+	+	+
Salmonella newport	+	+	+	+

Inhibition zones: +++: > 1.5 cm; ++ 0.5–1.5 cm; +: < 0.5 cm, and -: no activity.

The inhibition zones induced by CH-RH, CH-DH, EDBM-RH and EDBM-DH at 10 and 20 mg/mL against *Staphylococcus aureus* and *Kocuria rhizophila* are presented in **Figure V-15**. Hydrolysates at 20 mg/mL induced larger inhibition zones than hydrolysates at 10 mg/mL.

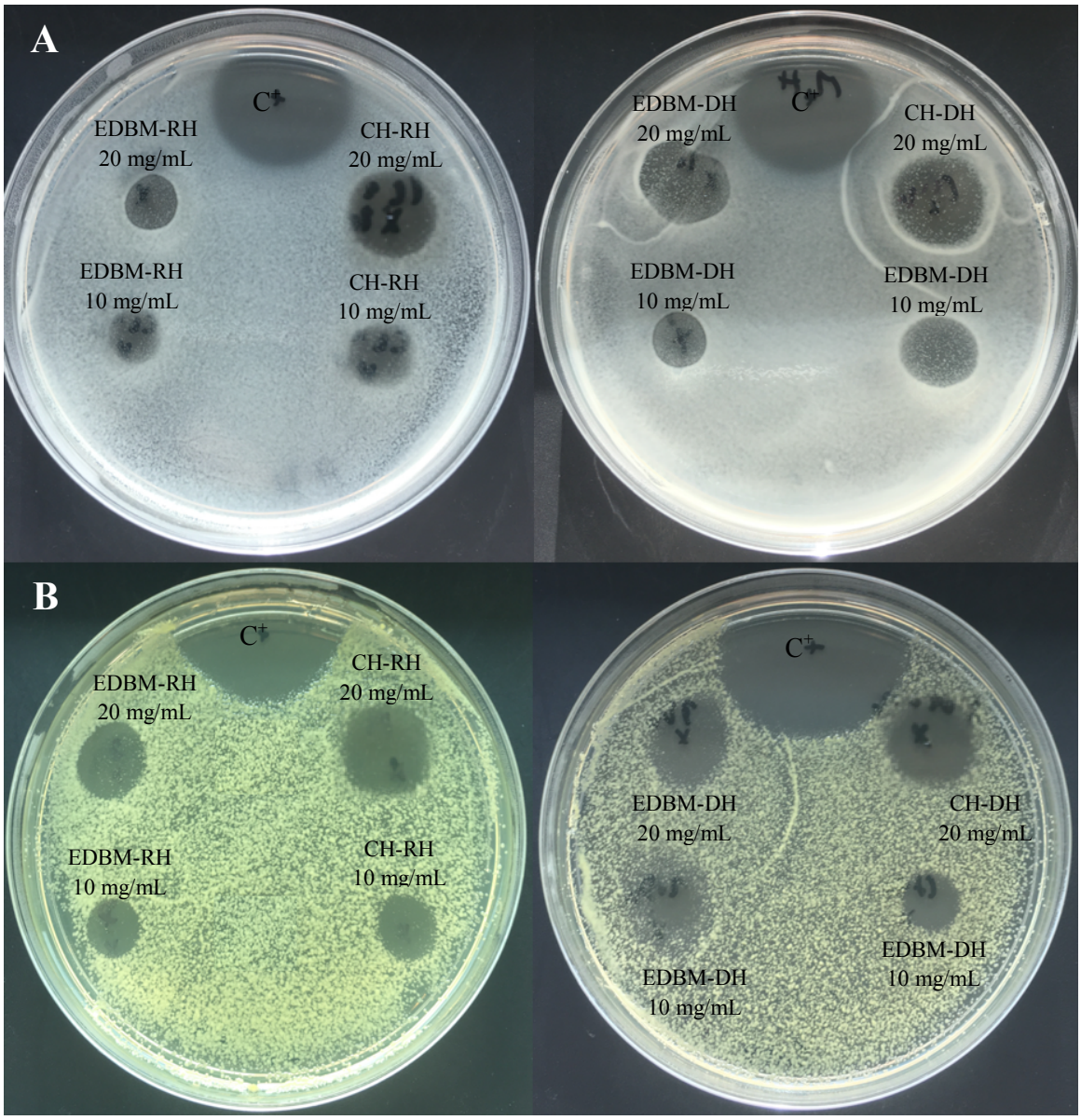

Figure V-15. Inhibition zones induced by 2 different concentrations (10 and 20 mg/mL) of raw and discolored bovine hemoglobin hydrolysates produced by two different hydrolysis processes on Muller-Hinton Agar overlaid with a suspension (10^6 strain per plate) of Staphylococcus aureus (**A**) and Kocuria rhizophila (**B**) and compared to a positive control (C^+: ampicillin).

V.5.1.2. Minimum inhibitory concentration (MIC)

MIC values of the hydrolysates presented in **Tableau V-6** are expressed in mg/mL due to the presence of a large peptide population. The results of MIC confirmed the findings previously demonstrated with the Agar Diffusion Method. Lower MIC values of hydrolysates were registered against *Staphylococcus aureus, Kocuria rhizophila* and *Listeria monocytogenes* (between 0.31 to 2.5 mg/mL) than *Micrococcus luteus*, *Escherichia coli* and *Salmonella newport* (between 5 to 10 mg/mL).

The antibacterial effect of CH-RH, CH-DH and EDBM-DH were statistically similar ($p > 0.05$) and corresponded to MIC values of 0.31 mg/mL against *Staphylococcus aureus* and *Kocuria rhizophila,* while EDBM-RH was statistically different ($p < 0.05$) and corresponded to MIC values of 1.25 and 0.62 mg/mL against *Staphylococcus aureus* and *Kocuria rhizophila*, respectively. CH-RH displayed important and statistically lower ($p < 0.05$) antibacterial activity than the other conditions against *Listeria monocytogenes* and *Salmonella newport* with MIC values of 1.25 and 5 mg/mL, respectively. The MIC value of 5 mg/mL against *Micrococcus luteus* obtained for CH-RH and CH-DH was statistically different ($p < 0.05$) from MIC value of 10 mg/mL for EDBM-RH and EDBM-DH. Identical antibacterial activity was also recorded for all hydrolysates against *Escherichia coli* with a MIC value of 10 mg/mL **(Tableau V-6).** Our findings are in line with previous findings reported by Abou-Diab *et al*. (2020a) and Zouari *et al*. (2020), who showed the inhibitory effect of bovine or porcine hemoglobin hydrolysates against *Staphylococcus aureus, Kocuria rhizophila*, *Listeria monocytogenes*, *Micrococcus luteus*, *Escherichia coli* and *Salmonella newport*.

Tableau V-6. MIC values of the bovine hemoglobin hydrolysates.

Bacteria Strains	Bovine Hemoglobin Hydrolysates			
	MIC			
	CH-RH	CH-DH	EDBM-RH	EDBM-DH
	mg/mL	mg/mL	mg/mL	mg/mL
Staphylococcus aureus	0.31 ± 0.0 [b]	0.31 ± 0.0 [b]	1.25 ± 0.0 [a]	0.31 ± 0.0 [b]
Listeria monocytogenes	1.25 ± 0.0 [b]	2.5 ± 0.0 [a]	2.5 ± 0.0 [a]	2.5 ± 0.0 [a]
Micrococcus luteus	5 ± 0.0 [b]	5 ± 0.0 [b]	10 ± 0.0 [a]	10 ± 0.0 [a]
Kocuria rhizophila	0.31 ± 0.0 [b]	0.31 ± 0.0 [b]	0.62 ± 0.0 [a]	0.31 ± 0.0 [b]
Escherichia coli	10 ± 0.0 [a]	10 ± 0.0 [a]	10 ± 0.0 [a]	10 ± 0.0 [a]
Salmonella newport	5 ± 0.0 [b]	10 ± 0.0 [a]	10 ± 0.0 [a]	10 ± 0.0 [a]

The minimum inhibitory concentration (MIC) of the peptide hydrolysates was determined in a microtiter plate assay system after 24 h of incubation at 37 °C. **[a-b]:** Population means for each bacteria strain within each row with different letters are significantly different, similar letter means no significant difference $p < 0.05$ (Anova, Tukey).

