Development of Biocatalytic Nanofibrous Membranes Using Different Modification Approaches for Continuous Proteolytic Reactors

by

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Abstract

Biocatalytic membranes (BMs) have promising applications in a diversity of fields including food, pharmaceutical and water treatment industries. Of particular relevance, Alcalase is a commercially important protease that has been applied for the production of peptides from the hydrolysis of proteins. In this study, two different approaches were applied for the modification of electrospun polyacrylonitrile nanofibrous membranes (EPNMs) for Alcalase immobilization. The first approach is alkali modification of EPNMs followed by EDC/NHS coupling for covalent bonding with Alcalase, whereas the other is based on polydopamine coating with or without glutaraldehyde grafting as a covalent linker. Immobilized Alcalase on these prepared BMs were studied and compared with free enzymes. It was found that the stabilities of Alcalase on BMs created using both approaches were improved, which enabled their reuse of 10 cycles with significant retention of enzymatic activity. A continuous reactor housing BMs were tested for hydrolysis of both model substrate, azo-casein and soybean meal protein (SMP). It was found that decreasing flux could improve the extent of hydrolysis and that a single-layer reactor can hydrolyze about 50% of the substrate to peptides with the molecular weight of 10 kDa or less. Hydrolysis of SMPs was demonstrated in a continuous five-layer BM reactor and both BMs showed excellent hydrolysis capacity. This study provides the groundwork for the development of high-efficiency BM for continuous and cost-effective protein hydrolysis for the production of value-added peptides.

Résumé

Les membranes biocatalytiques (MB) font l’objet d’applications prometteuses dans une diversité de domaines, notamment dans les industries alimentaires, pharmaceutiques et de traitement de l'eau. Tout particulièrement intéressante, l'Alcalase est une protéase commercialement importante qui a été appliquée pour la production de peptides à partir de l'hydrolyse des protéines. Dans cette étude, deux approches différentes de modification des membranes nanofibreuses de polyacrylonitrile électrofilées (EPNM) ont été étudiées pour l'immobilisation de l'Alcalase. La première approche consiste en une modification alcaline des EPNM suivie d'un couplage EDC / NHS pour une liaison covalente avec l’Alcalase. La seconde approche est basée sur un revêtement de polydopamine, avec ou sans greffe de glutaraldéhyde comme liant covalent. L'Alcalase immobilisée sur ces MB préparées a été étudiée et comparée avec des enzymes libres. Il a été constaté que les stabilités d'Alcalase sur les deux MB ont été améliorées après immobilisation et que les deux MB pouvaient être réutilisées. En outre, les deux MB ont été appliquées dans un réacteur continu pour l'hydrolyse des protéines. L'azo-caséine a été utilisée comme substrat modèle pour étudier les effets du flux et du nombre de couches de membranes sur l'hydrolyse des protéines. Il a été constaté que la diminution du flux pourrait améliorer l'étendue de l'hydrolyse et qu'un réacteur monocouche peut hydrolyser environ 50% du substrat en peptides ayant un poids moléculaire de 10 kDa ou moins. Des protéines de farine de soja ont été hydrolysées en tant que substrat dans un réacteur de MB à cinq couches et les deux MB ont montré une excellente capacité d'hydrolyse. Cette étude fournit une base de travail pour le développement de MB à haute efficacité pour l'hydrolyse continue et rentable des protéines, permettant une production de peptides à valeur ajoutée.

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Nomenclature

PAN	Polyacrylonitrile
BM	Biocatalytic membrane
EPNM	Electrospun PAN nanofibrous membrane
1-PANM	Alkali treated PAN nanofibrous membrane
2-PANM	Activated PAN nanofibrous membrane
PAN-BM	Alcalase-functionalized PAN electrospun biocatalytic membrane
PDA	Polydopamine
PM	PDA-coated PAN nanofibrous membrane
GA	Glutaraldehyde
GPM	GA-grafted PM
APM	Alcalase-immobilized PM
AGPM	Alcalase-bearing GPM
SMP	Soybean meal protein
EDC	1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride

NHS	*N*-Hydroxy succinimide
BCA	Bicinchoninic acid
BPA	Bradford protein assay
BSA	Bovine serum albumin
PBS	Phosphate buffer saline
SEM	Scanning electron microscopy
FT-IR	Fourier Transform Infra-Red spectroscopy

Statement of Contributions of Collaborators and Co-Authors

Chapter 3 – Immobilization of Alcalase onto Alkali Modified Polyacrylonitrile Nanofibers for Protein Hydrolysis

Aotian Li was thoroughly responsible for the literature review, all experiments, technical calculation, setup of equipment, the first draft of the manuscript and its revision during the review process. Professor Christopher Q. Lan provided supervision and helped with concept development, structuring and revision of the manuscript as well as the funding for this study. Dr. Trent C. Yang was responsible for the concept development and provided supervision and experimental facilities and chemicals. Fang Huang and Julian T. Dafoe provided technical supports and training related to this study. Professor Takeshi Matsuura and Dipak Rana helped with the revision of the manuscript and the training for the electrospinning facility.

Chapter 4 – Biocatalytic Nanofibrous Membrane Based on Polydopamine Coating for Continuous Protein Hydrolysis

Literature review, technical calculation, setup of equipment, all experiments were thoroughly performed by Aotian Li. He was also responsible for the first manuscript and its revision during the review process. Professor Christopher Q. Lan provided supervision and helped with structuring and revision of the manuscript as well as the funding for this study. Dr. Trent C. Yang provided supervision and experimental facilities. Fang Huang provided technical supports for this study. Erin Nguyen helped with some experiments. Professor Takeshi Matsuura and Dipak Rana helped with the revision of the manuscript and the training for the electrospinning facility.

Chapter 1:

Introduction

1.1. Introduction

Protein is one of the essential nutrition for humans and is very important for the growth of children. According to a study in 2012, approximately 8% of Canadians (7.14% and 8.34% of children and adults, respectively), are suffering from food allergy [1]. 52% of these children are allergic to the proteins from common dietary sources such as soy, eggs and milk. A solution to reduce the allergenicity is to hydrolyze proteins to small peptides or amino acids [2]. However, the hydrolyzed products are usually expensive in the market due to the use of enzymes, reducing their affordability to low-income families [3].

Although enzymes are effective and specific biocatalysts, some drawbacks of free enzymes including instability and difficulties in reuse, restricts their application and lead to their increasing cost [4]-[6]. Moreover, the separation of products from substrates and enzymes needs extra cost, which can increase the price of the products. Therefore, it is necessary to reduce the cost in the industry and the price of the hydrolyzed products for the health of people. In recent years, biocatalytic membranes (BMs) bearing immobilized enzymes have attracted tremendous interests due to the integration of the biological catalytic activity and membrane separation that offers numerous advantages including (1) high enzymatic activity and reaction specificity; (2) improvement of enzyme stability and reusability; and (3) real-time physical separation of reactants and products, which favours the completion of reaction [7][8].

Membrane matrix and enzyme are the two most important constituents of BMs. One of the choices of membrane matrix is electrospun nanofibrous membranes (ENMs), which have been widely used in adsorption, filtration and composite materials with good stability, mechanical and physical properties (*i.e.* high surface-to-volume ratio and porosity) [9].

Polyacrylonitrile (PAN) is one of the most important polymers used in electrospinning. Some studies have been reported for the successful enzyme immobilization on ENMs of PAN or co-polymers with PAN [10][11]. However, some modification methods involved the use of dangerous chemicals like dry HCl gas, which demands special facilities due to the risks associated with the use and storage of high-pressure HCl. Furthermore, most immobilized enzymes are applied in sugar and oil industries but rarely for protein hydrolysis up to now [12].

Enzyme immobilization was found to be a promising solution to improve the operational and storage stability of the enzyme and enable enzymes to be reused and recovered from solutions [13][14]. Proteases, such as Alcalase (which is a family of proteolytic enzymes), may be one of the enzymes that could benefit the most from immobilization, which may help reduce their autoproteolytic activity (auto-digestion) and sensitivity to pH values and temperature [15][16]. Alcalase has been found to produce desired bioactive peptides with antioxidant and emulsifying properties from various sources (e.g., meat emulsions) [17]. Further investigations on looking for a suitable modification of ENMs and biocatalytic nanofibrous membranes with immobilized Alcalase will provide the important groundwork for the manufacturing of high-value bioactive peptides with continuous biocatalytic membrane reactors.

In this thesis, Alcalase was selected to be the model enzyme and immobilized onto electrospun PAN nanofibrous membranes. The objective of this study was threefold: 1) develop different modification approaches of electrospun PAN nanofibrous membranes for the preparation of BMs; 2) investigate the effect of enzyme immobilization on the activity and stability of Alcalase on BMs; 3) demonstrate the application of different BMs in a continuous proteolytic reactor for hydrolysis of both model substrate azo-casein and proteins extracted from commercial soybean milk.

Chapter 2 is a literature review on the preparation and application of BMs, providing background information about enzyme immobilization, enzyme activity tests and electrospinning including common modification methods for ENMs. Chapter 3 reports and discusses the results of the first research focusing on the immobilization of Alcalase directly onto alkali modified PAN ENMs for soybean meal proteins (SMPs) hydrolysis. Chapter 4 is another research article investigating BMs preparation based on polydopamine coating for SMPs hydrolysis. Chapter 5 is a synthesis chapter, which summarizes the research results with an emphasis on comparing SMPs hydrolysis using continuous reactors made of the two different BMs. Recommendations for further investigations are also presented in this chapter.

1.2. References

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Chapter 2:

Literature Review – Preparation and Application of Biocatalytic Membranes

2.1. Introduction

Enzymes, as biocatalysts, have been powerful catalysts for industrial applications, replacing more and more traditional chemical catalysts for their high catalytic efficiency, mild reaction conditions and stereoselectivity in recent decades [1][2]. However, some drawbacks limit the application of enzymes in some industrial areas. Dissociative enzymes (alias: free enzymes) denature easily due to the change of their conformation during the production process under a harsh environment. They also inactivate even in optimum conditions because of their low stability. Furthermore, the addition of dissociative enzymes makes the separation or purification of products more complex, which makes the reuse of dissociative enzymes difficult, leading to the increase of costs in application [3]-[5]. Immobilized enzymes are those enzymes “anchored” onto/into carriers providing enzymes with some specific areas where enzymes can catalyze reactions. The process to immobilize enzyme is enzyme immobilization. Enzymes can be separated and reused after enzyme immobilization because of the properties of carriers. Moreover, the operational and storage stabilities of immobilized enzymes may improve for the protective effects of the carriers [6][7]. With enzyme immobilization, it is possible to combine the use of different enzymes or the use of enzymes and carriers, such as membrane for separation.

Biocatalytic membranes (BMs) combine the physical separation of reactants/products and the bio-catalysis reaction by “anchoring” enzymes in/on membranes [8]. Reactants binding to BMs can react with those immobilized enzymes, while some products can go through the pores of BMs selectively according to their differential sizes and/or other properties and leave the proximity of the enzyme involved, promoting the release of products. The mechanism of the bio-catalysis reaction is still dependent on the enzyme.

Integrating membrane separation and enzyme immobilization, BMs offer tremendous advantages including (1) selective catalysis reaction and extraction of products, in which reactants are selected to react with enzymes, while some products are allowed to leave the proximity of reaction site to change the reaction equilibrium favourably [9]; (2) relatively high activity since the matrix of BMs are usually with high porosity and surface-to-volume ratios, which help the transfer of reactants contact with enzymes and improve the selectivity and catalytic efficiency; (3) improvement of enzyme stability: immobilized enzymes usually exhibit better stability at high temperature, large pH range and longer storage time than free enzymes; (4) immobilized enzyme on BMs can be reused for reducing costs and suitable for the use of continuous reactors (or flow reactors).

2.2. Preparation of BMs

Enzyme immobilization is one of the most important steps for the preparation of BMs. There are mainly four ways to immobilize enzymes into/onto the membranes, *i.e.*, encapsulation, physical adsorption, covalent bonding and cross-linking [10].

2.2.1. Encapsulation of enzymes

Encapsulation of enzymes, which is also called entrapment, refers to the method to immobilize enzyme inside membranes during the fabrication of matrix membranes (**Fig. 2.1**). Two different strategies are commonly used.

1. Matrix entrapment

Enzymes are dissolved in the polymer solution followed by membrane casting or spinning and are therefore entrapped in the membrane matrix or fibers. Most enzymes are water-soluble and denature in organic solvents, which limits the application of this strategy, making it quite restrictive the selection of polymers to fabricate membranes. Nonetheless, many polymers or co-polymers have been studied for the preparation of BMs given that this strategy is able to be highly effective for enzymes like lipases, whose substrates and optimum activities are usually in mixed organic solvents. For example, Sakai et al. [11][12] have studied the entrapment of lipase in polystyrene (PS) electrospun fibers. The lipase-PS fibers showed 47-fold faster of transesterification of (*S*)-glycidol than free lipase and 77% of initial activity retained after reused 10 times. Some researchers reported that emulsification has protective effects to encapsulate enzymes in those polymers involving the use of organic solvents. Sorbitan monopalmitate was used as an emulsifier for entrapment of glucose oxidase (GOx) in polylactic acid (PLA) fibers during electrospinning [13]. GOx-PLA fibers performed 19-fold activity compared with PLA films prepared by casting with the same solution and 50% retention activity. However, 37% of the initial GOx activity lost due to the leaching during catalysis and reuse. Even though emulsions help broaden the choice of polymers for this strategy, leaching still a major problem limiting its development of applications.

2. Fouling Induced Entrapment

Luo et al. [14] reported fouling-induced enzyme immobilization on ultrafiltration (UF) membranes with three different modes, i.e., the normal mode (skin layer facing feed, the reversed mode (support layer facing feed), and the sandwich mode (the normal mode with an additional support layer between the skin layer and membrane holder). Feed solutions containing the appropriate concentration of enzymes were filtered through selected UF membranes using one of three aforementioned three modes using the dead-end configuration to create a fouling layer on the membrane surface. Results showed that the Sandwich mode produced the BM with similar enzyme loading, slightly better reusability, but much higher fluxes in comparison to the sandwich mode. Whereas the normal mode retained very little enzymes, which was quickly lost in the usability tests. It was thus hypothesized that entrapment of enzymes in the macropores of the support layer played a key role in the fouling induced enzyme immobilization while adsorption and hydrophobic interactions may also play roles since these would be the predominant mechanisms for the immobilization on membrane surface in the normal mode. This approach is simple and versatile since it is not restricted by solvents used in membrane fabrication. Nevertheless, BMs thus prepared suffered from enzyme leakage, which was argued to be overcome by the selection of UF membranes with suitable support pore size.

2.2.2.Physical adsorption

Enzymes can be physically adsorbed on the surface of membranes under mild conditions and simple operation (**Fig. 2.1**). However, physical adsorption is generally too weak to keep enzymes fixed on the surface of membranes under harsh industrial conditions (e.g., high temperatures, the change of pH values, high concentration of reactants/products or high ionic strength), which can lead to the leaking of enzymes from the surface of membranes. Ginka et al. [15] immobilized β-xylosidase by immersing polyamide membranes in enzyme solution via physical adsorption, obtaining 30% maximum immobilization yield and 6.8% activity yield. This immobilization process changed the enzymatic optimum temperature and pH and the effect of enzyme immobilization may be improved by making membranes or enzymes with opposite charges with pH adjustment in order to immobilize enzyme with ionic bonding. GOx was immobilized on poly (methyl methacrylate) (PMMA) fibers with embedded multi-wall carbon nanotubes (MWCNTs) by physical adsorption[16]. This prepared GOx-PMMA/MWCNT fibrous membrane was reported for the application in glucose sensing. Chen et al. [17] observed a high enzyme loading (332 mg/g-fiber) and 48% retention activity by physically adsorbing lipases on PMMA membranes. Pepsin-nylon membranes were successfully prepared by physical adsorption and studied for rapid digestion and purification [18].

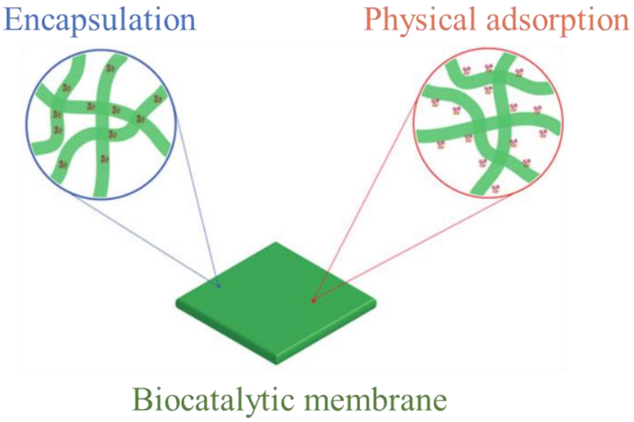

Fig. 2.1. Preparation of BMs by encapsulation and physical adsorption [10]

2.2.3.Covalent bonding

Compared with physical adsorption, covalent bonding can immobilize enzymes firmly onto/into membranes. This method takes advantage of the diverse functional groups (e.g., amino, carboxyl, thiol and hydroxyl groups) of enzymes. Covalent bonds can be formed between those functional groups of enzymes and pendent functional groups of membranes with or without modification using cross-linker reagents (**Fig. 2.2**), such as glutaraldehyde (GA), *N*-hydroxy succinimide

(NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), maleic anhydride, ethylenediamine, p-benzoquinone and tannin. Namely, the pendent functional groups can be from membranes themselves (e.g., polymers) or/and from the membranes after modification/functionalization.

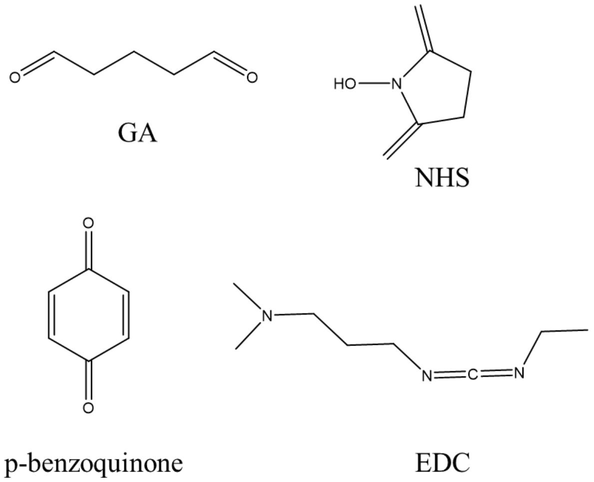

Fig. 2.2. Structures of cross-linker reagents commonly used for enzyme immobilization

As shown in **Fig 2.3**, Caze et al. [19] prepared a gel layer on the ceramic ultrafiltration membrane and covalently immobilized laccase on the gel layer of the membrane by glutaraldehyde grafting. Xu et al. [20] modified electrospun microfibrous membranes with amino groups for horseradish peroxidase immobilization via glutaraldehyde covalent bonding, which performed an outstanding capacity of biodegradation and bisphenol A removal. However, the strong covalent bonding between enzymes and membranes may restrict the conformation of the enzyme, reduce exposure of active sites of enzyme accessing to substrate molecules and cause additional steric hindrance and inhibition of the release of products, which may lead to the decrease of enzyme activity [9]. Yang et al. [21] immobilized alkaline phosphatase using DNA as covalent bonding on magnetic nanoparticles, which showed that immobilized enzyme with the

DNA length of 24 bases showed the best activity comparing with those with the length of 12, 33 and 42 bases. This study suggested that the length of covalent bonding may have a significant influence on immobilized enzyme activity and controllable length of the covalent bonding may help enhance enzyme stability and reduce additional conformation restriction at the same time.