V.5.2. Antifungal Activity

V.5.2.1. Agar diffusion method

Antifungal activity was investigated on CH-RH, CH-DH, EDBM-RH and EDBM-DH at two different concentrations (10 and 20 mg/mL). The hydrolysates were tested against *Paecilomyces* spp. (5332-9a), *Aspergillus niger* (3071-13), *Mucor racemosus* (LMA-722), *Penicillium crustosum* (27,159) and *Rhodotorula mucilaginosa* (27,173). A large growth inhibition zone was observed against *Paecilomyces* spp. and *Rhodotorula mucilaginosa* (**Figure V-16**). CH-RH, CH-DH and EDBM-DH at a concentration of 20 mg/mL were strongly active against *Paecilomyces* spp., inducing inhibition zones of 18 mm, 18.5 mm and 18 mm, respectively, while CH-RH, CH-DH, and EDBM-DH, at a concentration of 10 mg/mL, induced inhibition zones of 14 mm, 14.5 mm and 13.5 mm, respectively. Statistical analysis demonstrated that there was no significant difference ($p > 0.05$) between hydrolysates having the same concentration. However, EDBM-RH presented a smaller and statically lower ($p < 0.05$) inhibition zone than the other hydrolysates (14.5 mm at 20 mg/mL and 11 mm at 10 mg/mL). Statistical analysis demonstrated that there was no significant difference ($p > 0.05$) in the inhibition zone formed by CH-RH, CH-DH and EDBM-DH against *Rhodotorula mucilaginosa*. At 20 mg/mL, CH-RH presented an inhibition zone (18.5 mm) roughly larger than CH-DH (18 mm) and even roughly larger than EDBM-DH (17 mm).

At 10 mg/mL, CH-RH presented an inhibition zone (14 mm) similar to that of CH-DH (14 mm) and roughly larger that EDBM-DH (13 mm). While a statistical difference ($p < 0.05$) in the inhibition zone formed by EDBM-RH was noticed (13.5 mm at 20 mg/mL and 10 mm at 10 mg/mL). All hydrolysates, at 20 mg/mL, induced an inhibition zone against *Rhodotorula mucilaginosa* statistically higher ($p < 0.05$) than the positive control (11 mm). At a concentration of 20 mg/mL, CH-RH presented a clear activity against *Mucor racemosus* (inhibition zone of 15 mm) statistically similar ($p > 0.05$) to CH-DH (14 mm) and EDBM-DH (13 mm) but statistically higher ($p < 0.05$) than the one of EDBM-RH (11 mm). At 10 mg/mL, all hydrolysates were active against *Mucor racemosus* inducing roughly similar inhibition zones of 8, 8, 6 and 7 mm for CH-RH, CH-DH, EDBM-RH and EDBM-DH, respectively. CH-RH, CH-DH, EDBM-RH and EDBM-DH at a concentration of 20 mg/mL were active against *Penicillium crustosum* producing inhibition zones of 10, 11, 8 and 9 mm, respectively, and statistically higher ($p < 0.05$) than the inhibition zone at 10 mg/mL. Moreover, CH-RH, CH-DH, EDBM-RH and EDBM-DH at a concentration of 20 mg/mL were active against *Aspergillus niger* producing inhibition zone of 10, 11, 8 and 9 mm, respectively, and statistically higher ($p < 0.05$) than the inhibition zone at 10 mg/mL in comparison with negative control, which has no inhibition zone. The smaller inhibition zone noticed for EDBM-RH may be due to the lower peptide population recovered at the end of the process due to the precipitation of hem or peptide-hem associations in the dilute compartment as described by Abou-Diab *et al*. (2021b).

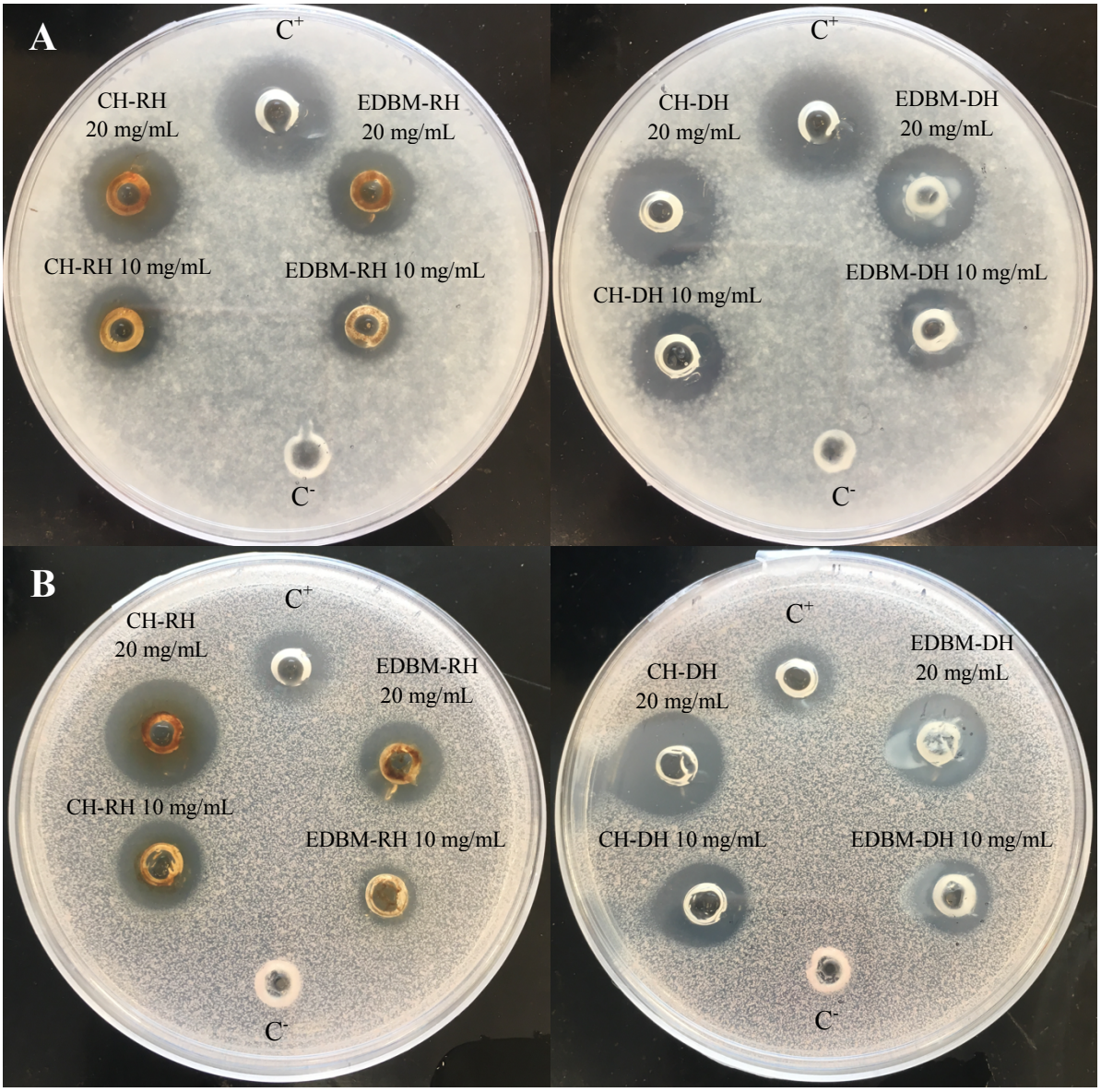

Figure V-16. Inhibition zones induced by 2 different concentrations (10 and 20 mg/mL) of raw and discolored bovine hemoglobin hydrolysates produced by two different hydrolysis processes on Potato Dextrose Agar (PDA) overlaid with a suspension (10^4 spores per plate) of *Paecilomyces* spp. (**A**) and *Rhodotorula mucilaginosa* (**B**) and compared to a positive (C^+: natamycin) and negative control (C^-: distilled water).

V.5.2.2. MIC and MFC of bovine hemoglobin hydrolysates

MIC of CH-RH, CH-DH, EDBM-RH and EDBM-DH against *Paecilomyces* spp. (5332-9a), *Aspergillus niger* (3071-13), *Mucor racemosus* (LMA-722), *Penicillium crustosum* (27,159) and *Rhodotorula mucilaginosa* (27,173), were determined by broth microdilution followed

by visual reading (**Tableau V-7**). All samples showed activity against the different fungal strains tested. The results of MIC confirmed the findings previously obtained with the Agar Diffusion Method. The MIC values recorded for hydrolysates against *Paecilomyces* spp. and *Rhodotorula mucilaginosa* (between 0.44 to 3.5 mg/mL) were considerably lower than the MIC values registered against *Mucor racemosus*, *Penicillium crustosum* and *Aspergillus niger* (between 3.57 to 7.15 mg/mL). The antifungal effect of CH-RH, CH-DH and EDBM-DH exhibited a broad spectrum of activity inhibiting *Paecilomyces* spp. and *Mucor racemosus,* with a statistically similar ($p > 0.05$) MIC values of 0.44 and 3.57 mg/mL, respectively. However, EDBM-RH exhibited a higher and statistically different ($p < 0.05$) MIC value to inhibit *Paecilomyces* spp. (0.89 mg/mL) and *Mucor racemosus* (7.15 mg/mL). EDBM-RH showed also higher and statistically different ($p < 0.05$) MIC value of 3.57 mg/mL to inhibit *Rhodotorula mucilaginosa.* The antifungal activity of CH-RH, CH-DH, EDBM-RH and EDBM-DH, against *Penicillium crustosum* and *Aspergillus niger* showed a statistically similar ($p > 0.05$) MIC value of 7.15 mg/mL.

MFC of bovine hemoglobin hydrolysates was ranged between 0.44 and 7.15 mg/mL for all fungal strains except *Penicillium crustosum* and *Aspergillus niger* that did not display any MFC value. As described by Pfaller *et al*. (2004), by extrapolation from the conventional definition used for bacterial testing, CH-RH, CH-DH, EDBM-RH and EDBM-DH acted with a fungicidal mechanism towards *Paecilomyces* spp*., Rhodotorula mucilaginosa* and *Mucor racemosus*, since MFC/MIC ratio was ≤ 4. Moreover, the hydrolysates inhibited the growth of *Penicillium crustosum* and *Aspergillus niger* after 48 h only since MFC/MIC ratio was not determined.

Tableau V-7. In vitro MIC and MFC ranges of CH-RH, CH-DH, EDBM-RH and EDBM-DH against filamentous molds and yeast as determined by broth microdilution followed by visual examination.

	Bovine Hemoglobin Hydrolysates (mg/mL)											
	CH-RH			CH-DH			EDBM-RH			EDBM-DH		
Fungal strain	MIC	MFC	MFC/ MIC	MIC	MFC	MFC/ MIC	MIC	MFC	MFC/ MIC	MIC	MFC	MFC/ MIC
Paecilomyces spp.	0.44 ± 0.0^{b}	0.44 ± 0.0^{C}	1	0.44 ± 0.0^{b}	0.44 ± 0.0^{C}	1	0.89 ± 0.0^{a}	0.89 ± 0.0^{B}	1	0.44 ± 0.0^{b}	1.78 ± 0.0^{A}	4
Aspergillus niger	7.15 ± 0.0^{a}	0.0 ± 0.0^{A}	ND	7.15 ± 0.0^{a}	0.0 ± 0.0^{A}	ND	7.15 ± 0.0^{a}	0.0 ± 0.0^{A}	ND	7.15 ± 0.0^{a}	0.0 ± 0.0^{A}	ND
Rhodotorula mucilaginosa	0.89 ± 0.0^{c}	1.78 ± 0.0^{C}	2	1.78 ± 0.0^{b}	7.15 ± 0.0^{B}	4	3.57 ± 0.0^{a}	14.3 ± 0.0^{A}	4	1.78 ± 0.0^{b}	7.15 ± 0.0^{B}	4
Mucor racemosus	3.57 ± 0.0^{b}	7.15 ± 0.0^{A}	2	3.57 ± 0.0^{b}	3.57 ± 0.0^{B}	1	7.15 ± 0.0^{a}	7.15 ± 0.0^{A}	1	3.57 ± 0.0^{b}	7.15 ± 0.0^{A}	2
Penicillium crustosum	7.15 ± 0.0^{a}	0.0 ± 0.0^{A}	ND	7.15 ± 0.0^{a}	0.0 ± 0.0^{A}	ND	7.15 ± 0.0^{a}	0.0 ± 0.0^{A}	ND	7.15 ± 0.0^{a}	0.0 ± 0.0^{A}	ND

Not determined: ND

For each strain, different lowercase letter indicates significant difference between conditions for MIC, different capital letter indicate significant difference between conditions for MFC, similar letter means no significant difference $p < 0.05$ (Anova, Tukey).

These results demonstrated that bovine hemoglobin hydrolysates, whatever their mode of production, had antibacterial and antifungal activities. Moreover, the demineralization-discoloration did not impact the antimicrobial capacity. However, a lower antibacterial and antifungal activity was noticed for EDBM-RH. This can be explained by that fact that this condition revealed less antibacterial peptides as described by Abou-Diab, *et al*. (2021b), who identified a lower peptide population in EDBM-RH compared to other conditions due to the precipitation during the demineralization in the diluate compartment. The α34-66, α33-45, α133-141 and α34-65 which are known for their antibacterial activity, did not appear in EDBM-RH, while they appeared in CH-RH, CH-DH and EDBM-DH. Therefore, the antibacterial and antifungal capacities of EDBM-RH was lower since at the same pH adjusted to 7 of the final hydrolysates, the global pHi of EDBM-RH had a value of +7, lower than the pHi of CH-RH, CH-DH and EDBM-DH with a value of: +15, +14 and +14, respectively. The antibacterial peptides are the most characterized because the focus for discovery of antimicrobial peptides has long been restricted to those with antibacterial activity. However, the mechanism of action of antibacterial and antifungal activities is roughly the same as reported by Yeaman, *et al*. (2003), Van Der Weerden, *et al*. (2013), Bahar and Dacheng,

(2013), and Kumar, *et al*. (2018). The charge, the conformation, the hydrophobicity, and the amphiphilic character of the peptide sequence are all elements influencing the antimicrobial potential. The charge of the antimicrobial peptide is an essential part of its activity. In general, it is described that cationic domains are essential for the establishment of interactions between the biological, negatively charged phospholipid membrane and the peptide through electrostatic interactions. The higher the charge of the peptide, the greater the antimicrobial potential of the peptide.

V.5.3. Antioxidant Activity

V.5.3.1. Antioxidant assay using the β-carotene bleaching method

The method is based on the loss of the yellow color of β-carotene due to its reaction with radicals which are formed by linoleic acid oxidation in an emulsion. β-carotene bleaching, measured by the decrease in the initial absorbance at 470 nm, is slowed down in the presence of antioxidants. The method is widely used in the antioxidant activity evaluation of different types of different compounds as described by Koleva al., [19]. In this study, the inhibition activity of lipid peroxidation was determined by assessing the ability of CH-RH, CH-DH, EDBM-RH and EDBM-DH to inhibit the oxidation of linoleic acid in this emulsified model.

The antioxidant activities of CH-RH, CH-DH, EDBM-RH and EDBM-DH at different concentrations using the β-carotene bleaching assay are presented in **Figure V-17**. The results were compared to the standard antioxidant, BHT (0.5 mg/mL) and synthetic bioactive peptide, neokyotorphin (NKT, 0.5 mg/mL). The oxidation of β-carotene was inhibited by CH-RH, CH-DH, EDBM-RH and EDBM-DH. However, different relative antioxidant activity (%) were noticed depending on concentration. A concentration of 20 mg/mL showed significantly ($p < 0.05$) higher antioxidant activity than the other concentrations. At a concentration of 2.5 and 20 mg/mL, there was no significant difference ($p > 0.05$) in antioxidant activity between CH-RH, CH-DH, EDBM-RH and EDBM-DH. While, at a concentration of 5 and 10 mg/mL, there was a significant difference ($p < 0.05$) in antioxidant activity, but the values were very close. The antioxidant activity of CH-RH, CH-DH, EDBM-

RH and EDBM-DH at a concentration of 20 mg/mL (90.92 ± 1.07; 89.93 ± 0.13; 89.73 ± 1.55; 91.49 ± 0.42%, respectively) were significantly lower ($p < 0.05$) but very close to that of the BHT (99.00 ± 0.31%) and NKT (94.41 ± 1,03%). Bleaching of β-carotene has been slowed down in the presence of bovine hemoglobin hydrolysates. Therefore, CH-RH, CH-DH, EDBM-RH and EDBM-DH could be considered as antioxidant allowing food protection from lipid peroxidation.