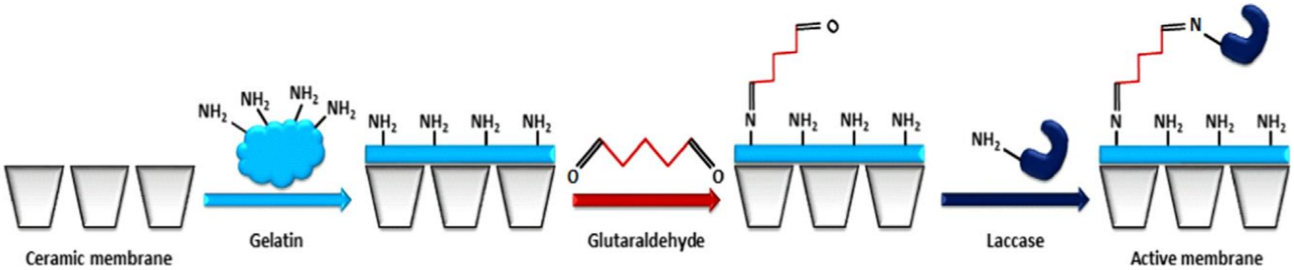

Fig. 2.3. Scheme of enzyme immobilization on ceramic membranes [19]

2.2.4.Cross-linking

This method can be regarded as an extension of the covalent bonding method. Enzymes can be immobilized using cross-linker reagents, which have more than two functional groups for bonding enzymes or/and bonding enzymes and membranes. Namely, cross-linking involves the cross-linking between enzymes and membranes and the cross-linking of enzyme aggregated/crystals. However, the direct contact of cross-linker reagents with enzymes, may cause the denaturing of the enzyme during the immobilization process. The cross-linking of enzyme aggregated/crystals was usually a method to prepare carrierless macroparticles [22]. Recently, this method has been developed to prepare BMs or even pure enzyme membranes. There are two main strategies to prepare biocatalytic fibrous membranes via cross-linking.

(1) Cross-linked enzyme aggregates coating (CLEA-coating): cross-linkers are used to form cross-linked enzyme aggregates (CLEAs) and these CLEAs are coated on the surface for

fibers with adsorption or covalent bonding (**Fig. 2.4**). These CLEA-coating layers were reported to be an important role in the improvement of thermal and environmental stability of enzymes and the prevention from enzyme leaching [23]. Kim et al. [24] demonstrated a study of trypsin immobilization on electrospun nanofibers with CLEA-coating, which performed good stability in rigorous conditions and good reusability of protein digestion for one week.

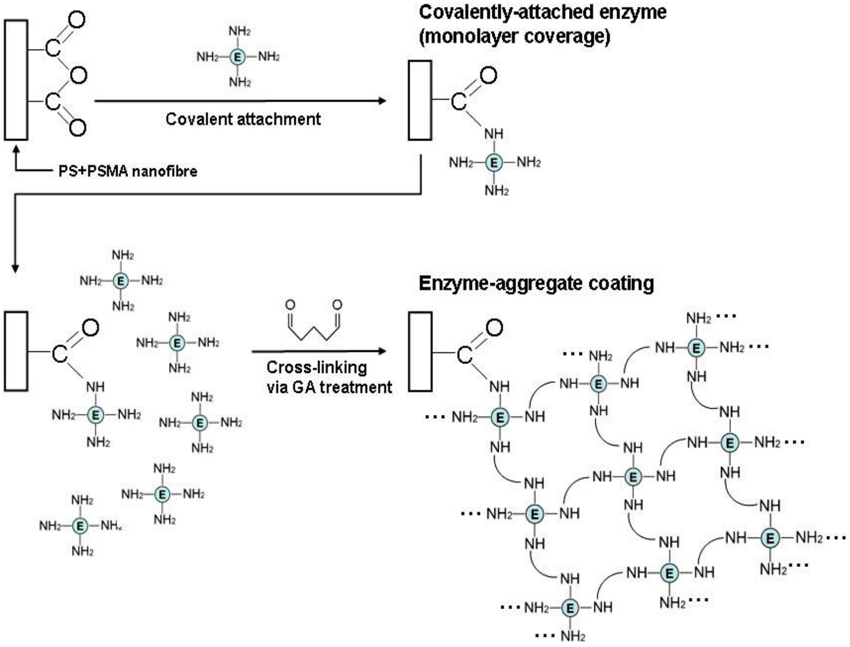

Fig. 2.4. Scheme of preparation of CLEA-coating biocatalytic nanofibrous membranes [23]

(2) Core-shell fibers/pure enzyme fibers: instead of cross-linking on the surface of the fibers, the solution of CLEAs might be even electrospun directly with cross-linkers to be fibrous membranes. In this case, a modified spinneret might be required for concentrically electrospinning the inner enzyme solution and the outer polymer solution at the same time (**Fig. 2.5)**. CLEAs are formed in the core of the fibers while the outer polymer solution helps the fabrication of fibers. The outer polymer shell is then washed away,

leaving the pure enzyme fibers available (**Fig. 2.6**). Cellulase fibrous membranes were generated by concentric electrospinning with polyethylene oxide (PEO) as the outer shell and glutaraldehyde as the cross-linker [25]. 24% retention activity was observed after washing away the water-soluble PEO shell. The low activity is likely due to the restricted contact and diffusion of enzymes.

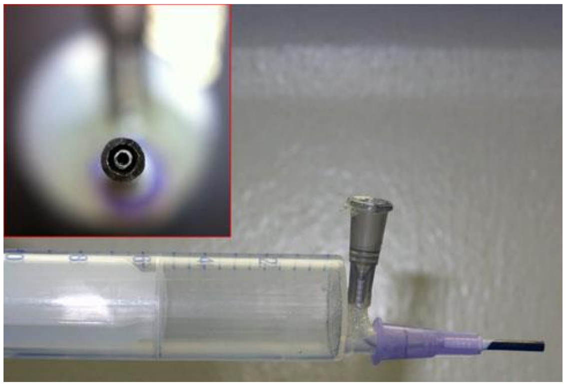

Fig. 2.5. A modified spinneret for concentrically electrospinning [10]

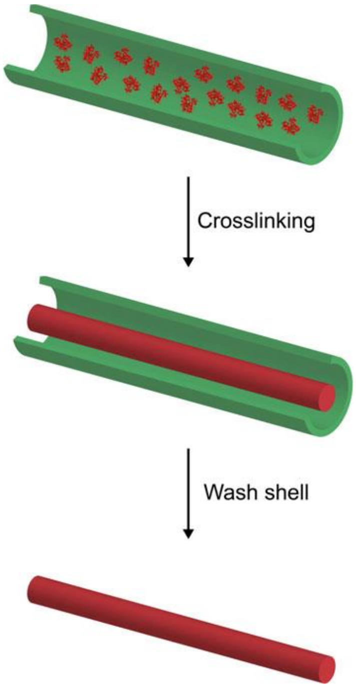

Fig. 2.6. Scheme of electrospun fiber with enzyme solution as the inner core solution [10]

2.3. Enzyme activity tests for BMs

2.3.1. Enzyme activity of BMs

Enzyme activity is usually used to indicate the catalytic efficiency of enzymes, which is quoted in unit per milliliter (U/mL), while specific enzyme activity is quoted as unites per milligram (U/mg). An enzyme unit (U) is involved in the definition of enzyme activity. Although a standard definition of one enzyme unit is given by the amount of enzyme that catalyzes the reaction of 1 μmol of substrate per minute or 1 nmol of substrate per minute due to the fact that 1 μmol of the substrate is sometimes a lot of materials in some cases, different definitions are used by different researchers. For example, Yang et al. used casein as the substrate for measuring enzyme activity of immobilized Alcalase, in which the activity was defined by the amount of Alcalase releasing 1 μg of tyrosine per minute with the measurement at 275 nm [26]. In contrast, Sheng et al. [27] measured the enzyme activity of immobilized Alcalase with casein as the substrate. The activity was defined by absorbance change per minute at 680 nm.

The use of different substrates also makes the definition of enzyme unit or enzyme activity, which may also change the value of Michaelis-Menten constant (K_m) due to different affinity of the enzyme to different substrates. Instead of using casein as substrate, du Plessis et al. carried out an azo-casein assay for enzyme activity test for immobilized Alcalase[28]. The activity was defined by the amount of liberated azo dyes per minute with a measurement at 340 nm. Žuža et al. [29] defined one unit of Alcalase activity by an increase in absorbance at 440nm of 1.0 in a 1-cm cuvette with the azo-casein assay. Moreover, substrates and/or definitions for the enzyme activity measurement are quite different from the use of different enzymes. Note that the different sources of the same enzyme may also make varied results. For example, Xu et al. [30]

tested laccase activity with the use of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate), while Zhang et al. [8] used dimethoxy phenol as the substrate for laccase activity test. Definitions and evaluation methods for the immobilization of a specific enzyme were quite different, making the comparison difficult.

2.3.2. Enzyme activity retention of BMs

Besides the different definitions of enzyme activity, enzyme activity retention (or retention activity) can be usually expressed by three different methods. All of them can be used to determine the success of enzyme immobilization [22].

(1) The immobilization yield can be ascribed to the percentage of total enzyme activity from the free enzyme which is immobilized (**Eq. 2.1**). Note that the immobilized activity can only be measured by subtracting the total residual enzyme activity which retains in free enzyme after immobilization from the total starting activity.

$$\text{Yield (\%)} = 100 \times (\text{immobilized activity} / \text{total starting activity}) \quad \textbf{(2.1)}$$

(2) The immobilization efficiency refers to the percentage of enzyme activity which can be observed from the immobilized enzyme (**Eq. 2.2**). Low efficiency is likely due to the deactivation or inaccessible to substrates for some reasons upon immobilization.

$$\text{Efficiency (\%)} = \text{observed activity} / \text{immobilized activity} \quad \textbf{(2.2)}$$

(3) Activity recovery is shown in **Eq. 2.3**, which determines the activity compared to that of total staring activity. The observed activity may be affected by many factors, such as the activity of the free enzyme, the assay of enzyme activity (*i.e.* substrate, substrate concentration and incubation condition) and the properties of the BMs (*i.e.* pore size, surface-to-volume ratio and hydrophobicity-hydrophilicity). In most cases, mass transfer diffusion of substrate and inhibition of the release of the product may cause varying retention activity.

$$\text{Activity recovery (\%)} = \text{observed activity} / \text{starting activity} \quad \textbf{(2.3)}$$

2.4. Applications of BMs

2.4.1. Application in the food industry

BMs have great prospect in the food industry for BMs can separate different substrates/products based on the size/molecular weight, extract products at the same time with reaction, which is especially suitable for the fermentation process for the production of drinks (*i.e.* juice, wine, milk), organic acids and antibiotics. In many cases, the product obtained by fermentation is present as a component of a complex solution where it needs to be separated and purified. For instance, the must which is pressed juice of grapes containing polyphenolic compounds for the organoleptic taste and color of wine (e.g. rosé wine), can be stabilized by the use of laccase-immobilized BMs due to the oxidization of polyphenols in solution [31]. During the maturation process, secondary fermentation of conversion of malic acid into lactic acid occurs. A BM reactor is used to control this conversion for the production of wines with good organoleptic properties.

2.4.2. Application in the pharmaceutical field

BMs have been commonly used to produce amino acids, antibiotics and anti-cancer medicines, but some reactions require the participation of coenzymes which limit the development of BMs for large scale production [32][33]. Recently, efficient and commercially viable production of bioactive peptides were studied quite a lot since bioactive peptides are with high value because of their special activity (*e.g.*, antioxidant) while BMRs are also suitable for these products and thus have a great prospect [34].

2.4.3. Application in environment protection

BMs have become widely applied in environmental protection in recent years, especially those BMs with immobilized laccase, protease and catalase. Lloret et al. [35] immobilized laccase with ultrafiltration membranes and obtained up to 90% removal of estrogenic from water. Caze et al. [19] modified ceramic membranes with gels followed by laccase immobilization, which proved the satisfied efficiency of antibiotic removal from water. Hou et al. [36] also applied BMs with immobilized laccase for bisphenol-A and obtained a good removal effect. Du Plessis et al. [28] prepared three different anti-biofilm BMs with two different proteases and amylase for bacterial removal and confirmed that there was no anti-biofilms formation observed on BMs.

2.5. Alcalase for production of peptides from agricultural wastes

Alcalase is a serine-type non-specific endoprotease from Bacillus licheniforms, which is a part of subtilisin-like proteases family with an Asp, Ser and His catalytic triad that catalyze the hydrolysis of peptide bonds for multiple types of proteins [37]. Models of Alcalase (**Fig. 2.7**) can indicate the catalytic triad at Asp32, His64 and Ser221, while a scheme of its protein hydrolysis mechanism is as shown in **Fig. 2.8**.

Alcalase has been applied for a wide diversity of different applications. These include the production of functional peptides from commercial proteins, agricultural wastes and dairy wastes such as egg white protein, soy protein isolates and cheese whey, for enhanced recovery or improved product olubility, stability, emulsification, assimilability, bioactivity (e.g., antioxidant), and reduced allergenicity to meet the increasing needs of proteins and peptides for different markets [26][27][38][39][40][41]. Gema et al. [38] found that potato proteins hydrolyzed by Alcalase acquired antioxidant and emulsifying properties. Li et al. [39] obtained protein hydrolysates with antioxidant activities and functional properties from Camellia oleifera seed cake. Sewczyk et al. [42] investigated the continuous hydrolysis of protein isolates and concluded that Alcalase-based biocatalytic membrane reactors allow the production of specific bioactive peptides from the hydrolysis of casein, sunflower and lupin protein isolates.

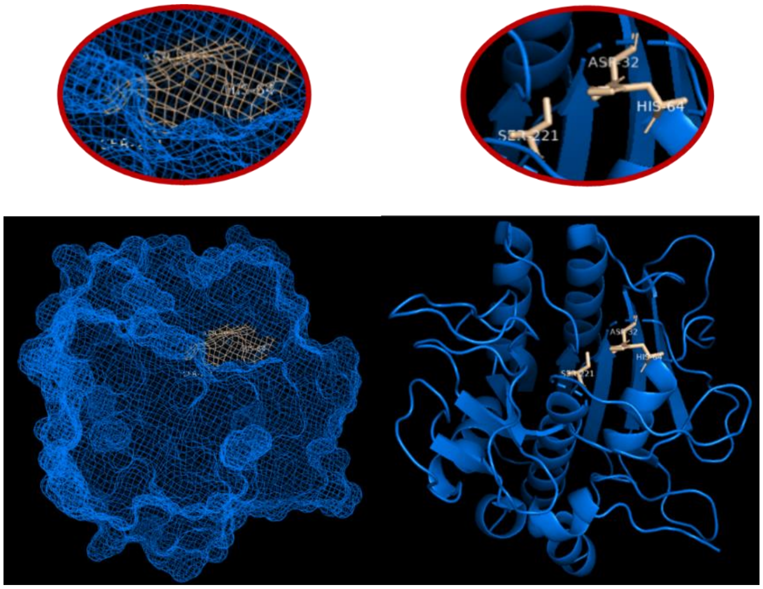

Fig. 2.7. Models of Alcalase structure (PDB: 1SCA)

Of particular relevance, soybeans have been an important crop for centuries that have been an important diet for both humans and animals as a relatively inexpensive source of proteins [43]. Consumption of soybean products has been growing explosively worldwide in recent decades. However, the low solubility limits its application in producing high-value products comparing with wheat proteins, which have been explored quite extensively commercially as nutrition supplements in various fields of sports for muscle/bodybuilding [44]-[46]. Several studies of bioactive peptides derived from soybeans have been investigated but large-scale production has been challenging due to the low water-solubility of SMPs, which are of high molecular weights [47][48]. It is therefore of great interest to explore the means to increase the efficiency of hydrolysis of this group of commercially important proteins.

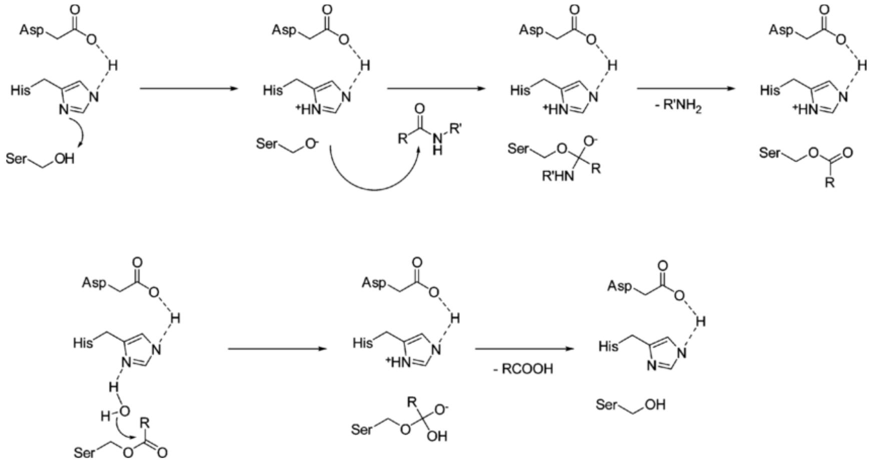

Fig. 2.8. Scheme of a peptide bond hydrolysis by the use of Alcalase [37]

2.6. BMs by electrospun nanofibrous membranes (ENMs)

2.6.1. Electrospinning

In the 1930s, electrospinning was reported as a method to prepare nanofibers by Formhals and electrospun nanofibers were developed from 2D to 3D by Simons who obtained a patent for the invention of non-woven fabrics using electrospinning technology [49][50]. Electrospinning is the process in which charged threads of polymer solutions or melts were drawn to form fibers with some hundred nanometers by the electric force under a high-voltage electric field generated between the polymer solution and a collector. Based on this principle, two common processes have been applied in electrospinning ENMs. A simple and effective process is needle-type electrospinning (**Fig. 2.9a**). This process allows the rapid formation of ENMs and the controllable orientation of nanofibers formation, such as aligned nanofibers [51].

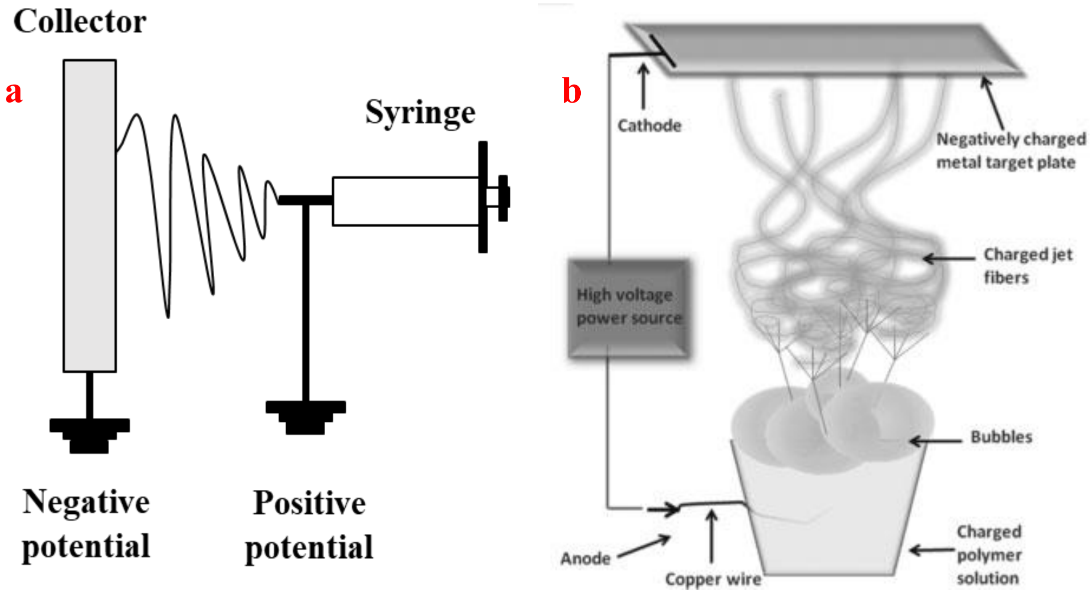

Fig. 2.9. Electrospinning of (a) Needle-type and (b) bubble-type

Another approach is bubble-type electrospinning, which involves multiple formations of electrostatically driven jets on the top collector from charged bubbles of polymer solution under the collector (**Fig. 2.9b**) [28]. This process requires a much higher voltage than that of needle-type electrospinning and may be difficult in forming aligned nanofibers. Nowadays, a variety of polymers has been used for the electrospinning process and **table 2.1** indicates some polymers commonly used for the preparation of ENMs.