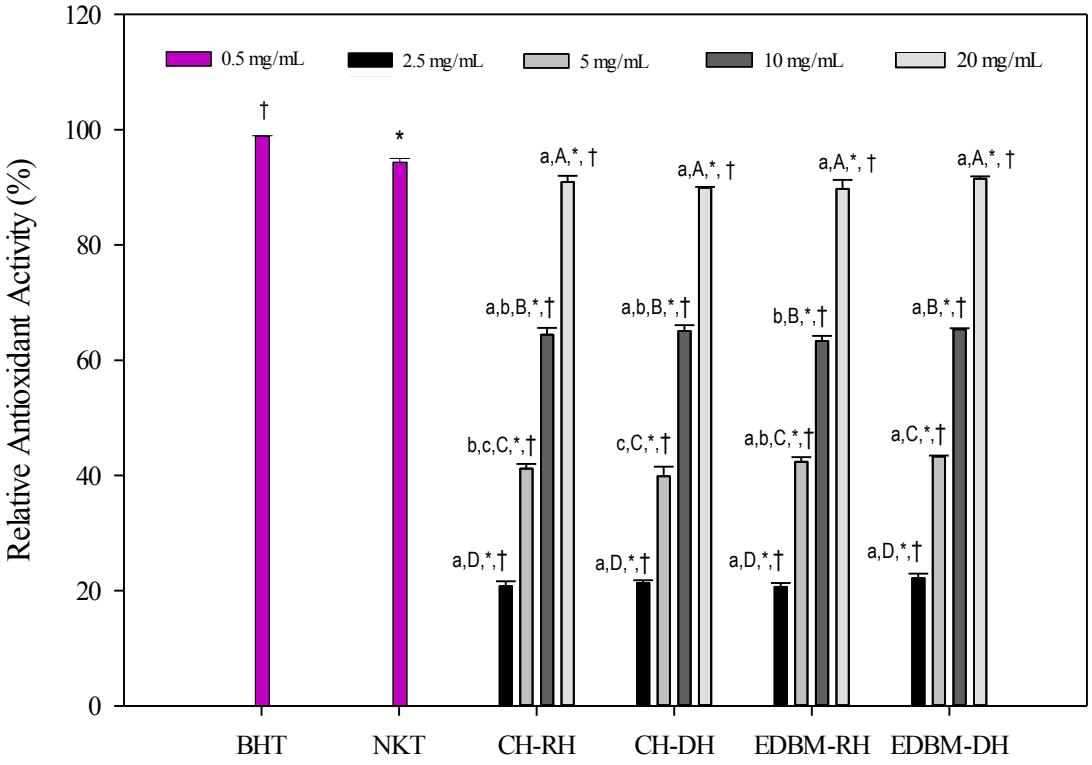

Figure V-17. β-carotene bleaching inhibition activity of bovine hemoglobin hydrolysates at different concentrations in CH-RH, CH-DH, EDBM-RH and EDBM-DH. Values with (*) symbol are significantly different from BHT; Values with (†) symbol are significantly different from neokyotorphin; values with different lowercase letter (a-b) within the same concentration are significantly different; values with different capital letter (A-B-C-D) within the same condition are significantly different ($p < 0.05$, Tukey).

V.5.3.2. DPPH radical scavenging capacity

The DPPH radicals have widely been used in the evaluation of antioxidant capacity, based on the ability of molecules to transfer one electron to reduce an oxidant. The DPPH radical scavenging activity method was done on CH-RH, CH-DH, EDBM-RH and EDBM-DH at different concentrations. The DPPH radical scavenging activities of CH-RH, CH-DH, EDBM-RH and EDBM-DH were expressed as the half-maximal inhibition concentrations (IC_{50}) and Trolox Equivalent Antioxidant Capacity (TEAC). The comparative IC_{50} and TEAC values of the tested phenols were shown in **Tableau V-8.**

The DPPH radical scavenging activity of CH-RH, CH-DH, EDBM-RH and EDBM-DH showed different ($p < 0.05$) IC_{50} and lower than Trolox. However, NKT showed an IC_{50} close to that of Trolox but statistically lower ($p < 0.05$). The TEAC of EDBM-RH and EDBM-DH were statistically similar ($p > 0.05$) but higher ($p < 0.05$) than CH-RH and CH-DH. In comparison with Trolox, the DPPH radical scavenging activity of all the hydrolysates expressed in TEAC, showed lower radical scavenging activity (TEAC value lower than 1). As expected, TEAC of NKT showed higher radical scavenging activity than the hydrolysates with a value closer to 1. These findings are coherent with recent works reported by Abou-Diab *et al*. (2020b) where bovine hemoglobin hydrolysates showed important DPPH scavenging capacity but lower capacity compared to Trolox due to the competition effect of bioactive peptides present in large numbers in the hydrolysates.

Tableau V-8. IC_{50} and Trolox Equivalent Antioxidant Capacity (TEAC) coefficients of bovine hemoglobin hydrolysates for the DPPH method.

DPPH	CH-RH	CH-DH	EDBM-RH	EDBM-DH	NKT	Trolox
IC_{50} (mg/mL)	2.52 ± 0.01^{b}	2.77 ± 0.03^{a}	2.39 ± 0.09^{c}	2.29 ± 0.04^{d}	0.58 ± 0.02^{e}	0.36 ± 0.01^{f}
TEAC	0.14 ± 0.002^{d}	0.13 ± 0.005^{e}	0.15 ± 0.001^{c}	0.15 ± 0.003^{c}	0.64 ± 0.01^{b}	1^{a}

[a-e]: Mean values within each row with different letters are significantly different, similar letter means no significant difference at a probability level of 0.05 (Anova, Tukey).

V.5.3.3. Antioxidant properties products by ABTS assay

The ABTS assay is a widely applied method for measuring the radical scavenging ability of antioxidants, especially those present in foods. The antioxidant capacities of CH-RH, CH-DH, EDBM-RH and EDBM-DH were measured at different concentrations, as their quenching capacity toward the ABTS radical cation. The results presented in **Figure V-18** clearly indicated that hydrolysates produced using EDBM exhibited a strong ABTS radical scavenging activity. The antioxidant activity of bovine hemoglobin hydrolysates increased with increasing peptide concentrations. A concentration of 20 mg/mL for CH-RH, CH-DH, EDBM-RH and EDBM-DH, showed significantly higher ($p < 0.05$) scavenging capacity than the other concentrations. At a concentration of 10 and 20 mg/mL, there was no significant difference ($p > 0.05$) in ABTS scavenging capacity between CH-RH, CH-DH, EDBM-RH and EDBM-DH. While, at a concentration of 2.5 and 5 mg/mL, there was a significant difference ($p < 0.05$) in ABTS scavenging capacity between CH-RH/CH-DH and EDBM-RH/EDBM-DH. Our findings are in line with previous works reported by Abou-Diab *et al*. (2020a), who reported that the ABTS scavenging activity increased with increasing peptide hydrolysates concentrations (2.5-20 mg/mL).