Table 2.1. A list of polymers used for Electrospinning

Polymer	**Reference**
Polyethylene oxide	[52], [53]
Polyacrylonitrile	[52], [53]
Cellulose acetate	[54]
Polysulfone	[55]
Polyvinylidene fluoride	[52], [56]
Polyvinylchloride	[53]
Polycarbonate	[52], [53]
Polyhexylisocynate	[52]
Polybutadiene	[52]
Matrimid	[57]

2.6.2. Surface modification of ENMs for preparation of BMs

Some polymers lack available functional groups and make ENMs formed by these polymers show chemical inertness. On one hand, the chemical inertness enables ENMs in some applications which require some resistance to chemicals. On the other hand, this also limits further expanded application of ENMs. For instance, enzyme immobilization onto/into ENMs usually requires available functional groups on membranes to prepare BMs. In this case, surface modification is necessary for ENMs for further use. There are many modification methods based on the polymers of ENMs. According to the type of mechanism, most of them could be categorized into the two different groups of modification approaches, i.e., chemical modification and compositing, and the former is more frequently adopted than the latter.

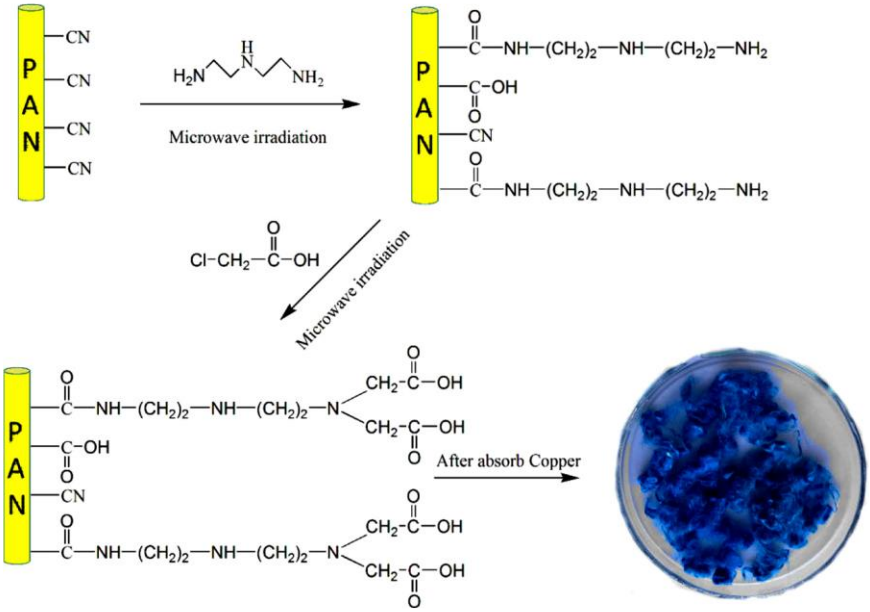

Fig. 2.10. Modification of PANENMs under microwave irradiation [58]

The chemical modification takes advantage of the functional groups of polymers themselves (*i.e.* nitrile, amino and hydroxyl groups) and enables them to react with other chemicals to change their surface properties. For example, Sheng et al. [58] modified the surface of PANENMs by two steps under microwave irradiation (**Fig. 2.10**) for adsorption of heavy metal ions from water, showing up to 250 mg/g and 100 mg/g capacity of Cu^{2+} and Hg^{2+} adsorption.

PANENMs were also modified by imidoesterification for enzyme immobilization (**Fig. 2.11**). Nitrile groups of PAN can react with ethanol with dry HCl gas bubbling in the solution so that amidination is allowed to react between the modified PANENMs and enzymes. However, this method requires the use of dry HCl gas as the catalyst, which means that high demand for facilities and purity of ethanol is necessary. This method may increase the cost and risk of manufacturing applications. Laccase was immobilized on PAN ENMs with this modification method and showed more than 85% of the trichlorophenol removal [30]. This method was also applied for protease immobilization onto PANENMs showing excellent anti-biofilm activity [28].

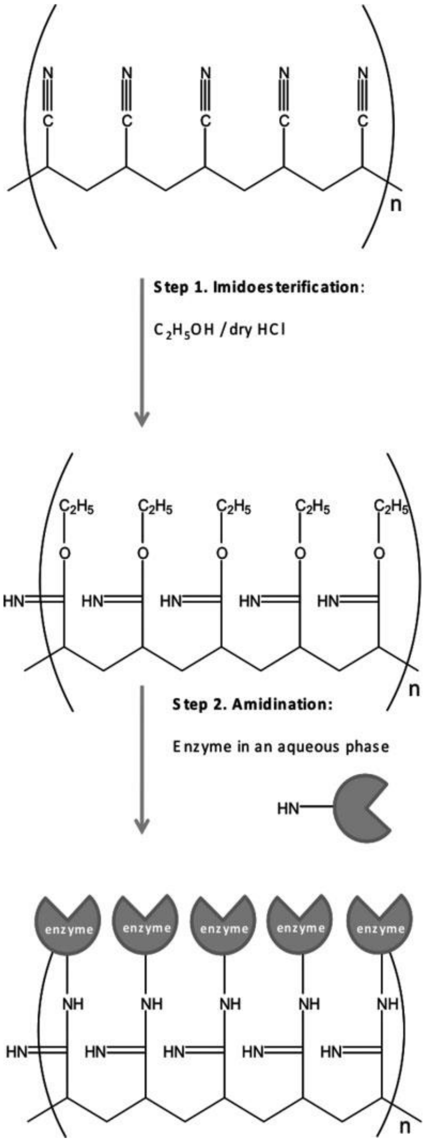

Fig. 2.11. Modification of PANENMs with imidoesterification [28]

Another approach, partial alkaline hydrolysis of PAN functional groups at polymer surface, has been applied in large scale production of commercially acceptable water-soluble additives and water absorbents [59]. In alkaline hydrolysis, some nitrile groups of PAN are converted to carboxyl groups by a series of steps with the participation of alkaline (**Fig. 2.12**). This method adopts a much milder condition than the aforementioned imidoesterification approach by avoiding the use of HCl. It enables converted carboxyl groups without the destruction of PANENMs structures for further use. Wei et al. [60] immobilized laccase on PANENMs with this method for bioremediation.

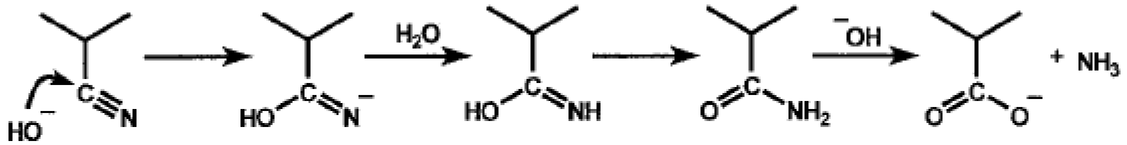

Fig. 2.12. Mechanism of alkali treatment of PAN [59]

Compositing is the method to modify/functionalize ENMs by the addition of other components in polymer solutions for electrospinning or after electrospinning to form a coating layer for further grafting. Yang et al. added metal-organic frameworks (MOFs-PVDF) in polyvinylidene solution and this modified membrane showed 99.99% rejection of NaCl rejection in membrane distillation [61]. Dopamine (DA) can self-polymerize under alkaline conditions and adsorb onto a wide range of materials to form a polydopamine (PDA) layer (**Fig. 2.13**). This coating layer allows further grafting due to the multiple functional groups and different tautomeric forms of PDA. Besides further grafting, the PDA coating layer also enables to improve properties of membranes (*i.e.* hydrophilicity, electrical and thermal conductivity and biocapacity). For example, Jiang et al. [62] immersed hydrophobic polyethylene membranes in DA solution for the formation of PDA coating layers, followed by grafting heparin with the o-benzoquinonyl groups of PDA. This hydrophobic membrane was transferred to be hydrophilic and be applied to plasma recalcification. Furthermore, PDA coating layers were also found to be of prevention from light and ultrasound waves and resistance of free radicals, which allow PDA coating layer to protect biological molecules from overheating and damage [63][64][65].

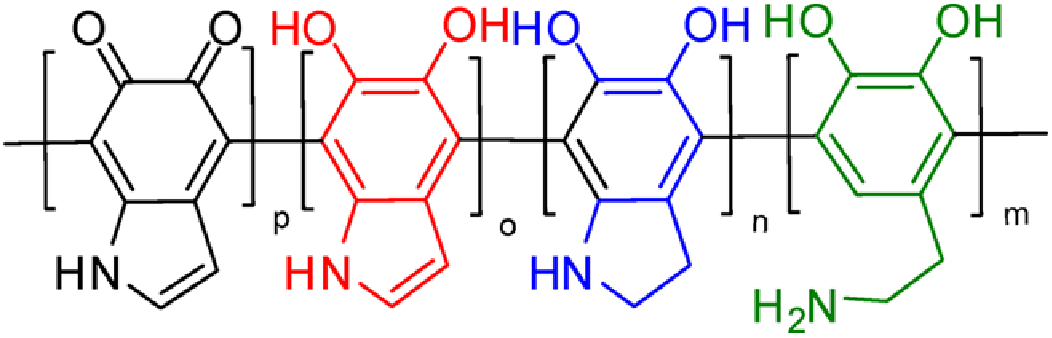

Fig. 2.13. Example of some tautomeric forms of PDA [66]

2.7. Challenges in BMs development for protein hydrolysis

While there are many approaches for the immobilization of enzymes on to membranes for the creation of BMs, these approaches are quite limited when it comes to protein hydrolysis. First of all, immobilization by physical adsorption suffers from significant enzyme leakage and is therefore not suitable for the targeted application of protein hydrolysis for peptides production, which requires a long-time continuous operation and demands high reusability of the BMs. Entrapment of enzymes in membrane matrices or the support layer both limits the accessibility of the active sites of enzymes from their substrates, i.e., proteins, which are of large molecular weights and therefore difficult to diffuse inside the pores. Covalent bonding and cross-linking are the most promising approaches for BMs development for protein hydrolysis, both have their own unique challenges.

Most commercial immobilized enzymes are currently developed for the conversion of sugar and oil engineering, but seldom protein [42]. Similarly, lipase immobilization has been studied for using different polymers as membrane matrix, but rarely Alcalase which has been proved to produce specific bioactive peptides with antioxidant and emulsifying properties [38]. Moreover, few electrospun nanofibrous membranes and their modification were studied for Alcalase immobilization. Further studies on biocatalytic nanofibrous membranes with immobilized Alcalase will be a foundation for the manufacturing scale production of high-value bioactive peptides. This thesis explored two different approaches for the modification of PANENMs for Alcalase immobilization, investigated the effects of pH, temperature and storage on the enzymatic activities and stabilities of the immobilized Alcalase in operation, and demonstrated the application of biocatalytic PANENMs for SMPs hydrolysis with a biocatalytic membrane continuous reactor.

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Chapter 3:

Immobilization of Alcalase onto Alkali Modified Polyacrylonitrile Nanofibers for Protein Hydrolysis

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Abstract

Proteases, such as Alcalase, are widely applied in the hydrolysis of proteins, which produces favourable effects including reduced allergenicity and enhanced water-solubility, stability, digestibility, and bioactivity (e.g., antioxidants). This study focuses on the immobilization of Alcalase onto polyacrylonitrile (PAN) nanofibers modified by 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide/*N*-hydroxy succinimide covalent bonding with Alcalase. Scanning electron microscopy and Fourier Transform Infra-Red spectroscopy were used to characterize the Alcalase-functionalized PAN biocatalytic nanofibrous membranes (PAN-BMs). Immobilization improved both the pH stability and thermal stability of the enzyme in comparison with free Alcalase. The immobilized Alcalase retained high activities in a broader range of pH, especially in the high alkaline region, and its thermal stability was considerably improved at 50℃ and 60℃. The immobilized enzyme could maintain 45% of its initial activity after 10 times repeated usage under optimum conditions of protein hydrolysis (i.e., 50℃ and pH 9.0). 53% of azo-casein (0.5 w/v%) was hydrolyzed to the molecular weight of 10 kDa or less with the use of a five-layer PAN-BM in a continuous proteolytic reactor at the flux of 6 $L/(h\cdot m^2)$ while 74% of the normalized extent of hydrolysis (NEH) was obtained. Hydrolysis of soybean meal proteins (SMPs) was tested in the same reactor and the NEH was 72%.

Key Words: Electrospinning; Nanofibers; enzyme immobilization; continuous immobilized enzyme reactor; protein hydrolysis; soybean meal proteins.

3. 1. Introduction

Enzymes are biocatalysts for a large variety of different applications with high catalytic efficiency, exceptional selectivity and superb biocompatibility [1][2]. They are the ideal choice of catalysts in many fields including food and pharmaceutical industries and environmental protections [3]-[5]. Alcalase is an important commercial proteinase (enzyme catalyzing the hydrolysis of proteins), which has been applied for a wide diversity of different applications. These include the production of functional peptides from commercial proteins, agricultural wastes and dairy wastes such as egg white protein, soy protein isolates and cheese whey, for enhanced recovery and/or improved product solubility, stability, emulsification, assimilability, bioactivity (e.g., antioxidant), and reduced allergenicity to meet the increasing needs of proteins and peptides for different markets [6]-[11]. Nevertheless, the industrial application of free enzymes faces some challenges due to instability and poor recyclability of this enzyme, resulting in a considerable increase in enzyme consumption and costs [12][13]. Immobilization of enzymes onto/into support materials is an appealing solution to overcome these challenges industry [14][15].

Thanks to the advances in material science and engineering, a large number of support materials have been made available for enzyme immobilization. There has been significant interest in enzyme immobilization using nanoscale materials as supports since they have large surface-to-volume ratio and therefore higher enzyme loading capacity and reduced mass transfer hindrance for better catalytic efficiency [16][17]. Among these nanoscale materials, electrospun nanofibrous membranes exhibit large surface area, high porosity and strong mechanical properties, and offer the potential of real-time separation of product from substrates and enzymes in order to reduce the cost in the industry [18]-[21]. Polyacrylonitrile (PAN) has been widely used in electrospinning for filtration, adsorption, and the fabrication of composite materials because of its good stability and mechanical properties (e.g., abrasion resistance, high tensile strength, and flexibility) [22][23]. Several investigations have been reported that lipases were immobilized on PAN nanofibers for organic contamination removal or transesterification [22][24][25]. A few studies are also available on protease immobilization on PAN nanofibrous membranes. Of particular relevance, Du Plessis [21] immobilized Alcalase on PAN nanofibers for anti-biofilm activity. Imidoesterification was used to modify PAN to make imidoester groups available for covalent binding of Alcalase. Nevertheless, the modification method involves the use of anhydrous HCl gas, which makes the operation relatively complex, unsafe and demanding on equipment.

In this research, we investigated the effectiveness of modifying electrospun PAN nanofibrous membranes (EPNMs) using the carbodiimide method to avoid the use of dry anhydrous HCl gas for PAN modification. This is a classical coupling reaction, which has been used by Li [26] for the successful immobilization of laccase onto PAN nanofiber. However, it has not been used for protease immobilization to our best knowledge. A continuous proteolytic reactor with the immobilized Alcalase was also set up in this research and tested for hydrolysis of azo-casein as a model substrate and SMPs which have high-molecular-weights and low-solubility, as a potential application of this biocatalytic nanofibrous membrane.

3. 2. Materials & Methods

3.2.1. Materials

PAN (average molecular weight 1.5 × 10^5 Dalton), Alcalase 2.4 L FG (Protease from Bacillus licheniformis, Novozymes), Azo-casein (Protease substrate sulfanilamide azo-casein), *N*, *N*-Dimethyl formamide (DMF, 99.8%), and *N*-Hydroxy succinimide (NHS, 98%) were purchased from Sigma-Aldrich Inc., St. Louis, MO. 1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC, 98+%) was obtained from Alfa Aesar, Ward Hill, MA. Bicinchoninic acid (BCA) protein assay test kit™, and Bovine serum albumin standards (BSA) were obtained from Thermo-Scientific, Rockford, IL. Defatted soybean meal samples (SBM) were obtained from Tri-county protein Corp., Winchester, Canada.

3.2.2. Preparation of PAN-BMs

An 8% (w/w) PAN solution was prepared by dissolving PAN in DMF at 50°C with stirring for 24 h until a light yellow, transparent solution was obtained. The PAN solution was then loaded into a plastic syringe with a stainless-steel needle. The applied voltage, the flow rate of the PAN solution, and the distance between the needle tip and aluminum foil collector were maintained at 20.0 kV, 1.1 mL/h and 25 cm, respectively. After the electro-spinning, the EPNM was dried at room temperature overnight before it was removed from the aluminum foil.

As shown in **Fig. 3.1**, the immobilization of Alcalase was carried out in three steps, i.e., alkali treatment of EPNM to produce alkali-treated PAN membrane (1-PANM), activation of 1-PANM to produce activated 1-PANM (2-PANM), and immobilization of Alcalase onto 2-PANM to produce the final Alcalase-functionalized PAN electrospun biocatalytic membrane (PAN-BM).

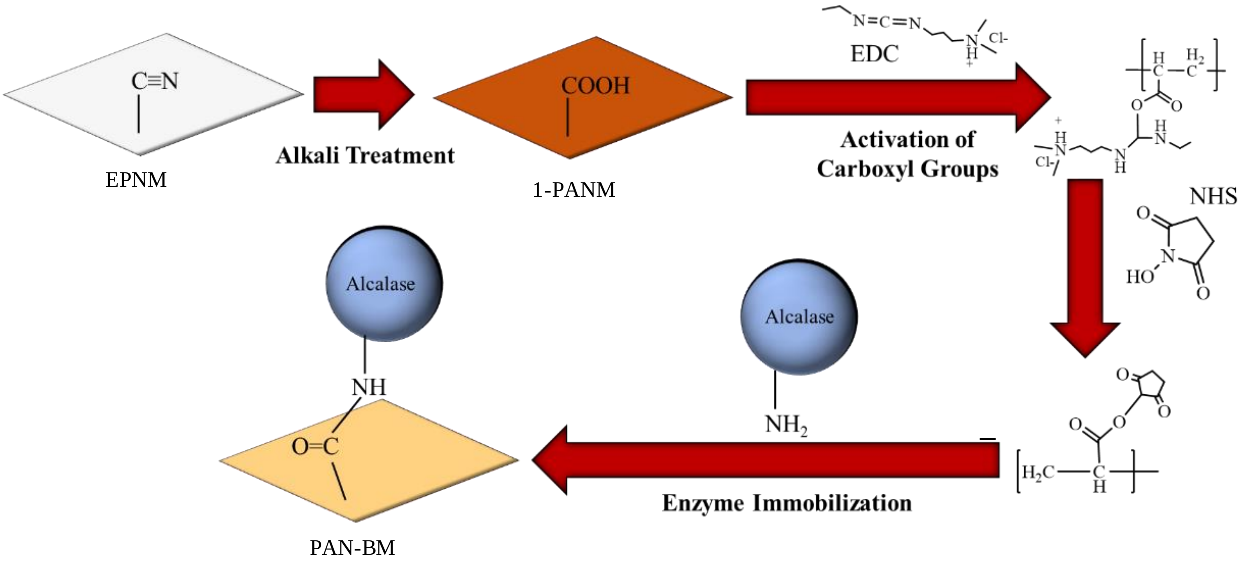

Fig. 3.1. Enzyme immobilization process with the carbodiimide method

Alkali treatment of EPNM: The prepared EPNM was alkali treated using the protocol proposed by Li et al with slight modifications [26]. Briefly, 8 g of NaOH was dissolved in 200 mL 95% (v/v) ethanol at 60°C with stirring. The PAN nanofibrous membrane (8 × 8 cm) was immersed into the as-prepared alkali solution and kept there for 100 min maintaining the temperature at 60°C with continuous stirring. The obtained 1-PANM was washed thoroughly using deionized water until pH neutrality and then air-dried at room temperature before activation.