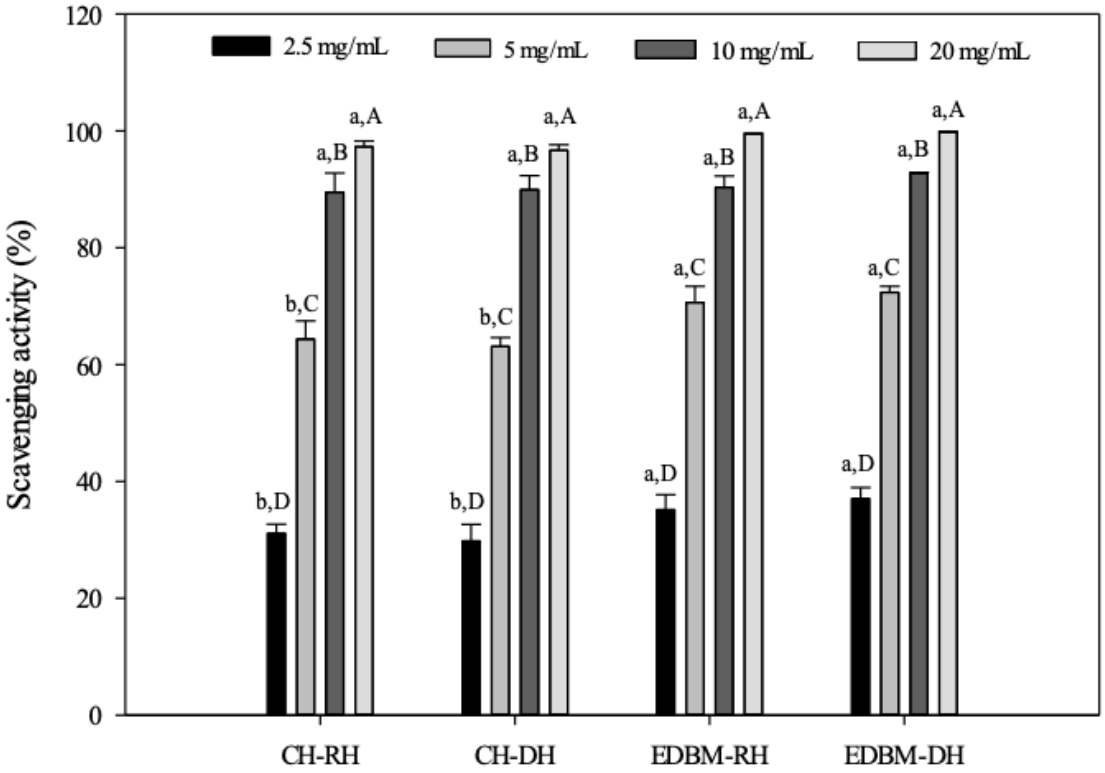

Figure V-18. ABTS scavenging activities of bovine hemoglobin hydrolysates in CH-RH, CH-DH, EDBM-RH and EDBM-DH at different concentrations. Values with different lowercase letter (a-b-c) within the same concentration are significantly different; values with different capital letter (A-B-C) within the same condition are significantly different ($p < 0.05$, Tukey).

Trolox was used as a reference molecule. The comparative IC_{50} and the relative values, expressed as TEAC are reported in **Tableau V-9**. To inhibit 50% of ABTS radicals, concentrations of hemoglobin hydrolysates between 3.43 and 4 mg/mL were needed. Statistical analysis showed that there was a significant difference ($p < 0.05$) of IC_{50} for bovine hemoglobin hydrolysates between all conditions even if the values were very similar. To inhibit 50% of ABTS radicals, a concentration of 0.56 mg/mL of NKT was needed; this value is very close to Trolox but is statistically different ($p < 0.05$). Regarding TEAC, the ABTS radical scavenging activities of CH-RH and CH-DH were similar but statistically lower ($p < 0.05$) than the ABTS radical scavenging activities of EDBM-RH and EDBM-DH. However, TEAC values of all hydrolysates were lower than TEAC value of Trolox. TEAC of NKT (0.89 ± 0.03) was close to Trolox.

Tableau V-9. IC_{50} and TEAC coefficients of bovine hemoglobin hydrolysates for ABTS method.

ABTS	CH-RH	CH-DH	EDBM-RH	EDBM-DH	NKT	Trolox
IC_{50} (mg/mL)	3.91 ± 0.02 [b]	4.00 ± 0.02 [a]	3.54 ± 0.01 [c]	3.43 ± 0.02 [d]	0.56 ± 0.02 [e]	0.50 ± 0.02 [f]
TEAC	0.12 ± 0.004 [d]	0.12 ± 0.005 [d]	0.14 ± 0.005 [c]	0.14 ± 0.005 [c]	0.89 ± 0.03 [b]	1 [a]

[a-e]: Mean values within each row with different letters are significantly different, similar letter means no significant difference $p < 0.05$ (Anova, Tukey).

V.5.3.4. Evaluation of total antioxidant capacity

The capacity of CH-RH, CH-DH, EDBM-RH and EDBM-DH as a reducing agent to produce Mo(V) from Mo(VI) and to form a green-colored complex, based on an oxidation-reduction reaction was carried out at different concentrations using the total antioxidant capacity method and expressed as TEAC (**Figure V-19**).

The antioxidant capacity increased with increasing concentration. The TEAC of CH-RH, CH-DH, EDBM-RH and EDBM-DH increased from 0.15 ± 0.00; 0.12 ± 0.01; 0.15 ± 0.01; 0.15 ± 0.00 mg/mL, respectively, at a concentration of 2.5 mg/mL to 0.31 ± 0.00; 0.31 ± 0.00; 0.33 ± 0.01; 0.33 ± 0.03 mg/mL at a concentration of 20 mg/mL, respectively. As expected, a concentration of 20 mg/mL showed significantly higher antioxidant activity than the other concentrations with the highest TEAC value. For the same concentration of 5, 10, and 20 mg/mL there was no significant difference ($p > 0.05$) in TEAC between CH-RH, CH-DH, EDBM-RH and EDBM-DH. Meanwhile, for the same concentration of 2.5 mg/mL, there was a significant difference ($p < 0.05$) in TEAC between CH-RH and CH-DH. Based on this test, bovine hemoglobin hydrolysates could be considered as an effective reducing agent and thus a primary antioxidant even at high temperature (95°C).

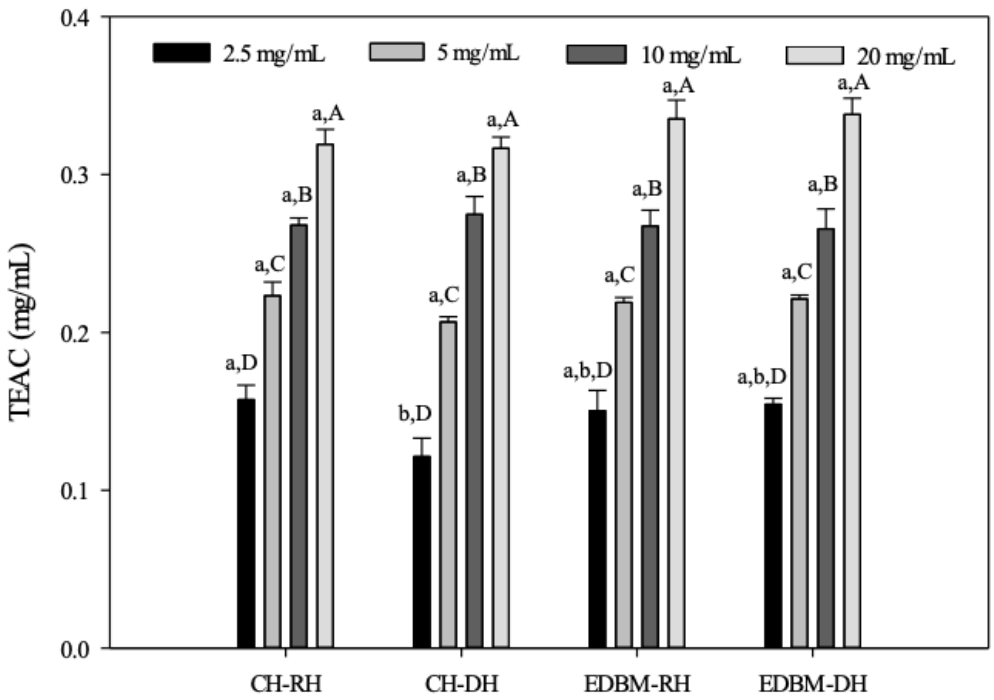

Figure V-19. Total antioxidant capacity of bovine hemoglobin hydrolysates in CH-RH, CH-DH, EDBM-RH and EDBM-DH at different concentrations. Values with (*) symbol are significantly different from BHT; Values with (†) symbol are significantly different from neokyotorphin; values with different lowercase letter (a-b) within the same concentration are significantly different; values with different capital letter (A-B-C-D) within the same condition are significantly different ($p < 0.05$, Tukey test).

After performing four antioxidant assays, based on different mechanisms, these results demonstrated that bovine hemoglobin hydrolysates, whatever their mode of production, had antioxidant capacities. The important antioxidant activity of CH-RH, CH-DH, EDBM-RH and EDBM-DH was based on the ability of peptides to reduce free radicals by hydrogen donation in a competitive reaction and/or their ability to transfer one electron to reduce an oxidant. It was important to mention that the demineralization and the discoloration did not impact the antioxidant capacity, whatever the method of producing the hydrolysates, the antioxidant activity was roughly the same. This is due to the resemblance in the peptide population between the conditions as described by Abou-Diab *et al*. (2021b). Moosmann and behl (2000) and Stadtman and Levine (2003) reported that two categories of amino acids (AA) are well known for their antioxidant activities: the phenolic AA, like tyrosine, tryptophan or phenylalanine, and the reducing AA (sulfur containing), such as methionine or

cysteine. In our study, based on roughly the same peptide population, it is possible to have the same phenolic and reducing amino acids content and therefore, the same antioxidant activities. The bioactive peptide sequences containing the phenolic and/or the reducing AA as: TSKYR, LSFPTTKTYFPHF, VLSAADKGNVKAAWGKVGGHAAEYGAE… were identified in CH-RH, CH-DH, EDBM-RH and EDBM-DH as reported by Abou-Diab et al. (2021b). Hydrolysates derived from EDBM are more interesting at the industrial scale since they are pure peptides with 85% less salt. Although the chemical antioxidant standards exhibited the highest antioxidant ability, natural antioxidants are of growing interest. Indeed, the incorporation of a natural protein hydrolysate with low salt content to foods could confer desirable nutritional and functional properties (Kim *et al*., 2007).

V.6. Conclusion

In this study we tested for the first time the biological activity of bovine hemoglobin demineralized and discolored-demineralized hydrolysates produced by EDBM. The results indicate that CH-RH, CH-DH and EDBM-DH, showed excellent bacterial growth inhibition against *Listeria monocytogenes, Staphylococcus aureus, Micrococcus luteus, Escherichia coli*, *Kocuria rhizophila* and *Salmonella newport*. Furthermore, they showed excellent fungal growth inhibition against *Paecilomyces* spp*., Aspergillus niger, Mucor racemosus, Penicillium crustosum* and *Rhodotorula mucilaginosa*. However, EDBM-RH showed lower antibacterial and antifungal activities compared to other hydrolysates due to loss of antimicrobial peptides after precipitation in the diluate compartment. Moreover, these results confirmed that CH-RH, CH-DH, EDBM-RH and EDBM-DH, whatever their mode of production, had antioxidant capacities. The demineralization and discoloration did not affect the antioxidant potential. The hydrolysates can act as inhibitors of lipid peroxidation, as direct scavengers of free radicals and as agents to chelate the transition metal ions that catalyze the generation of radical species.

The production of bovine hemoglobin discolored-demineralized hydrolysates by the new optimized « multiple-step » EDBM process fits perfectly within the concept of circular economy and could have a great importance at the industrial level. Indeed, bovine

hemoglobin from blood, a slaughterhouse waste, once hydrolyzed, allowed the bioproduction of pure active discolored peptides with a low concentration of mineral salts that could be used as bio-preservative alternatives to chemical additives. Therefore, they could be promising candidate to improve food safety and avoid food spoilage. However, further studies are required to investigate the *in vivo* effect of these hydrolysate on meat during food storage.

V.7. Acknowledgements

The Natural Sciences and Engineering Research Council of Canada (NSERC) financial support is acknowledged. This work was supported by the NSERC Industrial Research Chair METABIOLAC (Grant IRCPJ 499946-15 to Ismail Fliss), the NSERC strategic partnership grants for projects Program (Grant SPGP 5212082 to Laurent Bazinet) and the NSERC Discovery Grants Program (Grant SD 210829409 to Laurent Bazinet). This work was realized within the French-Canadian International Associated Laboratory on « Bioproduction of natural antimicrobials » (LIAAN).

V. Atteinte des objectifs et avancement des connaissances

Les objectifs fixés de cette dernière partie, consacrée à produire des hydrolysats d'hémoglobine bovine déminéralisés en effectuant simultanément un processus à multiple étapes à l'aide d'un système d'Électrodialyse avec Membranes Bipolaires, ont été atteints.

La configuration finale retenue était : + MB / MEA / MCP / MB -. Cette configuration a assuré la dénaturation et l'hydrolyse enzymatique de la solution d'hémoglobine bovine dans le compartiment d'acidification en abaissant le pH de la solution à pH 3; d'autre part, elle a permis l'inhibition de la réaction d'hydrolyse par alcalinisation de la solution à pH 9. Ces 2 étapes se sont accompagnées d'une augmentation de la minéralisation des solutions qui s'explique par la migration des ions nécessaires au transfert du courant et au maintien des conditions d'électro-neutralité des solutions. Cependant, et en simultané avec les étapes d'acidification et de basification par EDMB, le taux de minéraux a pu être réduit de 85%

grâce à la déminéralisation de la solution dans le compartiment central situé entre les compartiments acides et basiques. Par contre, un colmatage a encore été observé sur la membrane anionique en raison de la précipitation de l'hème. Ainsi, un procédé global « 4-en-1 » allant de l'hydrolyse de l'hémoglobine à la récupération des peptides bioactifs décolorés-déminéralisés à l'aide d'un système d'EDMB a été mis en place.

Ces résultats ont aussi concouru à répondre à l'objectif concernant l'élimination complète du colmatage. Le procédé global d'électrodialyse « 4-en-1 » a permis de transformer un sous-produit des abattoirs en un produit décoloré et déminéralisé contenant des peptides bioactifs sans ajout d'agents chimiques et sans colmatage sur les membranes.

Les résultats obtenus dans ce chapitre ont permis d'atteindre aussi l'objectif visant à étudier les activités biologiques des hydrolysats déminéralisés et décolorés-déminéralisés. Les hydrolysats ont montré des activités antibactérienne, antifongique et antioxydante importantes. Par contre, les hydrolysats déminéralisés du « 3-en-1 » ont montré des activités antibactérienne et antifongique inférieures par rapport aux autres hydrolysats dues à la perte de quelques peptides antimicrobiens après précipitation dans le compartiment du diluat. De plus, ces résultats ont confirmé que les hydrolysats d'hémoglobine, quel que soit leur mode de production, ont des capacités biologiques. La déminéralisation et la décoloration n'ont pas affecté leur potentiel bioactif.

Les résultats de cette étude ont permis l'avancement des connaissances sur la mise en œuvre d'un procédé complet basé sur un système d'EDMB en utilisant de manière optimale les propriétés des membranes bipolaires et monopolaires. La transformation de la solution d'hémoglobine bovine par EDMB en peptides bioactifs déminéralisés a été réalisée pour la 1ère fois dans un système unique utilisant une configuration spécifique optimale : a) les protons générés et retenus dans le compartiment d'acidification par l'emploi de membrane anionique ont dénaturé la solution d'hémoglobine et maintenu le pH acide constant durant son hydrolyse enzymatique; b) les ions hydroxyles générés dans le compartiment de basification par l'emploi de membrane cationique permsélective monovalente ont permis d'inactiver la pepsine; c) le compartiment central de diluat délimité par une membrane

anionique et cationique a assuré la réduction du taux de minéraux des hydrolysats d'hémoglobine par migration des ions dans les compartiments adjacents.

Chapitre VI

DISCUSSION GÉNÉRALE

VI. Discussion Générale

Les travaux présentés dans les précédents chapitres concernant la production des hydrolysats d'hémoglobine bovine par électrodialyse avec membranes bipolaires ont montré que :

- L'électro-acidification de l'hémoglobine bovine est influencée par le niveau de conductivité : les niveaux de conductivité plus hauts permettent une électro-acidification plus rapide, une plus faible résistance globale et une meilleure efficacité énergétique du système.

- L'électro-acidification de l'hémoglobine bovine est également dépendante de la valeur du pH : la dénaturation de l'hémoglobine a été améliorée à pH 3, induisant une dénaturation complète de l'hémoglobine et privilégiant un mécanisme de type « zipper ».

- Le mécanisme « zipper » produit une population peptidique variée ayant une plus grande concentration de peptides intermédiaires.

- Le colmatage des membranes est influencé par le niveau de conductivité : les niveaux de conductivité les plus hauts préviennent la formation de colmatage en lien avec les interactions électrostatiques avec les ions abondamment disponibles, limitant ainsi les interactions électrostatiques peptide-membrane.

- Le colmatage des membranes est aussi influencé par le type de membrane monopolaire utilisée dans le système d'EDMB : les membranes anioniques permettent une meilleure acidification, sans apparition de colmatage par diminution des liens électrostatiques peptides-membranes et une meilleure efficacité énergétique du système.

- L'activité enzymatique est impactée par le niveau de sel (force ionique et nature des sels) : les concentrations élevées en sel diminuent l'activité de la pepsine et induisent par conséquent un plus faible degré d'hydrolyse et une légère différence de population peptidique.

- L'électro-basification de la solution d'hémoglobine a un effet sur l'activité de la pepsine : les conditions alcalines, dues à la génération des ions hydroxyles par les membranes bipolaires, provoquent une inhibition complète de la réaction.

- Les différentes étapes de production des hydrolysats d'hémoglobine déminéralisés ont été effectuées en simultané dans un système d'EDMB à 3 compartiments : un taux de déminéralisation de 85% a été obtenu dans le compartiment de diluat.