Activation of 1-PANM: The 1-PANM was first immersed in a phosphate buffer saline (PBS) buffer (12 mM, pH = 6.5) for 30 min for impurities removal and then transferred into an EDC·HCl (8%, w/v)/NHS (4.8%, w/v) solution. After 3-h incubation at room temperature, the activated PAN nanofibrous membrane was removed and washed three times with PBS (pH 6.5).

Immobilization of Alcalase on 2-PANM: The 2-PANM was immersed in an Alcalase solution as purchased for immobilization at either 4°C or 25°C with shaking for different periods (e.g., 1, 3 or 24 h). Subsequently, the enzyme loaded PAN-BM was washed with PBS (pH 7.4), until no enzyme was detected in the rinsing solution, and then air-dried at 4°C.

3.2.3. Characterization of nanofibrous membranes

The morphology of EPNMs, 1-PANMs, 2-PANMs and PAN-BMs was investigated using scanning electron microscopy (SEM, JSM-7500F). The nanofiber surface was gold-sputtered (5 nm) before SEM imaging. Fourier Transform Infra-Red spectroscopy (FT-IR, Agilent Cary 630 FT-IR spectrometer) was used to investigate the functional groups of the EPNMs, 1-PANMs and PAN-BMs.

3.2.4. Determination of protein loading, enzyme activity & kinetic studies

The protein loading of the PAN-BMs was determined by BCA assay with BSA as the standard [27]. The amount of immobilized Alcalase onto the dried nanofibrous membranes was determined by comparing the absorbances at 562 nm to that of a BSA standard with a UV/VIS spectrophotometer (UV/VIS, Genesys 10S UV-Vis spectrophotometer) [21]. The absorbance of activated PAN nanofibers was set as blank for negative control.

The activity of the free and immobilized Alcalase was determined by azo-casein assay by the method reported by du Plessis et al with modification [21]. First, a 0.5% (w/v) azo-casein substrate solution was prepared by dissolving into PBS with stirring at room temperature. Afterwards, a sample of either a PAN-BM (1 × 2 cm) or a volume of free Alcalase solution whose protein content was equivalent to the amount of protein loading onto a 1 × 2 cm PAN-BM, was added into 500 μL of the azo-casein solution in a centrifuge tube for incubation for 20 min unless further indicated. 1 mL of 5% (w/v) TCA solution was then added to terminate the incubation after a predetermined period and precipitate the unreacted protein, followed by centrifugation (14, 000 × g) for 5 min. Then 400 μL of the supernatant as mentioned were transferred to 1 mL 0.1 M NaOH solution followed by vortex for 10 s. The absorbance of the transparent mixture was measured at 440 nm. The Alcalase activity is indicated by the change of absorbance at 440 nm due to the free azo-dye liberated by azo-casein hydrolysis. Namely, the increased amount of free azo-dye is used to represent the reduced amount of azo-casein hydrolyzed by free or immobilized Alcalase, and thus one unit of enzyme activity was defined as the 1 μmol of azo-dye per minute. **Eq. 3.1** was used to estimate the activity of Alcalase (V_{act}, U/mL) [28].

$$V_{act} = \Delta A(K/\varepsilon tL) \qquad \textbf{(3.1)}$$

Where ΔA, K, ε ($L \cdot mol^{-1} \cdot cm^{-1}$), t (min) and L (cm) refer to the change in absorbance, dilution ratio, extinction coefficient, incubation time and length of the optical pass, respectively.

Kinetic studies were carried out at 50°C in PBS solution (pH 9.0) by azo-casein assay. Different concentrations of azo-casein solution were prepared from 0.25 - 2% (w/v) as the substrate. **Eq. 3.2** is the Michaelis-Menten equation which was used to obtain the Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) by the fitting model of the software OriginLab.

$$V = V_{max}S/(K_m+S) \qquad \textbf{(3.2)}$$

where S is the substrate concentration (mg/mL).

3.2.5.Effects of temperature and pH on the activity of free & immobilized Alcalase

For tests of temperature effects, PAN-BM was allowed to hydrolyze azo-casein in PBS buffer (pH 9.5) for 20 min at different temperatures ranging from 20 to 60°C. To test the effects of pH, PAN-BM was incubated with substrate solution at pH varying from 7.5 to 10.5 at 50 °C for 20 min incubation. Enzymatic activity of free Alcalase was determined at the same conditions for comparison.

3.2.6. Stability of free and immobilized Alcalase

To test the thermal stability, aliquots of free and immobilized Alcalase in pH 9.5 PBS buffer were incubated at 50 or 60°C for a given time as specified in the text. The relative activities of the pre-incubated enzymes were then measured at different pre-incubation times with a time interval of 30 minutes. The initial activity of the sample without pre-incubation is set to be 100% relative activity since it offers the highest activity.

The thermal stability of free and immobilized Alcalase was also determined by storing them different temperatures ranging from 4 to 70°C for 48 h before the determination of relative enzymatic activity at 50 °C. The relative activity of free or Alcalase stored at 4°C for 48 hours was set as 100%. The reusability was studied by repeated usage of PAN-BM for 10 cycles. All samples were subjected to azo-casein assay at 50°C in the buffer (pH 9.0) for 20 min. The enzymatic activities of PAN-BMs in each cycle were compared with that in the first cycle.

3.2.7.Reusability of PAN-BMs

The reusability was studied by repeated usage of PAN-BMs for 10 cycles. All samples were subjected to azo-casein assay at 50°C in the buffer (pH 9.0) for 20 min. The enzymatic activities of PAN-BMs in each cycle were compared with that in the first cycle.

3.2.8. Effects of substrate flux and number of PAN-BM layers in a continuous reactor

A continuous reactor of PAN-BM was set up as shown in **Fig. 3.2**. A peristaltic pump (12000 Varioperpex, Bromma LKB, Sweden) was used to control the flux. Effects of different flux (6, 12, 31, 61 and 122 L/h·m^2) of 0.5% azo-casein solution (pH 9.0) with one layer of PAN-BM and one to five layers of PAN-BM the flux of 6 L/h·m^2 were tested at 50°C. The hydrolysate solutions were collected and tested at 440 nm as mentioned in ***3.2.5***. The hydrolysate was also analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% acrylamide (diluted from 30% AccuGel, 1.5 M Tris, 10% SDS, 10% ammonium persulfate, *N, N, N', N'*-tetramethyl ethylene diamine and deionized water) running gel in a Mini Protean system (Bio-Rad, Hercules, USA).

3.2.9. The hydrolysis of SMPs with PAN-BMs

1.4% (w/v) SMPs solution was prepared as follows. SMPs were first extracted from as received soybean meals under stirring at 50°C for one hour and mixture pH was maintained at 10 by adding 0.1 M NaOH solution. After it was cooled down at room temperature, the mixture was centrifuged at 3070 × g for 20 min and the supernatant was collected. Spin-X® UF concentrators (Corning, England) with 10 kDa molecular weight cut-off were used to one time concentrate SMPs at 15, 000 × g for 10 min. The pH of SMPs solution was adjusted to pH 9.0 with 0.1 M

HCl, which was subject to Bradford protein assay (BPA, Bio-Rad) at 595 nm to determine the protein concentration with BSA as the standard [29]. SMPs solution was subject to continuous hydrolysis in the continuous reactor with a five-layer PAN-BM at the flux of 6 L/h·m^2 under 50°C. The hydrolysate was collected and analyzed by BPA and SDS-PAGE, respectively.

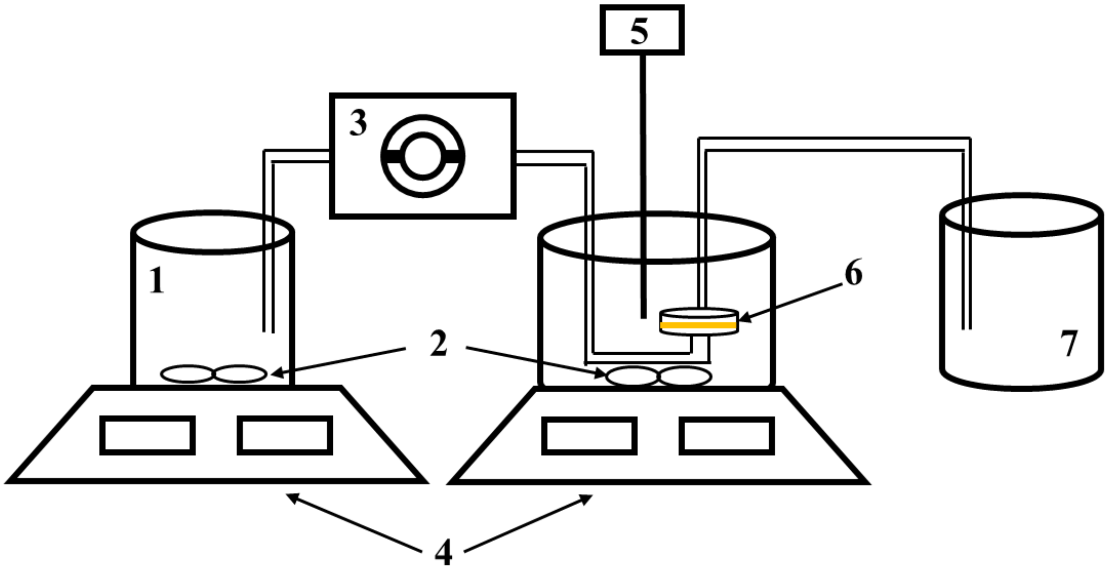

Fig. 3.2. Schematic of the continuous immobilized enzyme reactor

(1) reservoir; (2) stirring bars; (3) peristaltic pump; (4) magnetic stir with heating (5) electronic thermometer; (6) housing of PAN-BM; (7) hydrolysate collector

3. 3. Results & Discussion

3.3.1. Characterization of nanofibrous membranes by SEM and FT-IR

SEM images and digital photographs of EPNMs, 1-PANMs, 2-PANMs and PAN-BMs are given in **Fig. 3.3** to show the morphological changes of the nanofibrous membranes caused by modification, activation and immobilization. Color changed from white of EPNM (**Fig. 3.3a**) to reddish brown of 1-PANM (**Fig. 3.3b**). Some poly-conjugated intermediates, which were produced during the electro-cyclization process at the beginning of the alkali hydrolysis, likely contributed to this coloration [30]. In this reaction, hydroxyl ions attack the nitrile group and the participation of a small amount of water in the reaction leads to the cyclization followed by the formation of carboxylate groups [30]. The reddish-brown 1-PANM became yellow after the activation reaction in which it was converted to 2-PANM (**Fig. 3.3c**). This color change might have resulted from the consumption of the poly-conjugated intermediates because of the formation of semi-stable amine-reactive intermediates in the activation reactions. During this step, the carboxyl groups on the PAN membrane were first activated by EDC to form an unstable reactive ester, followed by the generation of semi-stable amine-reactive NHS ester due to the reaction with NHS [31][32]. The immobilization of Alcalase to 2-PANM to transform it to PAN-BM did not cause significant color change (**Fig. 3.3d**).

The structures of PAN nanofibers before and after alkali treatment (**Figs. 3.3a** and **3.3b**) displayed smooth fiber surfaces with a diameter distribution over 200 – 400 nm, indicating the relatively mild alkali treatment did not damage the structure of the PAN nanofiber. However, it can be noticed from **Fig. 3.3c** that adjacent nanofibers were adhered to each other after carboxyl activation and formed fibrous entanglements more easily than the ones before the activation. The formation of poly-conjugation and the inter-molecular forces of semi-stable amine-reactive esters after carboxyl activation likely contributed to the fibrous adhesion. Furthermore, small amounts of particles attached to the nanofibrous surface sporadically were visible on PAN-BM (**Fig. 3.3d**). This was likely due to the conglobation of aggregated Alcalase due to intermolecular forces [21].

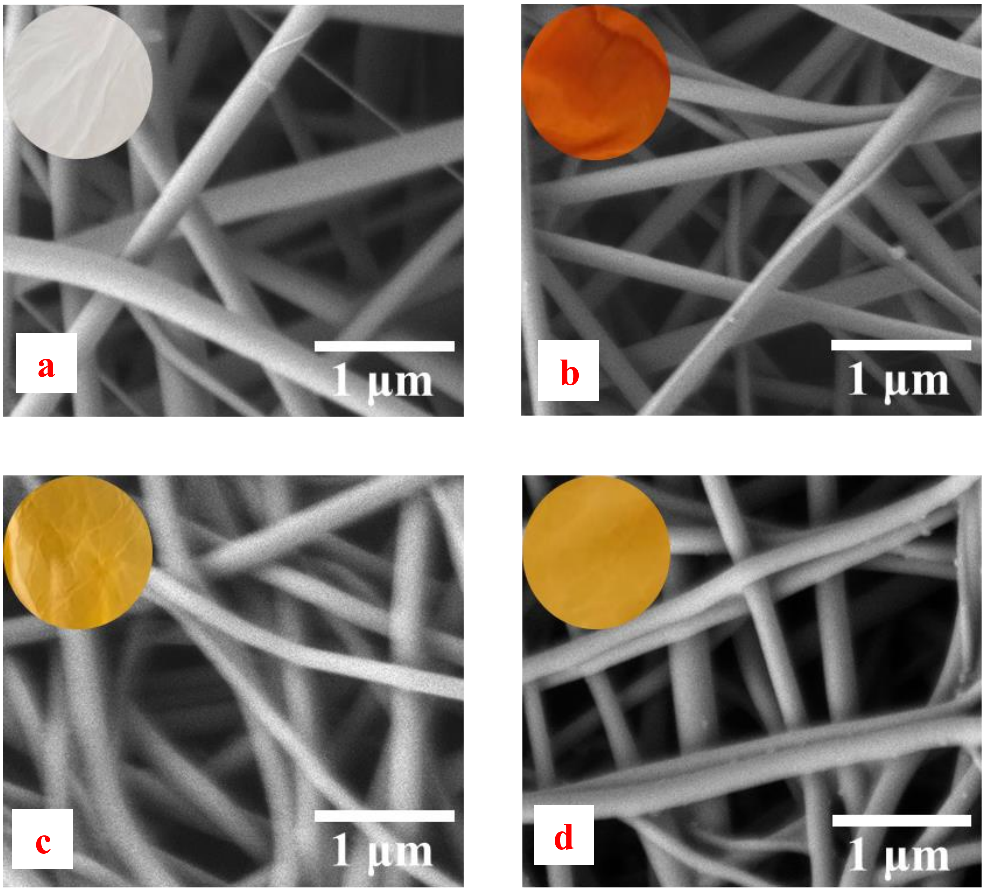

Fig. 3.3. SEM and digital images (inserts) of
(a) EPNM, (b) 1-PANM, (c) 2-PANM and (e) PAN-BM

The presence of functional groups with different characters confirmed the successful preparation of PAN-BMs using FT-IR, as indicated in **Fig. 3.4**, in which EPNMs, 1-PANMs and PAN-BMs are compared. A characteristic peak at 2243 cm^{-1} was observed on the spectra of all nanofibers representing the presence of a nitrile group (-C≡N). Two new peaks after the alkali treatment appeared at 1662 cm^{-1} and 1565 cm^{-1} which indicate the formation of $-CONH_2$ and -COONa groups respectively [26]. 2-PANMs were not characterized by FT-IR since they contain semi-stable ester, whose characterization is difficult by FT-IR. A peak at 3400 cm^{-1} of PAN-BMs shows the formation of N-H groups at the site of enzyme immobilization [21]. The immobilization of Alcalase led to the appearance of other three new peaks at 1671, 1583 and 1071 cm^{-1}, among which 1583 cm^{-1} was assigned to C=O vibration, 1671 cm^{-1} to the vibration of N-H bonds in protein backbone and to the stretching of C-N bonds, which were formed in the process of enzyme immobilization. C-N bonds of Alcalase and between Alcalase and membrane were also proved by the peak at 1071 cm^{-1} [26]. This observation concluded the successful preparation for PAN-BMs.

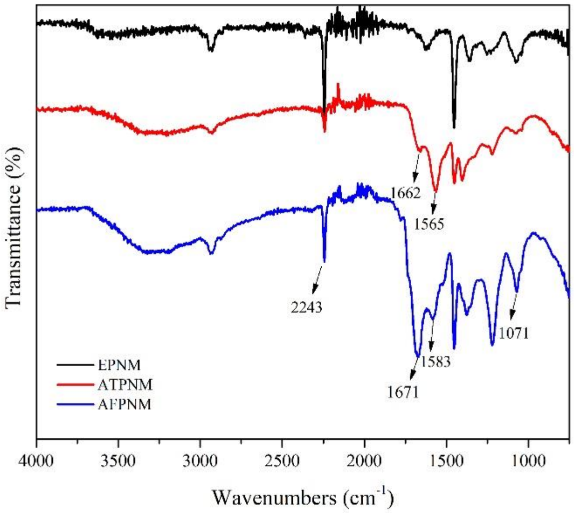

Fig. 3.4. FT-IR spectra of EPNM, 1-PANM and PAN-BM

3.3.2.Effects of immobilization temperature and contact time

Immobilization was conducted under five different combinations of different immobilization temperature and contact time. Results shown in **Table 3.1** indicate that higher temperature and longer contact time resulted in more Alcalase loading onto the PAN nanofibers, while the specific activity becomes less. Immobilization at higher temperatures likely facilitated enzymes to replace the semi-stable NHS esters to form a covalent C-N bond, which linked enzyme to the

PAN membrane faster than at lower temperatures. However, instability of Alcalase and auto-proteolytic activity during the long immobilization period were also stronger at a higher temperature, lowering the retained specific enzyme activity. As a result of these two competing effects, the combination of 25°C and 24 h gave the highest specific PAN-BM activity (106.01 U·g^{-1}-membrane, assayed by incubating at 30°C and pH 9.5 for 20 min). This combination has also the advantage of convenience and was therefore chosen for all the experiments thereafter. It is worth noting that, judging from the data presented in **Table 3.1**, there is room for further optimizing the loading temperature and contact time.

Table 3.1. Summary of effects of different temperatures and contact times on Alcalase immobilization (n = 3)

Immobilization temperature (°C)	**Contact time (h)**	**Specific activity (U·mg^{-1}-protein)**	**Protein loading (mg·g^{-1}-membrane)**	**PAN-BM Specific Activity (U·g^{-1}-membrane)**
4	3	6.31 ± 0.638	16.09 ± 0.466	101.56
4	24	6.07 ± 0.585	17.06 ± 0.206	103.53
25	1	6.27 ± 0.334	13.60 ± 0.903	85.24
25	3	6.52 ± 0.743	16.15 ± 0.132	105.25
25	24	4.66 ± 0.152	22.78 ± 0.683	106.01

3.3.3. Kinetic studies of free and immobilized Alcalase

Kinetic studies were carried out at different substrate concentrations using PAN-BMs and the results are shown in **Fig. 3.5**. Experimental data were fitted to the Michaelis-Menten equation to determine the kinetic parameters of free and immobilized Alcalase, which are listed in **Table 3.2**. The Michaelis-Menten constant (K_m) of immobilized Alcalase (3.17 g/L) was 10% higher than that of free Alcalase (2.88 g/L) correlating to a lowered affinity between the immobilized enzyme and substrate. The maximum reaction rate (V_{max}) of immobilized Alcalase (36.91 umol·min^{-1}·mg^{-1} -protein) was 24% lower than that of free Alcalase (49.11 umol·min^{-1}·mg^{-1} -protein), indicating fewer active sites available for immobilized enzymes in comparison to free enzymes. The decrease of the active sites of immobilized enzyme accessible to the substrate was likely due to the additional steric hindrance and the partial loss of conformation flexibility when the enzyme was anchored onto the surface of the nanofibrous membrane [33]. In addition, while free enzymes could collide with substrate protein molecules in an agitated reaction mixture, substrate proteins must diffuse to the membrane surface to access the immobilized enzymes. The slow diffusion process of large substrate proteins might also contribute to the lower V_{max} and higher K_m of immobilized Alcalase in comparison to free enzyme.

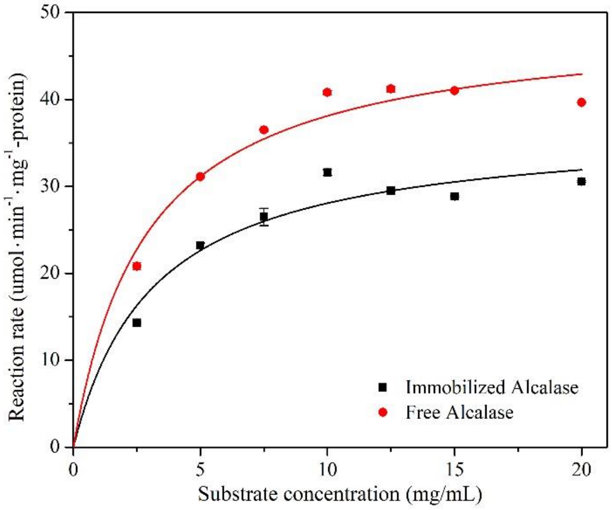

Fig. 3.5. Michaelis-Menten plots of free and immobilized Alcalase

(Symbols, experimental data; solid lines, non-linear fitting curves, n = 3)

Table 3.2. Kinetic parameters of free and immobilized Alcalase (n = 3)

Materials	K_m ($mg \cdot mL^{-1}$)	V_{max} ($U \cdot mg^{-1}$ -protein)	**Activity Retention (%)**
Free Alcalase	2.88 ± 0.583	49.11 ± 2.470	–
Immobilized Alcalase	3.17 ± 0.800	36.96 ± 2.449	58%

3.3.4. Effects of temperature and pH on the activity of free & immobilized Alcalase

Temperature and pH may have a significant influence on Alcalase activity and extreme temperature and/or pH might cause the denaturation of the enzyme. Therefore, an attempt was made to find the optima of temperature and pH in protein hydrolysis catalyzed by free or immobilized Alcalase. **Fig. 3.6a** shows the relative activities of free and immobilized Alcalase in a temperature range of 20 to 60°C. Herein the relative activity is defined as the ratio of the activity to the highest activity obtained for a given pH or temperature. For example, in **Fig. 3.6a**, a series of experiments were performed at pH 9.5 using the immobilized enzyme and the highest activity of 25.02 ± 0.005 $U \cdot mg^{-1}$ -protein was obtained at 60°C. On the other hand, the activity went down to 2.37 ± 0.133 $U \cdot mg^{-1}$ -protein at 20°C. Hence the relative activities are 100 and 9.5% at 60 and 20°C, respectively. A similar series of experiments were conducted for the free enzyme at pH 9.5 and the data plotted in **Fig. 3.6a**.

For both immobilized and free Alcalase the relative activity kept increasing with temperature until 60°C. The increasing rate of immobilized Alcalase activity was over that of free enzyme starting at 50°C. For free Alcalase, both the enzymes and the substrate proteins could move freely inside the reaction mixture. However, for the immobilized Alcalase, substrate proteins must diffuse to the membrane surface to access the active sites of Alcalase. The diffusion of large substrate proteins across the stagnant film on the membrane surface is understandably slow and most likely limiting. As a result, the overall reaction rate of hydrolysis catalyzed by the immobilized Alcalase is more sensitive to temperature than that of the free Alcalase. Since the highest activities were attained for both immobilized and free enzyme at 60°C, incubation experiments should have been done at even higher temperatures. However, temperatures higher than 60°C were not tested due to the limitation of our facility.

Buffer pH plays an important role in enzymatic reaction since it may affect the dissociation of ions and therefore the charge the enzyme's active site and may also cause reversible and even irreversible change of enzyme protein configuration. The effect of pH on Alcalase activity was studied and the results were shown in **Fig. 3.6b**. A series of experiments were performed at 50°C over a pH range of 7.5 to 10.5 at an incubation period of 20 min. The highest activity was obtained at pH = 9.0, which was 23.79 ± 0.518 $U \cdot mg^{-1}$-protein. Hence, the relative activity at pH 9.0 is set equal to 100 %. On the other hand, for the free enzyme relative activity was 100 % at pH 8.5. It is interesting to note that the pH range of high relative activities was significantly broadened for the immobilized enzyme. The three-dimensional structure of immobilized Alcalase may have been distorted, resulting in less structural changes in enzyme structure and activity caused by the pH variation [6]. Hence, PAN membrane-immobilized Alcalase, with its less susceptibility to the pH change, is more suitable than the free enzyme in practical applications.

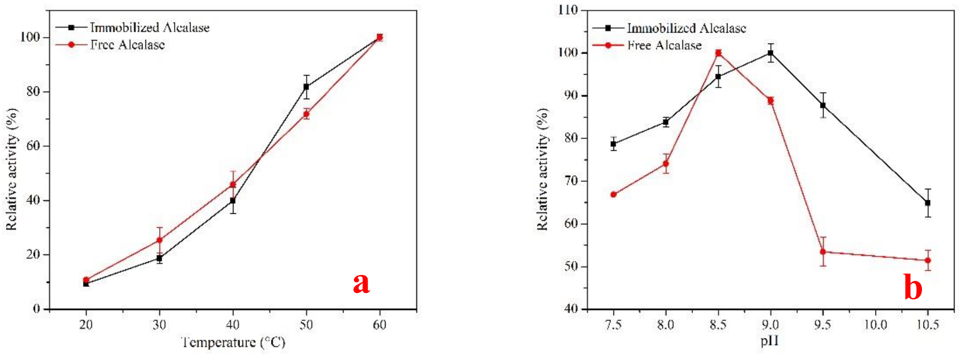

Fig. 3.6. Effect of (a) temperatures and (b) pH values on enzyme activity of free and immobilized Alcalase

3.3.5. Thermal stability of free and immobilized Alcalase

The thermal stability of enzymes is important for the practical application. At 50°C (**Fig. 3.7a**), immobilized Alcalase retained about 86% of its initial activity, whereas free Alcalase maintained only 41% after 210 min of incubation. Both free and immobilized Alcalase showed worse thermal stability at 60°C than 50°C. **Fig. 3.7a** and **b** also indicate that immobilized Alcalase is thermally more stable than the free enzyme and both free and immobilized Alcalase were less stable at 60°C than 50°C, which are expected. Nevertheless, the present work showed better thermal stability of immobilized Alcalase compared with a previous result from the literature for Alcalase immobilization in which the relative activity decreased approximately to 20% after 210 min incubation [7]. The better thermal stability of immobilized Alcalase is likely because immobilization strengthens the rigidity of Alcalase and protects it from unfolding [7]. Furthermore, since less inactivation at 50°C of both free and immobilized Alcalase, it can be concluded that 50°C is a better incubation temperature with a significantly longer enzyme lifetime than 60°C.

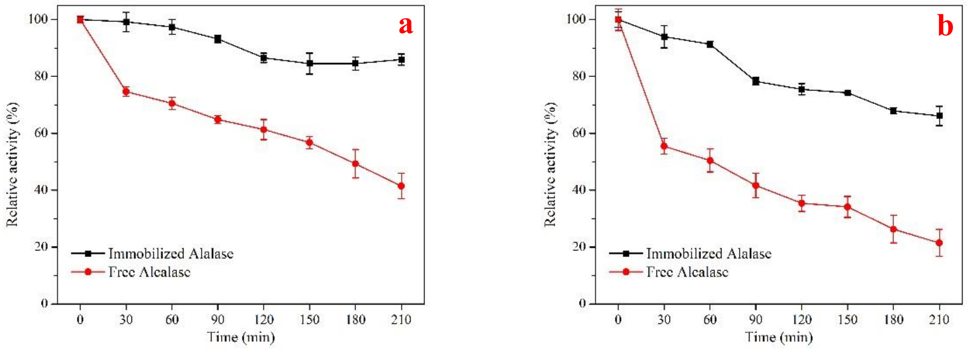

Fig. 3.7. Thermal stability of free and immobilized Alcalase at (a) 50°C and (b) 60°C

To further the thermostability investigation, free and immobilized Alcalase were stored at different temperatures (4, 20, 30, 40, 50, 60 and 70°C) in buffer solution at pH 9.5 for 48 h before testing the enzymatic activities at 50°C for 20 min. For both free and immobilized enzymes, the activity was the highest when stored at 4°C, therefore the relative activity at 4°C was set equal to 100 %. **Fig. 3.8** illustrates that free Alcalase lost activity significantly and was inactivated completely when stored 40°C or above, while immobilized Alcalase retained 24, 14, 9 and 7% of its initial activity when it was stored for 48 h at 40, 50, 60, and 70°C, respectively. This study once again confirmed that immobilized Alcalase has better thermostability, a significant characteristic beneficial to potential industrial applications, especially in the continuous hydrolysis of proteins.

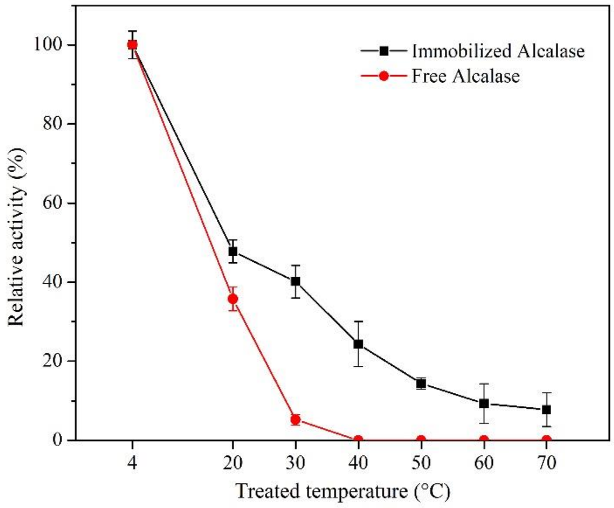

Fig. 3.8. Relative activity of free and immobilized Alcalase stored at different temperatures for 48 hours

3.3.6.Reusability of PAN-BMs

The reusability is one of the essential indications of operational stability in practical applications and high reusability could reduce costs in operation greatly. More importantly, good reusability is essential for continuous operation [34]. Hence, hydrolysis experiments were performed at 50°C, pH 9.0 in 10 consecutive cycles (20 min for each cycle). The results (**Fig. 3.9**) show that PAN-BM retained about 88% of its original activity after one operation cycle and over 50% of its original activity after six cycles. After ten cycles of continuous usage, the relative activity of PAN-BMs dropped to about 45%, indicating better reusability of PAN-BMs than the previous results from the literature, where immobilized Alcalase remained approximately 20% and 40% of their initial activity after being reused for 10 times [7][21]. The better reusability of PAN-BMs may benefit from the better biocompatibility due to the membrane modification and the improved rigidification of immobilized Alcalase.

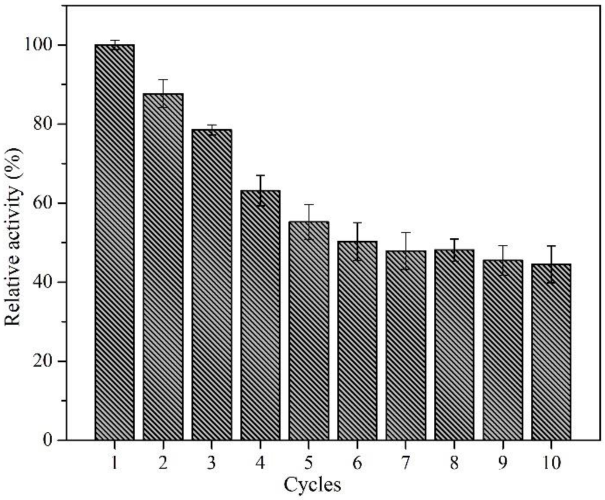

Fig. 3.9. Reusability of PAN-BM

3.3.7.Effects of feed flux and number of PAN-BM layers in the continuous reactor on protein hydrolysis

The results of studies on the effects of feed flux on the extent of hydrolysis of 0.5% azo-casein solution are shown in **Fig. 3.10a**. The hydrolysates of the free enzyme at a dosage approximately 100 times of that used for the hydrolysis experiments (37^{o}C, pH 9.0) with a prolonged incubation time of 24 hours was opted to ascribe the 100% hydrolysis of the substrate as a benchmark for comparison. The normalized extent of hydrolysis (NEH) is defined as the ratio of the absorbance at 440 nm, which measures the concentration of the azo-dyes released in the hydrolysis of azo-casein substrate, of the hydrolysate solution collected at each flux to that of the aforementioned benchmark. While the NEH was approximately 29% at a flux of 6 L/h·m^2, it decreased to around 14% when the flux increased to 12 L/h·m^2 and levelled off at around 4% and 2% at 61 and 122 L/h·m^2, respectively.

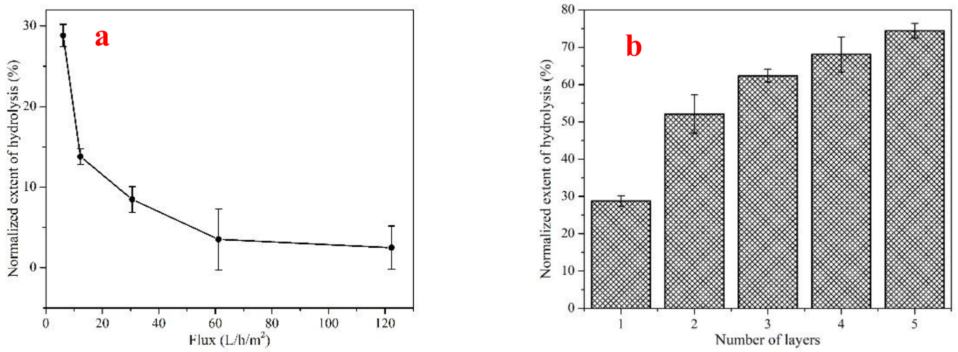

Fig. 3.10. The normalized extent of hydrolysis with different (a) flux and (b) layers of membranes

The result in **Fig. 3.10b** shows the effects of the number of PAN-BM layers in the reactor on the NEH of 0.5% azo-casein feed solution at a flux of 6 L/h·m^2. While it was 29% with a one-layer reactor, the NEH increased to 52% with a two-layer reactor and around 74% with a five-layer reactor. Increasing the number of PAN-BM layers caused the increase of the number of enzymes and the contact time of the substrate and enzymes and therefore the extent of hydrolysis when other conditions were the same.

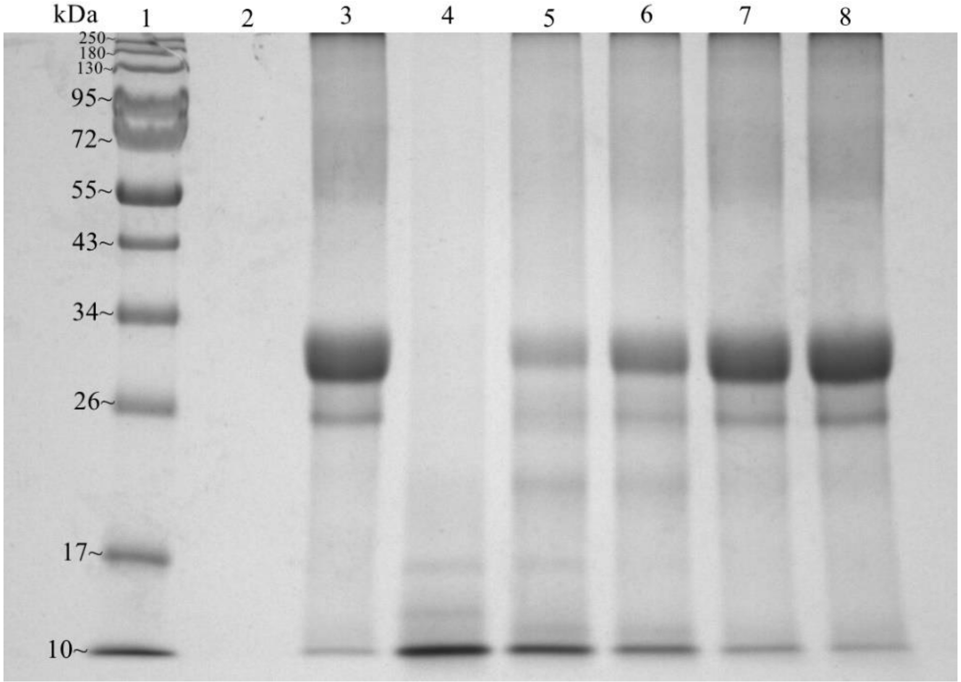

Fig. 3.11. SDS-PAGE of hydrolysates obtained with a one-layer PAN-BM reactor at different feed fluxes (Lane 1, molecular weight marker; Lane 2, negative control; Lane 3, positive control; Lane 4 – 8, hydrolysates obtained with the one-layer membrane at 6, 12, 31, 61, 122 L/h·m^2, respectively.)

The SDS-PAGE image in **Fig. 3.11** shows the molecular profiles of peptides in the hydrolysates from a single layer PAN-BM reactor at different feed fluxes**.** The negative control (Lane 2) was a PBS solution at pH 9.0 while the positive control (Lane 3) was 0.5% azo-casein PBS solution at pH 9.0. As shown in **Fig. 3.11**, the positive control gel was characterized by a predominant band of a molecular weight slightly smaller than the 34 kDa marker. For hydrolysis of azo-casein using a continuous single-layer PAN-BM reactor at a flux of 6 L/h·m^2, the band characteristic of azo-casein almost disappeared completely while only an obvious band at 10 kDa appeared, indicating complete hydrolysis of the substrate azo-casein to small peptides of 10 kDa or smaller molecular weight. On the other hand, the characteristic band appeared when the flux increased to 12 L/h·m^2 and its intensity increased with flux and the gel bands spectra were almost identical to that of the positive control when the flux was 61 or 122 L/h·m^2, indicating lack of hydrolysis at those high fluxes, apparently due to the extremely small residence time. It is of interest to mention that a band of molecular weight between 17 and 26 kDa appeared at flux 12 and 31 L/h·m^2, indicating a possibility of producing peptides in that molecular weight if conditions are fine-tuned. These results suggest that there is a possibility of controlling the NEH and even the molecular weight range of hydrolysates in the continuous reaction by controlling the flux.