- Une précipitation peptidique sur les membranes anioniques durant la déminéralisation a été induite par le changement du pH de la solution due à une désalcalinisation partielle de la solution, une partie des OH^- a migré du compartiment diluat vers le compartiment d'acidification adjacent en traversant la membrane anionique.

- La décoloration de la solution d'hémoglobine influence le colmatage membranaire : la séparation de l'hème des peptides en ajustant le pH à 4,5 par utilisation du HCl électro-généré avant la déminéralisation a permi d'éviter le colmatage membranaire.

- Les hydrolysats d'hémoglobine bovine ont montré d'excellentes activités antibactérienne, antifongique et antioxydante : quel que soit le mode de production des peptides bioactifs cela n'impacte pas leurs activités biologiques.

- La charge est un élément influençant le potentiel antimicrobien.

L'ensemble de ces observations permet de valider notre hypothèse selon laquelle, l'électrodialyse avec membranes bipolaires permet de réaliser en simultané dans un système d'électrodialyse les différentes étapes nécessaires à la production d'un hydrolysat (dénaturation et hydrolyse pepsique de l'hémoglobine bovine en milieu acide, inhibition de l'activité enzymatique à pH basique, déminéralisation et décoloration du produit final) sans ajout d'agents chimiques, sans colmatage sur les membranes, tout en aboutissant à un hydrolysat final à faible concentration en sels minéraux et ayant des activités antibactériennes, antifongiques et antioxydantes.

Le système complet d'EDMB à trois compartiments, a été utilisé d'une manière innovante. Un ajustement cible et bien étudié de la valeur de la conductivité de départ était suffisant pour le bon déroulement du procédé puisque la conductivité de la solution initiale d'hémoglobine était trop faible pour pouvoir être traitée dans un système d'électrodialyse. Le sel ajouté au départ est ainsi réutilisé au complet dans le système pour la production d'acide et de base nécessaires. De l'étape « 3-en-1 », il est ressorti trois produits d'intérêt au lieu d'un seul dans le cas de l'hydrolyse conventionnelle (contrôle) : les hydrolysats d'hémoglobine avec pepsine active (compartiment d'acidification), les hydrolysats d'hémoglobine avec pepsine inactive (compartiment de basification), et les hydrolysats d'hémoglobine avec pepsine inactive déminéralisés (compartiment de diluât). Ces étapes préliminaires étaient nécessaires pour la préparation des solutions qui servent à alimenter les différents compartiments avant que le système puisse fonctionner à pleine capacité avec les 3 compartiments simultanément (3-en-1) et pour la production des acides et des bases très purs *in situ*, réutilisables pour le nettoyage du système (acide et base électro-générés) ou pour la décoloration de la solution d'hémoglobine (acide électro-généré). La génération *in situ* et la réutilisation des composés dangereux pour l'environnement (acides et bases) suppriment les inconvénients et les risques liés au transport, au stockage, à l'utilisation et à l'élimination de ces matières.

La limite principale de l'EDMB appliquée à la production de peptides bioactifs à partir de l'hémoglobine bovine reste le coût relativement élevé de l'équipement, ainsi que l'achat et le renouvellement des membranes bipolaires et membranes monovalentes cationiques

permsélectives. Cependant, l'absence du colmatage obtenu en fin du projet suite à l'optimisation des conditions permettra d'augmenter la durée d'utilisation ainsi que les performances de ces membranes. De plus, les avancées des recherches consacrées aux membranes ont eu pour effet d'augmenter la durée de leur fonctionnement.

En ce qui concerne l'étape de déminéralisation des hydrolysats, qui constitue une étape importante de purification des peptides, elle représente une amélioration majeure par rapport au procédé conventionnel, puisqu'elle permet d'éliminer jusqu'à 85% des minéraux introduits lors des étapes précédentes. L'avantage de la configuration utilisée dans ce présent travail est que les flux ioniques sont contrôlés par le positionnement des membranes bipolaires et mono-polaires. Ainsi, les minéraux qui sont retirés lors de la déminéralisation jouent un rôle important dans les compartiments adjacents d'acidification et de basification, puisqu'ils permettent le transfert du courant, et augmentent la conductivité des solutions garantissant ainsi une bonne performance du système. Cependant, la diminution du pH expliquée par une désalcalinisation partielle de la solution dans le compartiment de diluat a provoqué la précipitation de l'hème ou les associations peptides-hème sur la membrane anionique. Ce phénomène constitue une limitation de l'EDMB puisque certains peptides ont été perdus. En effet, une autre étape supplémentaire a été proposée pour éviter ce problème de colmatage.

Au niveau technologique, il n'y a plus de limites spécifiques puisque le colmatage membranaire a été résolu en effectuant une étape supplémentaire de décoloration en utilisant l'acide électro-généré et sans ajout de produits chimiques. De plus, la décoloration, qui consiste à séparer l'hème (partie non protéique) de la globine (partie protéique) de l'hémoglobine à l'extérieure du système d'EDMB est importante pour augmenter la valeur commerciale potentielle du produit final. En effet, la présence d'hème, responsable de la couleur rouge de l'hémoglobine et contenant du fer, rendait difficile la valorisation des produits dans certaines applications, dues à l'oxydation du fer. Le produit final décoloré-déminéralisé obtenu présente un très faible risque de détérioration suite à l'incorporation des peptides bioactifs de l'hémoglobine dans la formulation d'aliments destinés à la consommation humaine ou animale.

La mise en évidence des activités antibactériennes et antioxydantes, et pour la première fois de l'activité antifongique des peptides, a permis de confirmer que des peptides bioactifs déminéralisés et décolorés-déminéralisés peuvent être produits par l'EDMB tout comme en hydrolyse conventionnelle. La réduction en sel des hydrolysats n'a pas influencé les capacités biologiques. Les activités antimicrobiennes et antioxydantes sont spécifiques aux peptides dépendamment du mode de production. Par conséquent, ils pourraient être des candidats prometteurs pour améliorer la sécurité alimentaire et éviter le gaspillage des aliments.

Au vu de l'ensemble des observations faites au cours de ce travail, le système d'EDMB peut donc être considéré comme un procédé alternatif, innovateur et vert pour produire un mélange de peptides bioactifs purs. Au sein d'une seule et même unité, les différentes étapes de dénaturation, contrôle de l'hydrolyse enzymatique, inactivation de la pepsine, déminéralisation et décoloration du produit final, ont eu lieu en simultanées et sans ajout de produits chimiques grâce à la génération électrique contrôlée des espèces ioniques désirées.

Conclusion

Les résultats obtenus dans la présente thèse ont montré qu'il était possible de développer une nouvelle méthode innovatrice pour produire des hydrolysats d'hémoglobine bovine avec une faible concentration en sels minéraux et ayant des activités biologiques remarquables suite à l'hydrolyse enzymatique de la protéine par la pepsine en utilisant l'électrodialyse avec membranes bipolaires (EDMB).

Tout au long de cette étude, de nouvelles connaissances ont été acquises sur les impacts des différents paramètres électro-dialytique comme l'empilement des membranes, le type de membrane employée, la nature et la cause du colmatage ainsi que l'impact de la conductivité et du pH sur les paramètres d'EDMB et d'hydrolyse. Ainsi, dans un premier temps, il a été démontré que l'électro-acidification et l'hydrolyse enzymatique étaient faisables en utilisant l'EDMB. Le choix de la conductivité de départ, du pH de milieu réactionnel et le type des membranes mono-polaires ont été des facteurs déterminants pour l'optimisation du procédé. Aussi, deux configurations ont été appliquées pour déterminer la meilleure d'entre elles : la configuration d'ED nommée « EDBM-AEM », formée par un empilement de 3 membranes anioniques et de 2 membranes bipolaires et la configuration d'ED nommée « EDBM-MCP », formée par un empilement d'une membrane anionique, 2 membranes monovalentes cationiques permsélectives et de 2 membranes bipolaires. L'EDBM-MCP a permis la production d'hydrolysats contenant une faible concentration en sels minéraux mais avec la présence d'un colmatage sur la MCP, alors que l'EDBM-AEM a permis la production d'hydrolysats sans colmatage mais avec une concentration en sel similaire à celle de l'hydrolyse conventionnelle (contrôle). Le phénomène de colmatage des peptides ou des associations peptides-hèmes sur les membranes cationiques a été un obstacle important à surmonter. D'un point de vue mécanisme enzymatique, l'hydrolyse en EDMB a montré un mécanisme, nommé « Zipper », identique à celui obtenu en contrôle. Ce mécanisme a permis d'obtenir une large population peptidique qui était similaire au contrôle et cela sans utilisation de produits chimiques. Suite à l'identification des séquences peptidiques par spectrométrie de masse, il a été démontré que l'EDMB produisait des peptides bioactifs, dont la