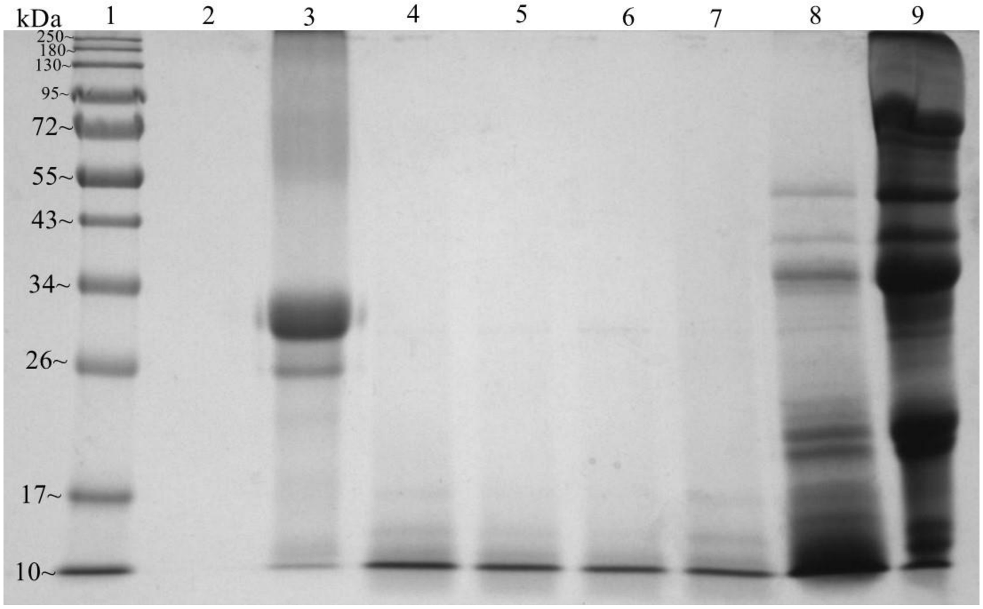

Fig. 3.12. SDS-PAGE images of hydrolysates from multilayer PAN-BM reactor with azo-casein or SMPs: Lane 1, molecular weight marker; Lane 2, negative control (PBS at pH 9.0); Lane 3, positive control for lane 4 – 8 (0.5% azo-casein at pH 9.0); Lanes 4 – 7, hydrolysates at 6 L/(h·m^2) with 2, 3, 4, 5 layers of PAN-BM, respectively; Lane 8, hydrolysates of 1.4% SMPs at pH 9.0 using a five-layer PAN-BM reactor; and Lane 9, positive control for lane 8 (1.4% SMPs at pH 9.0)

The SDS-PAGE image featuring the peptide profiles of hydrolysates of azo-casein obtained with multilayer PAN-BM reactors at 6 L/h·m^2 is shown in **Fig. 3.12**, together with that of the hydrolysate of SMPs hydrolysis with a five-layer PAN-BM reactor at the same flux. These results (Lanes 4 – 7) indicate that all the original azo-casein in the feed was hydrolyzed to peptides with 10 kDa or smaller molecule-weight, no matter how many layers a reactor had.

Table 3.3 also features the peptide profiles in terms of the percentage of peptides corresponding to individual molecular weight markers and the data were obtained from the analysis of the corresponding images in **Fig. 3.12** using UltraQuant. No peptides were detectable by SDS-PAGE at 34 kDa marker, which is characteristic of azo-casein, no matter how many layers the reactor had. While 13% was the band of the 17 kDa with the single-layer reactor, peptides at this band were also undetectable with reactors of 2 or more layers of PAN-BM. Nonetheless, around 50% was the band of 10 kDa, confirming that the overwhelming peptides in these hydrolysates were of 10 kDa or less molecular weight.

Table 3.3. The molecular weight distribution of peptides in hydrolysates of azo-casein from a single layer reactor at different flux and multilayer reactors at 6 L/h·m^2 flux

Marker (kDa)	Positive control	Single-layer at a varied flux (L/h·m^2)					Multilayer at 6 L/h·m^2			
		6	12	31	61	122	2 layers	3 layers	4 layers	5 layers
34	35%	–	13%	23%	34%	35%	–	–	–	–
26	7%	–	17%	17%	8%	8%	–	–	–	–
17	–	13%	8%	–	–	–	–	–	–	–
10	3%	42%	15%	9%	6%	5%	51%	53%	50%	53%

3.3.8. Hydrolysis of SMPs with PAN-BMs

The peptide profile of the hydrolysate of SMPs obtained by hydrolysis with a five-layer PAN-BM reactor at 6 L/h·m^2 is also shown qualitatively in **Fig. 3.12** (Lanes 8 and 9). The SDS-PAGE image of the positive control (Lane 9), that is, 1.4% SMPs solution at pH 9.0, indicates that SMPs are composed of proteins with a wide range of molecular weights. In the meantime, the bands in Lane 8 show that most of the large molecular weight proteins in SMPs were hydrolyzed to a certain extent and a significant portion of the product peptides were of 10 kDa. It is understood that another portion of peptides was of molecular weights less than 10 kDa and was not detectable in the SDS-PAGE analysis.

Analysis of Lanes 8 and 9 using the UltraQuant software indicate that 33% proteins of the SMPs were in the range of 26 – 95 kDa while only 9 % remained after going though five-layer PAN-BM reactor. In the meantime, proteins in the 10 kDa band increased dramatically from 3% to 50% after hydrolysis, indicating that most of the high-molecular-weight SMPs were hydrolyzed to low-molecular-weight peptides. Measurement of the SMPs solution before and after hydrolysis using the BPA showed that the protein concentration of SMPs dropped from 14.0 ± 0.09 mg/mL to 3.9 ± 0.03 mg/mL after going through the immobilized enzyme reactor. These results suggest that approximately 72% (n = 3, RSD = 0.7%) of SMPs were hydrolyzed to be peptides smaller than the lower limit of the BPA measurement, i.e., 3000 – 5000 Da [35].

3. 4. Conclusion

Electrospun PAN nanofibers were fabricated, modified by alkali-treatment and carboxyl activation, and subjected to Alcalase covalent immobilization. The presence of different functional groups demonstrated by FT-IR confirmed the successful immobilization of Alcalase onto the PAN nanofibers. At 25°C and 24 h of contact time, 22.78 mg/g-membrane of Alcalase was loaded, exhibiting an enzyme activity of 106.01 U/g-membrane. It was found that immobilized Alcalase retained higher enzyme activities in a broader range of pH and were more stable thermally than free Alcalase. PAN-BMs could maintain over 40% of its original activity after ten times of reuse at the optimum incubation conditions. The immobilization method herein described is not complex and therefore has a good potential for practical applications in the industry. In particular, significant improvements in the stability of Alcalase immobilized onto PAN nanofibers also demonstrate the possibility of decreasing costs in the industrial protein hydrolysis process. The higher extent of hydrolysis was obtained with more layers of membranes or smaller feed flux with a continuous PAN-BM reactor. High-molecular-weight SMPs were largely hydrolyzed to low-molecular-weight peptides in this reactor, which may have an attractive prospect in the application of SMPs hydrolysis to produce high-value products with elevated solubility and other functionalities.

3. 5. References

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Chapter 4:

Nanofibrous Biocatalytic Membrane Based on Polydopamine Coating for Continuous Protein Hydrolysis

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Abstract

Biocatalytic membrane (BM) combines the physical separation of reactants/products and the biocatalysis reaction by “anchoring” enzymes into/onto membranes. This research focused on the preparation of BMs by immobilizing Alcalase onto electrospun polyacrylonitrile nanofibrous membranes (EPNMs) based on polydopamine coating. The EPNMs and Alcalase-bearing BMs were characterized using scanning electron microscopy and Fourier Transform Infra-Red spectroscopy. Immobilization improved both pH and thermal stability of Alcalase compared with free Alcalase. The prepared BMs exhibited better storage stability than free enzymes in a period up to 60 days and could be reused up to 10 cycles. Inserting a spacer provides additional benefits including increased protein loading, enzymatic activity retention, and enzyme stability. Using a continuous single-layer BM reactor, 60% of the normalized extent of hydrolysis of model substrate azo-casein (5 mg/mL) was hydrolyzed to peptides of 10 kDa or smaller under the optimized conditions at a flux of 6 $L\cdot h^{-1}\cdot m^{-2}$, while 69% of soybean meal proteins were hydrolyzed with a five-layer continuous BM reactor at the same flux.

Key Words: Biocatalytic membranes; electrospun nanofibrous membranes, enzyme immobilization; protein hydrolysis; soybean meal proteins.

4.1. Introduction

Biocatalytic membranes (BMs) bearing enzyme integrates the catalytic functions of immobilized enzymes and the separation capacity of membranes and allows enzyme reuse, continuous processing and real-time separation of reactants and products to change the economics of the process towards a more viable situation [1][2]. With these functions, a wide range of traditional applications, including foods, pharmaceuticals and the environment, can be improved by the use of BMs to satisfy the growing demand of the society [2]. Enzyme immobilization is one of the most important steps for BMs preparation [3]. There are different strategies for enzyme immobilization into/onto membranes, which mainly include entrapment, physical adsorption and covalent bonding. Entrapment can immobilize enzymes and enable long retention of enzyme activity by avoiding conformation change, but it usually involves limited accessibility of high-molecular-weight substrates to enzymes and the presence of enzymes during the support material synthesis which limits the type of materials [4]. Physical adsorption is easy to operate under mild conditions, but the weak bindings between enzymes and membranes cause frequent enzyme leaking. Covalent bonding is strong and stable but requires suitable functional groups on the membrane surface for enzyme immobilization and may lead to the loss of enzyme activity and challenges in membrane regeneration [5][6].

Electrospun PAN nanofibrous membranes (EPNMs) have a large number of applications in filtration, separation, adsorption and composite material preparation, owing to their large surface area, chemical inertness, hydrophilicity and strong mechanical properties (e.g., high tensile strength and abrasion resistance) [7]-[12]. BMs can be fabricated by electrospinning with PAN and used to be carriers of immobilized enzymes. Xu et al. [13] immobilized laccase on modified

EPNMs and successfully applied them to remove 2,4,6-trichlorophenol from water samples. EPNMs were also modified with the use of imidoesterification and amidination reactions to immobilize Savinase, Alcalase and α-amylase and used as BMs for anti-biofilm activity [14].

Dopamine can adhere to diverse solid surfaces strongly after self-polymerizing in alkaline aqueous solutions exposing to the air to form a polydopamine (PDA) coating layer [15]. Thanks to its good dispersibility, hydrophilicity and biocompatibility, PDA coating is a well-adopted modification method with simple operations to functionalize a variety of materials and enhance material properties [16]. For example, Deng et al. [17] functionalized Fe_3O_4 magnetic nanoparticles surface with PDA and decorated MIL-101(Fe) on PDA coating layers for extracting sulfonylurea herbicides from water and vegetable samples. Some researchers have improved the properties of EPNMs based on PDA coating modification. For instance, Huang et al. [18] reported that a 100% increase of tensile strength (~10 MPa) and Young's module (~ 160 MPa) were obtained after PDA coating on EPNMs, while sustaining the same flexibility as before PDA coating. Wang et al. [19] modified electrospun PAN/hyperbranched polyethyleneimine membranes with PDA decoration and observed low water contact angle (0°), high oil contact angle (162°) and excellent capacity of oil/water separation (98.5% oil rejection).

It is reasonable to expect PDA to be an ideal bridging material for BMs preparation linking enzymes to the membrane surface by covalent bonding. PDA provides multiple functional groups, which makes it a suitable material for enzyme immobilization, given that the residual catechol and quinone groups on PDA can couple covalently with aldehyde- or amino-containing compounds via Michael addition and/or Schiff-base reaction [20]-[22]. Zhang et al. [23] studied the mechanisms of the immobilization of five common enzymes (laccase, glucose oxidase, lipase, pepsin and dextranase) onto different commercial porous flat-sheet and hollow fibre membranes based on PDA coating. Nonetheless, the stability and reusability of immobilized enzymes on electrospun nanofibrous membranes coated by PDA, can be further explored in order to apply them in the industry. Based on our knowledge, few explored the enzyme immobilization on PDA-coated electrospun nanofibrous membranes and biocatalytic nanofibrous membranes based on PDA coating have not been applied in a continuous biocatalytic reactor. Therefore, Alcalase (one of the important proteases has been found the ability to produce peptides with specific properties, such an antioxidant and emulsifying [24][25]) was covalently immobilized onto EPNMs based on PDA coating followed by grafting with glutaraldehyde (GA) to provide more flexibility and reduce steric hindrance for better accessibility of the active sites of the enzymes. In this work, we investigated the effects of incubation conditions on the activity of immobilized Alcalase, stability and reusability of the prepared BMs. Furthermore, the prepared BMs were applied in a continuous proteolytic reactor to hydrolyze soybean meal proteins (SMPs), whose solubility in water is low due to their large molecular weights.

4.2. Materials & Methods

4.2.1. Materials

Polyacrylonitrile (average molecular weight 1.5×10^5 Dalton), Alcalase 2.4 L FG (Protease from Bacillus licheniformis, Novozymes), Azo-casein (Protease substrate sulfanilamide azo-casein), *N*, *N*-Dimethyl formamide (DMF, 99.8%), dopamine hydrochloride (DA), glutaraldehyde (50% in H_2O) and Bradford protein assay reagents (BPA-1) were purchased from Sigma-Aldrich Inc. Bradford protein assay reagents (BPA-2) were purchased from Bio-Rad and Bovine serum albumin standards (BSA) were obtained from Thermo-Scientific, Rockford, IL. Defatted soybean meal samples (SBM) were supplied by Tri-county protein Corp., Winchester, Canada.

4.2.2. Fabrication of EPNMs

EPNMs were fabricated by the method described in ***Chapter 3.*** In brief, a PAN solution (8% w/w, PAN/DMF) was prepared by shaking the PAN/DMF mixture at 50°C for 24 h. The PAN solution was electrospun with the following parameters: the applied voltage (20.0 kV), the flow rate of the PAN solution (1.1 mL/h), the distance between the needle tip and the aluminum foil collector (25 cm). After the electrospinning, the EPNM was dried and stored at room temperature overnight before PDA coating.

4.2.3. Preparation of BMs based on PDA coating

The BMs were prepared in three steps, i.e., PDA coating, GA grafting, and Alcalase immobilization. These steps are described schematically in **Fig.4.1,** where monomers of PDA were used for simplifying the self-polymerization process of DA since the structure of PDA is complex and diverse.

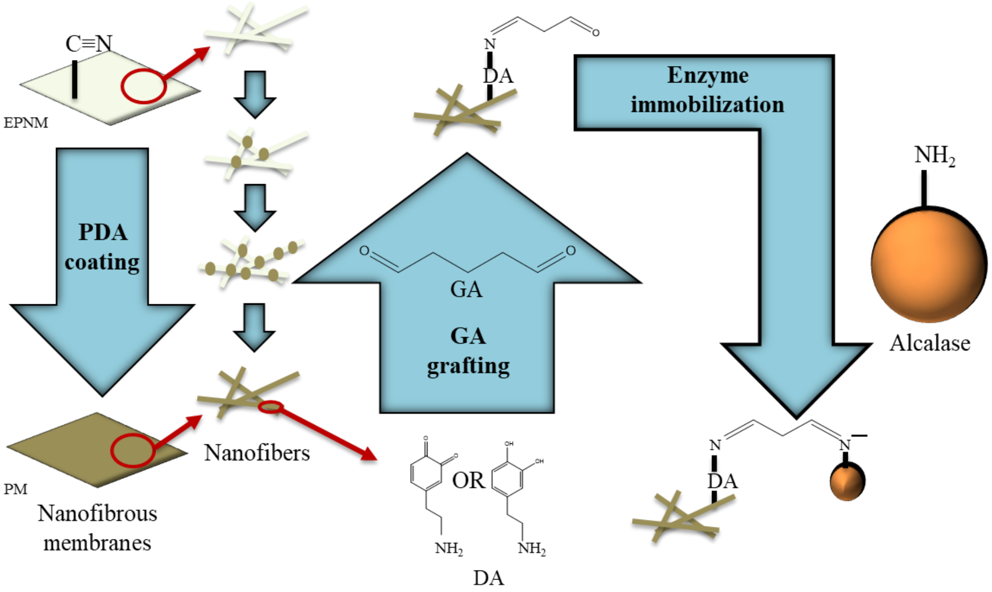

Fig. 4.1. Enzyme immobilization process based on PDA coating

PDA coating on EPNMs: The fabricated EPNM was carefully cut into an 8 × 8 cm piece and then peeled off from the aluminum foil. The EPNM piece was then immersed completely in the PDA coating solution, which was prepared by adding 0.2 g DA to 200 mL Tris-HCl buffer (10 mM, pH = 8.5), followed by shaking at 160 rpm for 8 h at room temperature. The obtained PDA-coated membrane (PM) was rinsed five times using deionized water (DI water) and stored in DI water at 4°C.

GA grafting of PMs: PMs were first air-dried at room temperature before use. GA was grafted on PMs based on the Schiff-base reaction and/or Michael addition [23]. As-purchased GA solution was diluted to 5% with phosphate buffer saline (PBS, 50 mM, pH = 8.0). PMs were placed in an as-prepared solution and grafted at 29°C with gentle shaking for 3 h. Afterward, the GA-grafted-PDA-coated membranes (GPMs) were transferred to DI water and washed thoroughly before the following step.

Immobilization of Alcalase on GPMs or PMs: GPMs were immersed in an Alcalase solution, which was 10 times diluted from the purchased Alcalase using PBS (50 mM, pH = 8.0), followed by shaking at 160 rpm for 3 h at 25°C. Subsequently, the Alcalase-bearing-GPMs (AGPMs) were washed several times with PBS (pH = 7.4), until no enzyme was detected in the washing solution by BPA-1. Likewise, Alcalase-immobilized-PDA-coated membranes (APMs) were prepared from PMs without the GA grafting step.

4.2.4. Characterization of nanofibrous membranes

The morphology of EPNMs, PMs and AGPMs were investigated using scanning electron microscopy (SEM, JSM-7500F). The nanofiber surface was gold-sputtered (5 nm) before SEM imaging. Fourier Transform Infra-Red spectroscopy (FT-IR, Agilent Cary 630 FT-IR spectrometer) was used to investigate the functional groups of the EPNMs, PMs, GPMs and AGPMs.

4.2.5. Determination of protein loading, enzyme activity & enzymatic reaction kinetics

Determination of protein loading: The protein loading of the APMs and AGPMs was evaluated using BPA-1 with BSA as the standard [26]. A microplate spectrophotometer (Epoch-BioTek, Agilent) was used to measure the absorbance at 595 nm and the protein loading was calculated by **Eq. 4.1** [13].

$$M_E = (C_0V_0 - C_RV_R - C_WV_W) / M_{membrane} \qquad \textbf{(4.1)}$$

Where M_E is the protein loading (mg/g-membrane); C_0, C_R and C_w are enzyme concentrations (mg/mL) in the original solution, the remaining solution and the washing solution, respectively;

V_0, V_R and V_W are volumes (mL) of the original solution, the remaining solution and washing solution, respectively; $M_{membrane}$ is the mass (g) of the membrane.

Azo-casein assay: The activity of the free and immobilized Alcalase was determined by the hydrolysis of the azo-casein assay in PBS (pH values were the same as the incubation pH values which were indicated in the following sections) [14]. The standard method for the activity measurement is as follows unless otherwise stated. Free and immobilized Alcalase whose protein contents were approximately equal were incubated in the azo-casein PBS solution (0.5% w/v, 0.5 mL) with shaking (210 rpm) for 10 min at a predetermined temperature (e.g., 50°C). 400 µL of the incubated solution was transferred to 1 mL of 5% (w/v) trichloroacetic acid solution (TCA), followed by centrifugation (14, 000 × g) for 5 min. Then 400 µL of the supernatant was transferred to 1 mL 0.1 M NaOH solution followed by vortex mixing for 10 s. The absorbance of the solution finally obtained was measured at 440 nm. The Alcalase activity is evaluated by the change of absorbance at 440 nm due to the free azo-dye liberated by azo-casein hydrolysis. Namely, the increased amount of free azo-dye is used to represent the reduced amount of azo-casein hydrolyzed by free or immobilized Alcalase. Herein, one enzyme unit (U) was defined as the amount of Alcalase to liberate 1 µmol of azo-dye per minute under the incubation condition and **Eq. 4.2** was used to calculate the activity of Alcalase (V_{act}, U/mL) [27]. The negative control corresponded to a solution with no enzyme.

$$V_{act} = \Delta A(K/\varepsilon t L) \qquad \mathbf{(4.2)}$$

Where ΔA, K, ε ($L \cdot mol^{-1} cm^{-1}$), t (min) and L (cm) refer to the change in absorbance, dilution ratio, extinction coefficient, incubation time and length of the optical pass, respectively.

Kinetic studies: Kinetic studies were carried out using different concentrations of azo-casein solutions from 0.25 - 2% (w/v) as the substrates at 50°C in PBS solution (pH = 8.5) for 10 min. **Eq. 4.3** is the Michaelis-Menten equation which was used to obtain the Michaelis-Menten constant (K_m) and the maximum reaction rate (V_{max}) by the fitting built-in model of the OriginLab.

$$V = V_{max}S/(K_m+S) \qquad \textbf{(4.3)}$$

where S is the substrate concentration (mg/mL).

4.2.6. Effects of pH and temperature on the activity of free & immobilized Alcalase

To investigate the effect of pH, free Alcalase, APMs and AGPMs were allowed to hydrolyze azo-casein in PBS buffer (pH values varied from 7.5 to 10.5) for 5 min at 50°C. To know the effect of temperature, free Alcalase, APMs and AGPMs were incubated in 0.5% azo-casein PBS solutions (pH = 8.5) at a predetermined temperature, ranging 20 - 70°C for 10 min. The activity of immobilized Alcalase was assayed by azo-casein assay with the aforementioned pH values and temperatures and compared with that of free Alcalase at the same incubation condition to obtain the effects of pH and temperature.

4.2.7. Stability of free and immobilized Alcalase

The thermal stability at 50 and 60°C of free and immobilized Alcalase was conducted in 0.5% azo-casein PBS solution (pH 8.5) recorded at 210 min intervals. Thermal stabilities at different temperature were evaluated by the relative activity. The initial enzyme activities at each temperature were set as 100% and the relative enzyme activities at different time intervals were then calculated. Free and immobilized Alcalase were also treated in PBS (50 mM, pH 7.4) at a temperature ranging from 4 to 70°C, for 48 h before incubation in order to further investigate their thermal stability.

The storage stability of Alcalase was evaluated by storing them at 4°C in PBS (50 mM, pH 7.4) at regular intervals up to 60 days before incubation. The activities of the free and immobilized Alcalase were then measured with the aforementioned standard azo-casein assay.

4.2.8. Reusability test

The reusability of BMs was studied by repeated usage for 10 cycles. The experiments were carried out with the aforementioned standard azo-casein assay (50°C, pH 8.5) for each cycle and washed three times by PBS (pH 8.5) before reuse. The relative activities of BMs were recorded after each cycle by comparing the activities with that in the first cycle.

4.2.9. Effects of substrate flux and AGPMs layers in a continuous system for protein hydrolysis

A dead-end, continuous system for protein hydrolysis is illustrated schematically in **Fig. 4.2**. A peristaltic pump (12000 Varioperpex, Bromma LKB, Sweden) was used to control the flux. The effect of the flux on the hydrolysis was studied by adjusting flux of 0.5% azo-casein PBS solution (pH 8.5) to 6, 12, 31, 61, 122 $L \cdot h^{-1} \cdot m^{-2}$ (given as the flux through the membrane) for one layer of BMs. As well, another test was carried out in the same condition for one to five layers of BMs at a flux of 6 $L \cdot h^{-1} \cdot m^{-2}$, respectively. For both, the hydrolysis temperature was 50°C. The hydrolysate solutions were then collected and subjected to the azo-casein assay. The hydrolysate was also analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis

(SDS-PAGE) using 12% acrylamide (diluted from 30% AccuGel, 1.5 M Tris, 10% SDS, 10% ammonium persulfate, *N*, *N*, *N'*, *N'*-tetramethyl ethylene diamine and deionized water) running gel in a Mini Protean system (Bio-Rad, Hercules, USA).

4.2.10. Hydrolysis of SMPs with AGPMs in the continuous system

SMPs solution was prepared as follows. SMPs were first extracted from as received soybean meals under stirring at 50°C for one hour, while maintaining the pH at 10.0 by adding 0.1 M NaOH solution. After cooling down to room temperature, the suspension was centrifuged at 3070 × g for 20 min and the supernatant was collected. Spin-X® UF concentrators (Corning, England) with 10 kDa molecular weight cut-off were used to one-time concentrate SMPs at 15, 000 × g for 10 min. The pH of SMPs solution was adjusted to pH 8.5 with 0.1 M HCl. BPA-2 was used to determine the protein concentration of as-prepared SMPs solution with BSA as standards [28]. SMPs solution was subjected to continuous hydrolysis with a five-layer AGPMs at a flux of 6 $L \cdot h^{-1} \cdot m^{-2}$ and a temperature of 50°C. The hydrolysate was collected and analyzed by BPA-2 and SDS-PAGE, respectively.

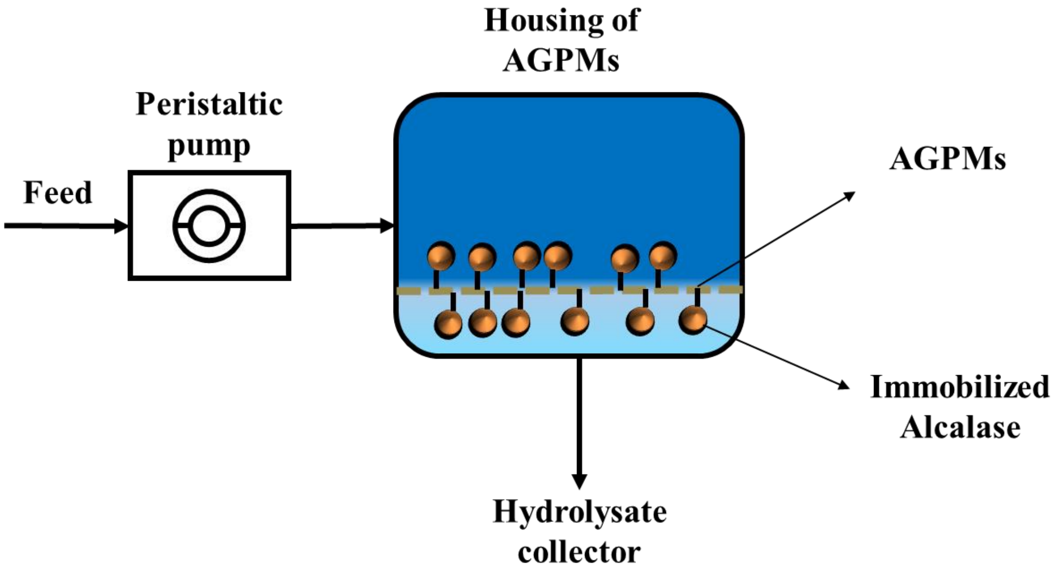

Fig. 4.2. Schematic of the continuous system for protein hydrolysis

4.3. Results & Discussion

4.3.1. Characterization of nanofibrous membranes by SEM and FT-IR

SEM images and digital photographs (insert images) of EPNMs, PMs and AGPMs in **Fig.4.3** show the structural and surface morphological change of the nanofibrous membranes caused by PDA coating and immobilization. **Fig. 4.3a** shows the typical SEM image of untreated EPNMs whose nanofibers are randomly distributed with the diameter in the range of about 200 – 400 nm and with a smooth surface. The white color EPNMs changed to black-brown due to the self-polymerization of DA after PDA coating, as shown in the insert images of **Fig. 4.3a** and **b**. The SEM images of **Fig 4.3a** and **b** imply that the PDA layer was coated on the surface of each nanofibers, which differs from the reported PDA coating skin layers covering flat sheet membranes [18][29]. This is likely due to the highly porous and interconnected structure of EPNMs, which promote the quick diffusion of DA monomer into the membrane matrices to form a uniform PDA layer. It is worth noting that the surfaces of PDA coated nanofibers are rougher than those before PDA coating. For example, some visible cracks on the surface of nanofibers and self-aggregated PDA particles can also be observed on the surface of nanofibers, which is compatible with other reported results [30][31]. Moreover, it can be observed in **Fig. 4.3b** that adjacent nanofibers were adhered to each other after PDA coating and formed more entanglements than that before PDA coating. This change might have resulted from the adhesion property of PDA due to its catechol- and amine-like structure and the formation of poly-conjugation and the inter-molecular forces of the abundant aromatic groups of PDA [15]. **Fig. 4.3c** indicates that the GA grafting and enzyme immobilization steps would not damage the surface of nanofibers.

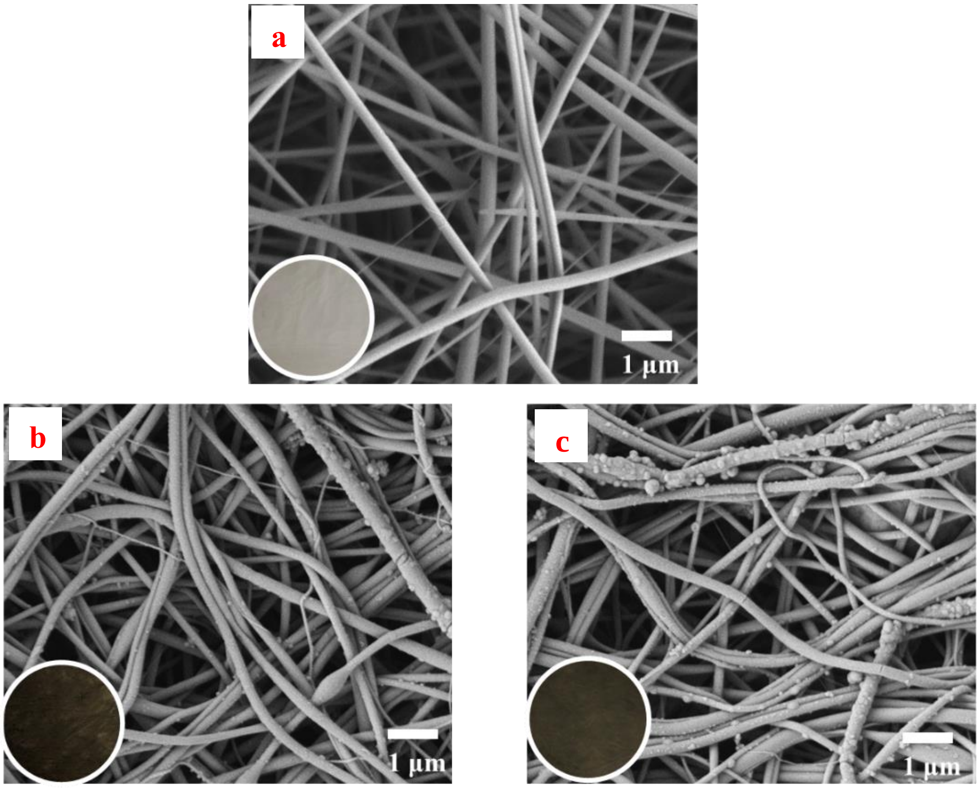

Fig. 4.3. SEM images and digital images (insert images) of (a) EPNMs; (b) PMs; (c) AGPMs

The results of FT-IR are shown in **Fig. 4.4**. The peak at 2243 cm^{-1} indicates the stretching vibration of C≡N, the characteristic peak of PAN. New peaks at 3361 and 1630 cm^{-1} represent the stretching and bending vibration of N-H from the PDA layer, implying the successful preparation of PMs [20]. After the GA grafting step, extra peaks appeared at 2875, 1712 and 960 cm^{-1}, represent the stretching vibration of aldehydic C-H and C=O, and the out-of-plane bending vibration of CH [23]. Thus, GA was successfully grafted on PMs and ready for the covalent immobilization of Alcalase. These extra peaks became weaker or disappeared after enzyme immobilization, which implies the success of covalent enzyme immobilization due to the consumption of aldehydic groups for the Schiff-base reaction for enzyme immobilization. A stronger peak at 3361 cm^{-1} may also confirm this result for the additional amino groups from enzymes.

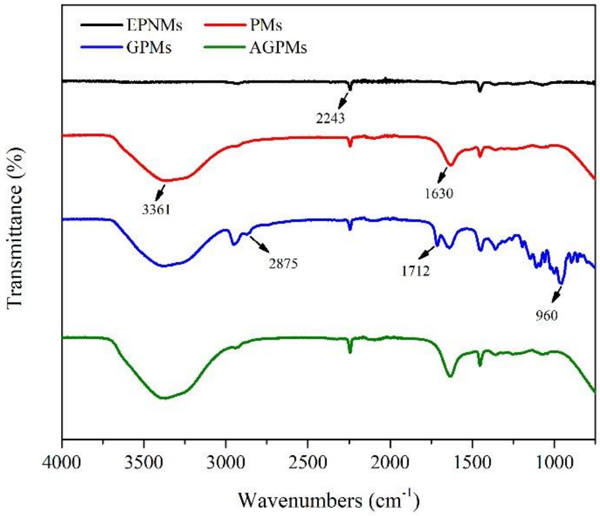

Fig. 4.4. FT-IR spectra of EPNMs, PMs, GPMs and AGPMs

4.3.2. Kinetics of free and immobilized Alcalase

Experiments were carried out at different initial substrate concentrations to study the enzymatic reaction kinetics of AGPMs, APMs and free Alcalase. Experimental data were fitted non-linearly to the Michaelis-Menten model (**Fig. 4.5**) to determine the kinetic parameters, which are presented in **Table 4.1**. The Michaelis-Menten constant (K_m) of APMs is 4.7 g/L, which was 25% higher than free Alcalase (3.5 g/L). This was likely caused by the conformational changes of the enzymatic protein when it was covalently linked to the coated PDA layer, which resulted in the decrease of the affinity of the substrate to the active sites [32]. Furthermore, upon the completion of hydrolysis, enzymes have to change their conformation once more for the release of products according to both the induced-fit model and key-lock theory of enzymatic reactions [33]. This conformation change for the product release also affects the overall affinity of the enzyme-substrate complex, and therefore the value of K_m.

Table 4.1. Summary of kinetic parameters of free and immobilized Alcalase (n = 3)

Materials	**K_m ($g \cdot L^{-1}$)**	**V_{max} ($U \cdot mg^{-1}$ -protein)**	**Protein Loading ($mg \cdot g^{-1}$ -membrane)**	**Activity Retention (%)**
AGPMs	2.5 ± 0.52	23.8 ± 1.11	76.4 ± 2.94	74
APMs	4.7 ± 0.85	21.6 ± 1.24	55.3 ± 2.12	24
Free Alcalase	3.5 ± 0.65	70.7 ± 3.57	–	–

The V_m of APM is 21.6 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ -protein, 33.7% of that of free Alcalase (70.7 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ -protein), indicating a significant decrease of the number of active sites accessible to substrates. This could be attributed to the additional steric hindrance caused by the immobilization of Alcalase that led to activate site blockage and substrate diffusion limitation [34].

While free enzymes can collide with substrate molecules freely in the solution, substrate proteins must diffuse to the membrane surface and even into the polymer matrix to access the active sites of the immobilized enzymes. The slow diffusion process of large protein molecules may also affect both the K_m and V_{max} of the immobilized enzyme in comparison to free enzyme.

The benefits of introducing GA molecules as spacers are obvious. The protein loading of AGPM (76.4 mg·g^{-1} -membrane) is 138% of that of APM. These results suggest that grafting of GA on the PDA coating made more anchoring sites available for the loading of enzymatic proteins. Furthermore, the V_m and K_m of AGPM were 110% and 53% of APM, respectively, indicating the introduction of GA molecules as spacers made more active sites available for substrates and more significantly, increased the affinity of the substrate-enzyme complex. It is worth noting that the K_m of APGM (2.5 g·L^{-1}) is 71% of that of the free enzyme (3.5 g·L^{-1}), corresponding to a higher affinity of the immobilized Alcalase with the substrate than that between the free Alcalase and substrate. This rather unexpected result suggests that conformational changes of the enzyme in association with the covalent bonding with the GA spacer enhanced the affinity of the active sites of the enzyme to the substrate and that the extent of the affinity enhancement was more than the affinity reduction caused by the diffusion limitation in association with the attachment of enzymes onto the membrane surface through the spacer [10]. This is of particular interest in the context of the aforementioned substantial decrease of substrate affinity when Alcalase was covalently bonded to the PDA coating without the spacer. As a result of the enhancement of affinity and mitigation of V_m reduction, AGPM exhibited activity retention of 74% in reference to the free Alcalase, which is 3.1 times of that of APM (24% activity retention).

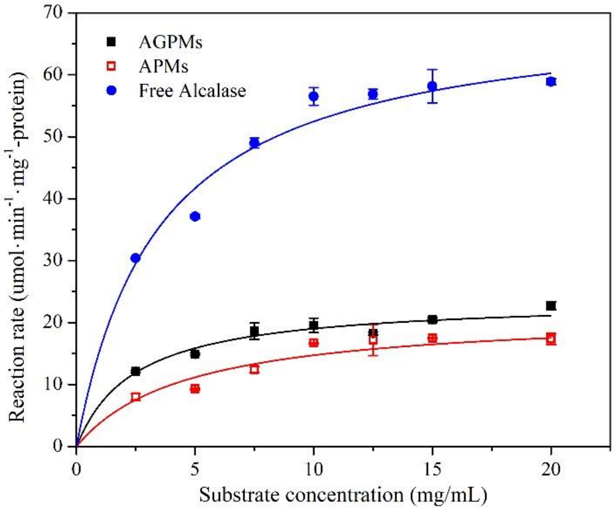

Fig. 4.5. Michaelis-Menten plots of AGPMs, APMs and free Alcalase

(symbols, experimental data; solid lines, non-linear fitting curves)

4.3.3. Impacts of immobilization on pH and temperature optima of enzymatic activity

Reaction buffer pH plays an important role in the conformation of enzymes, which may exert a significant influence on the enzyme activity through reversible or irreversible changes of enzyme structure and dissociation of ions of functional groups in enzyme's active sites. A series of experiments were performed at 50°C and an incubation period of 5 min in 0.5% azo-casein PBS solution over a pH range of 7.5 to 10.5. **Fig. 4.6a** reports the relative enzymatic activities of free Alcalase, APM, and AGPM, which are defined as the enzyme activities of them at a given pH normalized by the activity of each of them at their corresponding optimal pH, i.e., pH 8.5, pH 9.0 and pH 8.5 for free Alcalase, APM, and AGPM, respectively. The pH optimum shifted from pH

8.5 of the free enzyme to pH 9.0 of APM and the optimal peak of APM flattened significantly in comparison to that of the free enzyme, representing a broadened optimal pH range. The optimal pH of AGPM was the same as that of the free enzyme, i.e., pH 8.5, while the optimal peak was also significantly flattened.