néokyotorphine (α137-141), qui ont montré plusieurs activités biologiques à la même concentration que l'hydrolyse contrôle. En se basant sur les résultats obtenus ci-dessus, une nouvelle configuration d'EDMB a donc été mise en œuvre pour former un système intégré à 3 compartiments pour la production d'un hydrolysat d'hémoglobine bovine déminéralisé. Plusieurs opérations en simultanées ont été réalisées dans le système d'EDMB : la dénaturation de la solution d'hémoglobine bovine par électro-acidification, l'hydrolyse enzymatique par la pepsine, l'inhibition de la réaction enzymatique par électro-basification et la déminéralisation des hydrolysats. Le taux de minéraux a pu être réduit de 85% grâce à la déminéralisation de la solution dans le compartiment central situé entre les compartiments acides et basiques. Par contre, le colmatage n'a pas été éliminé dans le compartiment du diluât à cause de la précipitation de l'hème sur la membrane anionique. La solution proposée pour éliminer cette précipitation a été d'intégrer une nouvelle étape supplémentaire de décoloration avant la déminéralisation pour séparer l'hème de la globine. La décoloration a été effectuée en utilisant l'acide électro-généré sans ajouts d'acides chimiques. Par conséquent, des hydrolysats d'hémoglobine bovine décolorés et déminéralisés à 85% ont été récupérés et cela sans aucun colmatage sur les membranes.

Notre projet présente donc une contribution originale aux niveaux des avancées scientifiques :

- La mise en place d'un procédé global d'EDMB à multiple étapes « 4-en-1 » permettant la dénaturation de l'hémoglobine bovine, l'hydrolyse enzymatique, l'inactivation de la réaction enzymatique, la décoloration et déminéralisation des hydrolysats finaux en simultanés.

- Le colmatage membranaire qui constituait une limitation critique de l'application du procédé, a été supprimé par la décoloration suite au procédé « 4-en-1 ». Cela a eu un impact positif majeur sur les performances du système et la durée de vie des membranes.
- La production de peptides bioactifs décolorés et déminéralisés ayant des activités antibactériennes, antifongiques et antioxydantes importantes représente un intérêt

particulier puisque ces peptides peuvent se substituer à des composés synthétiques utilisés aujourd'hui dans des denrées de consommation courante et dont les effets peuvent être négatifs sur le long terme.

- L'activité antifongique des hydrolysats d'hémoglobine bovine a été démontrée pour la première fois dans ce projet. Cette caractéristique biologique présentera un intérêt particulier pour la conservation des aliments tels que la viande, les poissons, le poulet… dans le cadre de la sécurité alimentaire.

- La génération d'acide et de base *in situ* peut être utilisée pour le nettoyage du système ou pour d'autres étapes spécifiques à chaque substrat comme la décoloration.

- La décoloration évite les changements organoleptiques suite à l'incorporation de peptides bioactifs de l'hémoglobine dans la formulation d'aliments destinés à la consommation humaine ou animale dus à l'oxydation du fer. Ce qui augmente la valeur commerciale potentielle du produit final.

- La quantité de sel ajoutée à l'hydrolysat initial au départ était suffisante et a été réutilisée dans les autres compartiments au cours des différentes étapes du procédé. De plus, le transfert d'un électron au niveau des électrodes abouti à la réalisation de trois opérations simultanément, permettant une utilisation efficace du courant électrique.

- L'électricité est la seule énergie nécessaire au fonctionnement du système d'EDMB, considéré comme une source d'énergie propre. De plus, aucun effluent n'est généré puisque tous les flux dans les 3 compartiments sont utilisés ou réutilisés pour produire les peptides déminéralisés.

- La nouvelle application verte d'EDMB et la production d'une population peptidique ayant des propriétés biologiques, à partir d'un déchet des abattoirs, s'inscrivent parfaitement dans le concept d'économie circulaire. En effet, l'hémoglobine du sang

bovin, une fois hydrolysée, permet la bio-production de peptides actifs pouvant ensuite être utilisés dans la viande ou les produits carnés pour augmenter leur conservation et leur innocuité.

Étant donné le nombre croissant d'études portant sur l'hémoglobine, protéine dérivée d'un coproduit des abattoirs, ses hydrolysats présentent un grand intérêt pour les professionnels de l'agro-alimentaire, de la santé et de la biopharmaceutique dans les pays qui sont les principaux producteurs de viande (États-Unis, Brésil, etc.). La présente thèse a permis d'initier la faisabilité et l'optimisation du système d'EDMB, appliqué pour la première fois, à la production de peptides bioactifs à partir d'hémoglobine bovine. Les résultats obtenus sont extrêmement encourageants quant à la faisabilité de la technologie pour appliquer les différentes opérations. Dans une optique de production continue et intégrée, ce projet ouvre de nombreuses perspectives, tant pour la recherche que d'un point de vue industriel. Plusieurs axes de réflexion peuvent alors être engagés :

- Une étude *in vivo* est nécessaire afin de confirmer les effets observés *in vitro*. Il serait intéressant de tester dans cette étude *in vivo* l'effet des hydrolysats décolorés-déminéralisés récupérés sur l'oxydation des lipides, l'inhibition de la croissance des bactéries, des moisissures et des levures sur une matrice de viande pendant le stockage.

- À la lumière de ces travaux, et étant donné l'intérêt potentiel de ce procédé au niveau industriel, il serait intéressant d'effectuer une étude économique des coûts.

- Une analyse comparative du cycle de vie (ACV) sera importante à réaliser pour évaluer l'impact environnemental réel associé à la production de peptides bioactifs et sa contribution aux catégories d'impact : changement climatique, santé humaine, qualité des écosystèmes et consommation des ressources, selon deux scénarios différents : (1) procédé d'EDBM (scénario I) et (2) méthode conventionnelle (scénario II).

- Le procédé a été développé à l'échelle laboratoire. Avant son application en industrie, il sera nécessaire de réaliser des tests à plus grande échelle dans les conditions les plus proches possibles de celles que l'on retrouvera en industrie. Par conséquent, la montée en échelle devrait être envisagée, en premier lieu vérifier la faisabilité du procédé d'EDMB à « multiples-étapes » pour une concentration d'hémoglobine de 2% (p/v), puis l'augmenter jusqu'à 10% (p/v) et peut être encore plus. Par la suite, le système peut être généralisé sur le cruor bovin.

- Le nouveau procédé innovateur d'EDMB à « multiples-étapes » peut être appliqué sur d'autres coproduit d'abattage animal, comme sur le sang dérivé du porc, du poulet, de la dinde, de la brebis, de gibier sauvage ou même sur le sang humain.

- D'autres bio-activités peuvent être testées comme des activités : anti-cancéreuse, anti-inflammatoire, antidiabétique, hypocholestérolémie, anti-ostéoporose, antirides, anti-virales, émulsifiante, etc.

- Ce nouveau procédé peut aussi être appliqué sur une autre source de protéine abondante pour permettre une plus grande production de produits à haute valeur ajoutée. Il pourrait être envisagé, pour la filière végétale, de reprendre la stratégie globale de production de peptides bioactifs sans ajouts de produits chimiques et d'appliquer ces derniers sur des matrices alimentaires ou végétales.

- La pepsine porcine P6887 de Sigma est très efficace mais elle coûte très cher. Sa récupération avant son inactivation suite à un cycle d'hydrolyse et sa réutilisation représentent un intérêt majeur pour les professionnels du domaine. Suite à la mise en place de notre procédé d'EDMB réussi, des recherches et des stratégies plus avancées peuvent avoir lieu pour répondre à cette problématique.

Suivant ces différentes perspectives, nous constatons que notre projet ouvre encore plusieurs volets pour le futur. La science et la recherche ne s'arrêteront jamais. Ce projet met en valeur l'innovation et la valorisation d'un produit à haute valeur ajoutée dans le but d'améliorer

notre écosystème. Nous avons ainsi pu participer à l'avancement des connaissances nécessaire pour le développement et la préservation des ressources et de l'environnement.

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