The shift of pH optimum from 8.5 of free Alcalase to 9.0 of APM might be tentatively explained by the fact that the immobilized enzymes were very close to the polydopamine groups on the coating surface, which was negatively charged and thus impulsive to the OH^- from the bulk solution [35][36]. Therefore, AMP needs higher bulk pH (i.e., higher OH^- concentration) than the free enzyme to achieve the optimal ionic state in the active sites. On the other hand, the introduction of GA spacer between the enzymatic protein and the PDA coating in AGPM helped avoid such a scenario to prevent the shift of pH optimum.

The immobilization of enzymes might promote the rigidification of the three-dimensional structure of enzymes and change their pendent groups and microenvironment, which reduces the structural changes and activity loss caused by the pH variation [37]. Hence, both AGPMs or APMs are both less susceptibility to pH change, offering an enlarged range of pH range of stable enzymatic activities than the free enzyme.

Temperature is another important factor affecting enzyme stability and activity. **Fig. 4.6b** presents the relative activities of free Alcalase, APMs and AGPMs (pH 9.0). Reported are the relative enzymatic activities, i.e., the enzymatic activities of free Alcalase, APM, or AGPM at varied temperatures normalized by those of the same enzyme or BMs at 60°C. The free enzyme exhibited higher relative activity than the immobilized enzyme in the temperature range from 20 to 60°C. This could be ascribed to the diffusion limitation of substrates to active sites of enzymes immobilized on membrane surface and matrix, which is stronger at lower temperatures. It is interesting to notice that AGPMs retained almost 97% relative activity at 70°C, while that of free enzyme and APM were 90% and 80%, respectively. These results suggest that while both the free enzyme and APM became unstable at 70°C, while the AGPM maintained stable at 70°C. In other words, the results suggest that the introduction of GA as a spacer also improved the rigidification and stability of the enzyme.

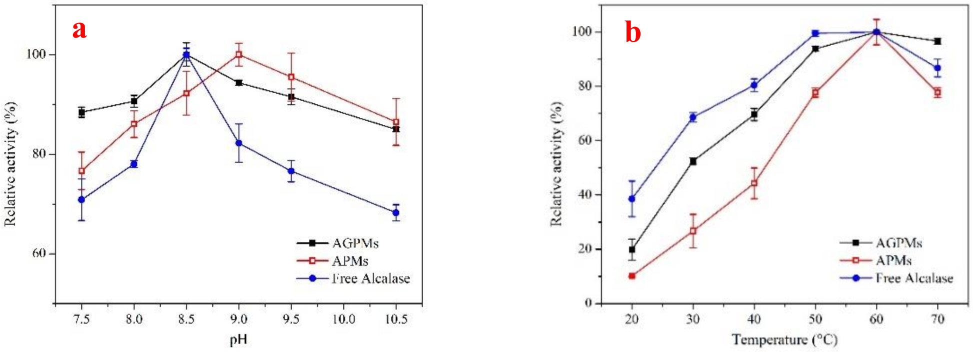

Fig. 4.6. Effect of (a) pH and (b) temperature on enzyme activity of free and immobilized Alcalase

4.3.4. Thermal stability of free and immobilized Alcalase

The thermal stabilities of free Alcalase and BMs were studied at 50 and 60°C and recorded every 30 min. As shown in **Fig. 4.7**, at 50°C, AGPMs retained approximately 95% of its initial activity after 90 min of incubation, while APMs maintained 91% of its initial activity, which were higher than that of the free Alcalase (66%). After 210 min, AGPMs retained 88% of relative activity that is higher than that of APMs (78%) and free Alcalase (44%). Although the thermal stability was reduced at the higher temperature of 60°C (210 min, **Fig. 4.7b**), AGPMs and APMs showed better thermal stabilities (70% and 54%, respectively) than that of free Alcalase (23%). The thermal stability of immobilized Alcalase of this work (e.g., AGPMs maintained 90% and 75% of their initial activities at 50 and 60°C, respectively) is superior to that of a case study reported in the literature, in which the relative activity of immobilized Alcalase decreased to 86% and 70% at 50 and 60°C after 180 min incubation, respectively [38]. The enhanced thermal stability of immobilized Alcalase could be ascribed to the rigidification and additional steric hindrance of immobilized Alcalase, which protects the enzyme from unfolding under harsh environment [39] [40]. Because of less inactivation at 50°C of both free and immobilized Alcalase, a conclusion can be made that 50°C is a better incubation temperature with a significantly longer enzyme lifetime than 60°C.

The thermal stability of free Alcalase and BMs were also investigated by treating them at different temperatures (4, 20, 30, 40, 50, 60 and 70°C) in PBS solution (pH 7.4) for 48 h before being subjected to the standard azo-casein assay at 50°C and pH 8.5. As shown in **Fig. 4.7c**, results demonstrated remarkable improvement of BMs over free Alcalase at all the tested storage temperatures while the two different BMs, i.e., APM and AGPM, did not show a significant difference from each other. For both free and immobilized enzymes, the activity was best retained when stored at 4°C, therefore all activities at other (higher) temperatures were normalized by the activity at 4°C to get the relative enzymatic activity. The result shows a significant loss of free Alcalase activity in the 48-hour storage, resulting in complete inactivation at 40°C or above. However, AGPMs and APMs retained over 60% and 16% of its initial activity at 40 and 50°C, respectively, after the same period of storage. This test once again demonstrates that AGPM had better thermal stability than APM, which confirms the benefits of the GA spacer in the immobilization of Alcalase on PDA coating. The improved thermal and storage stability is a valuable feature to potential industrial applications, especially in continuous hydrolysis of proteins [20].

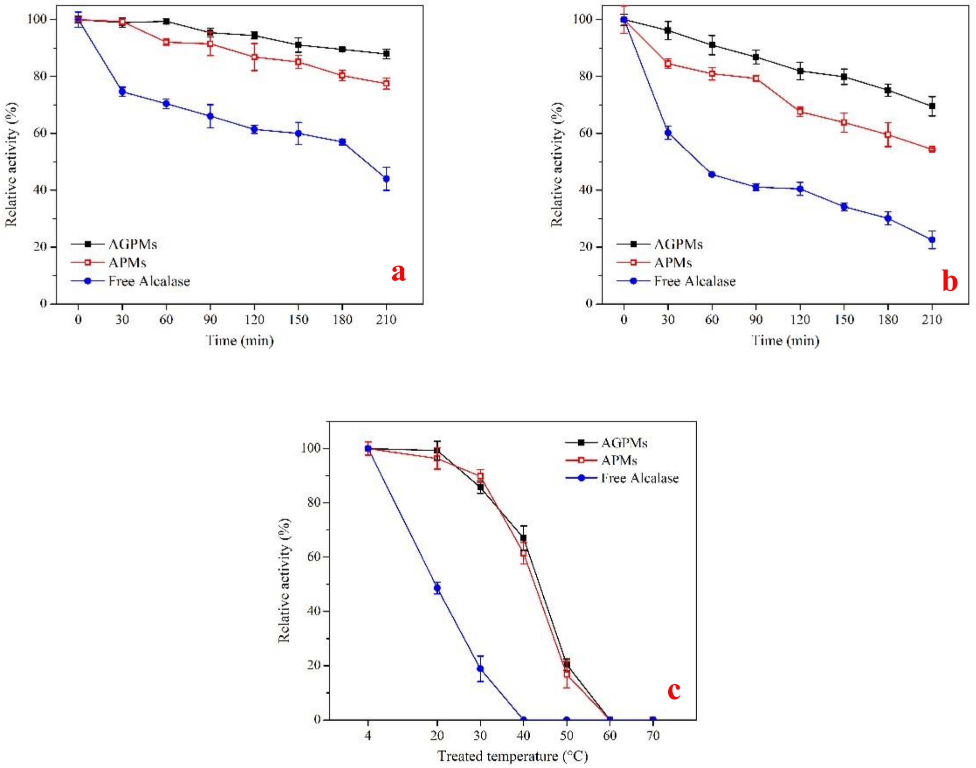

Fig. 4.7. Thermal stability of free Alcalase, AMP, and AGMP (a) at 50°C, (b) at 60°C and (c) at different temperatures for 48 hours

4.3.5.Storage stability of free and immobilized Alcalase

The storage stability is one of the features that are crucial for practical applications of enzymes and BMs. For estimating the storage stability, all samples were stored in PBS solution (50 mM, pH 7.4) at 4°C for scheduled days before the azo-casein assays (pH 8.5, 50°C, and 10 min). The stability tests on long term storage at 4°C show that immobilization improved the stability of Alcalase. For example, the relative activities free enzyme, APMs and AGPMs decreased after storing for 25, 35 and 45 days, respectively, to approximately 90% at 4°C. After 60-day storage, the relative activity of the free enzyme dropped to 76%, while AGPMs maintained 87% of its initial activity. The improvement of the storage stability may have been contributed by the enhanced rigidification of enzymes after immobilization, which helps slow down enzyme unfolding under non-optimal conditions as more linkages/forces are to be broken for enzymes losing the three-dimensional structure [41][42]. It is also beneficial to the enhanced storage stability that the conformation change of enzymes was limited by their additional steric hindrance and less mobility and flexibility after immobilization [43][44]. Furthermore, the protective effects of membranes can increase the enzyme resistance to environmental factors and protect immobilized enzymes, such as slower diffusion of change of ions in solution caused by pH vibration and electrostatic interaction between the membrane and the bulk solution [45]. AGPMs with enhanced stability than free Alcalase and better activity retention than APMs, would be a potential BM for practical applications.

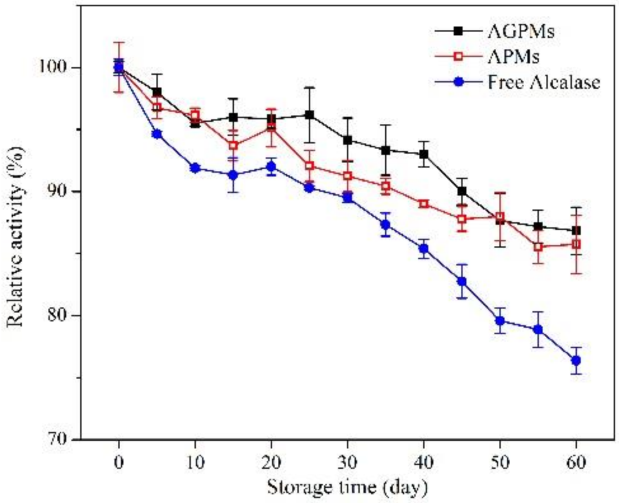

Fig. 4.8. Storage stability of free and immobilized Alcalase at 4°C

4.3.6. Reusability test

Besides the thermal stability and storage stability, the reusability of BMs is also one of the important aspects of practical applications due to its economic significance for continuous operation [46]. Hence, azo-casein assays were carried out at 50°C, pH 8.5 in 10 cycles. **Fig. 4.9** shows that AGPMs retained about 84% of its original activity in the second cycle and APMs showed a similar degree of relative activity (82%). However, AGPMs still maintained over 22% of its original activity after ten repeated reuses while APMs lost the activity completely after seven operation cycles. A significant relative activity decrease of BMs in the first four cycles may suggest that multiple layers of immobilized Alcalase on BMs were adsorbed on the PDA coating layers via relatively weak π–π stacking and/or van der Waals interaction, which may lead to enzyme leakages after several utilizations at a high temperature (50°C) and rinses [47]. AGPMs had more stable covalent bonding and GA spacers between enzyme and PDA coating layers in comparison to APMs, which improved their reusability. The loss of activity of both nanofibrous membranes was also caused by the thermal stability and denaturation of enzymes, which affect their capacity to be reused.

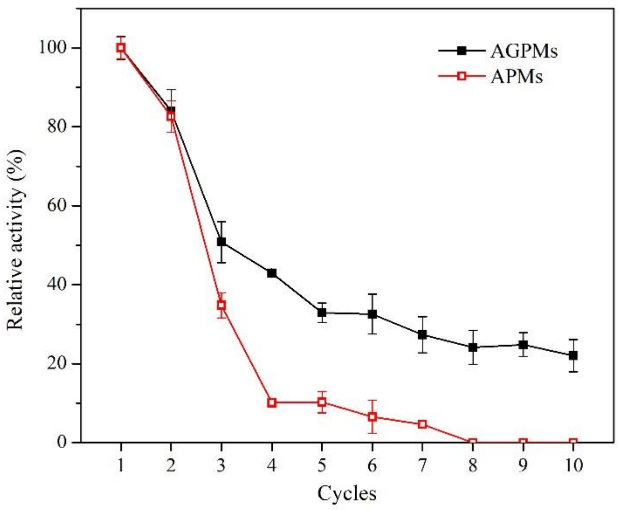

Fig. 4.9. Reusability of AGPMs and APMs

4.3.7. Effects of substrate flux and AGPMs layers in the continuous system on protein hydrolysis

Studies on the effects of feed flux on the extent of hydrolysis of 0.5% azo-casein using a continuous AGPM reactor have been carried out and the results are shown in **Fig. 4.10a**. In this experiment, the hydrolysis of 0.5% azo-casein solution by the free enzyme at a dosage 100 folds of that in the free enzyme tests reported in the previous sections with a prolonged hydrolysis time of 24 hours is assigned to be 100% as a benchmark for comparison. The normalized extent of hydrolysis (NEH) is defined as the ratio of the absorbance at 440 nm of the samples collected from the continuous hydrolysis reactor at a given flux to that of the aforementioned benchmark. 52% of NEH was observed at a flux of 6 L·h^{-1}·m^{-2}. A decrease to around 27% occurred when the flux increased to 12 L·h^{-1}·m^{-2} levelled off at around 6% at 122 L·h^{-1}·m^{-2}.

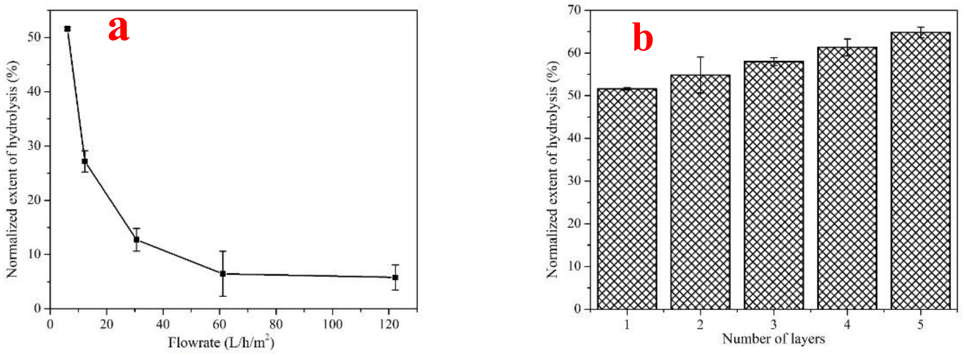

Fig. 4.10. The normalized extent of hydrolysis with different (a) flux and (b) layers of membranes

The results of studies on the effect of the number of AGPMs layers in the continuous reactor on the NEH of 0.5% azo-casein feed solution at a flux of 6 L·h^{-1}·m^{-2} are shown in **Fig. 4.10b**. The NEH was 52% with a one-layer reactor, which increased to 65% with a five-layer reactor. The increase of the amount of enzyme and contact time of the substrates and enzymes in association with increasing the number of AGPM layers may lead to higher NEH at the same flux.

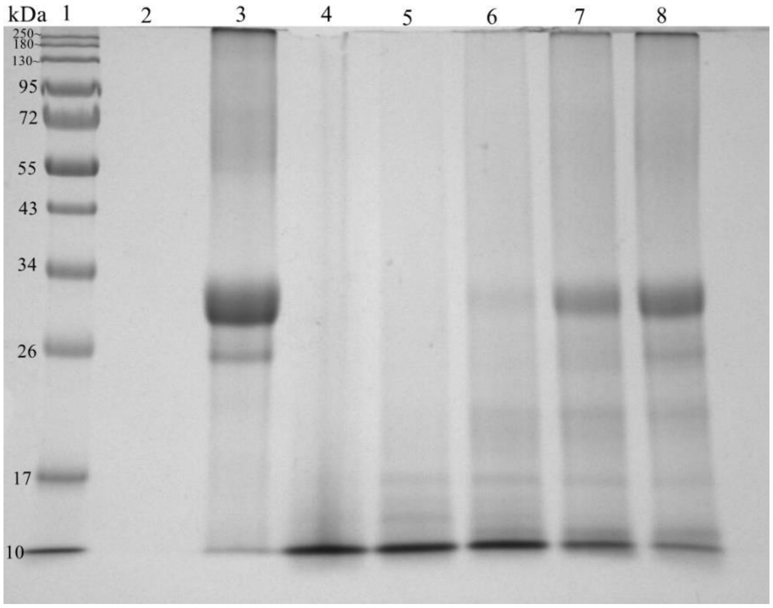

Fig. 4.11. SDS-PAGE of hydrolysates obtained with a one-layer AGPM reactor at different feed fluxes: Lane 1, molecular weight marker; Lane 2, negative control; Lane 3, positive control; Lane 4 – 8, hydrolysates obtained with the one-layer membrane at 6, 12, 31, 61, 122 L·h^{-1}·m^{-2}, respectively

The SDS-PAGE image of hydrolysis of 0.5% azo-casein at varied flux with a single-layer reactor (**Fig. 4.11**) shows the molecular weight profiles of peptides thus produced. Lane 2 was the negative control with a PBS solution (pH 8.5) whereas lane 3 with 0.5% azo-casein PBS solution (pH 8.5) was a positive control, which was characterized by a predominant band of a molecular weight slightly smaller than the 34 kDa marker. As shown in **Fig. 4.11**, the band characteristic of azo-casein almost disappeared after hydrolysis with a continuous single-layer AGPM reactor at 6 and 12 L·h^{-1}·m^{-2}. This suggests the substrate azo-casein was completely hydrolyzed to small peptides of 10 kDa or smaller. In comparison, the characteristic band reappeared when the flux increased to 31 L·h^{-1}·m^{-2}. The intensity of the characteristic band increased with increasing flux and this band was almost identical to that of the positive control when the flux was 122 L·h^{-1}·m^{-2}, implying lack of hydrolysis at those high fluxes, apparently due to the extremely small residence time. Nonetheless, the peptide profile at lane 8 shows that hydrolysis still occurred when the flux was 122 $L\cdot h^{-1}\cdot m^{-2}$ due to the clear band marks below 26 kDa compared with the positive control. It is of interest to note that the band of 17 kDa and the band between 17 and 26 kDa appeared at each flux, indicating a possibility to produce specific peptides of these molecular weights if conditions (e.g., flux) are fine-tuned.

The intensities and areas of the bands in each lane in **Fig. 4.11** were evaluated by the UltraQuant software for semi-quantitative analysis of the peptides in each band. The percentages of the peptides represented by individual bands in a lane in the gel image on the basis of total peptides in the corresponding lane were obtained (**Table 4.2**). the sum of all the obtained bands is always less than 100% since only the bands corresponding to the markers are counted. For instance, the predominant band of the positive control was between the markers of 26 and 34 kDa, which was off both markers and the sum of the four bands is thus only 45% of the total.

As presented in **Table 4.2**, the positive control has only 2% peptides at 10 kDa. After the hydrolysis in a single layer AGPM reactor at a flux of 6 $L\cdot h^{-1}\cdot m^{-2}$, however, 34 kDa were detected by UltraQuant, implying complete hydrolysis of the original substrate to a certain extent. At the same time, the 10 kDa band increased from 2% in the positive control lane to 60% in the hydrolysates from the single-layer reactor at 6 $L\cdot h^{-1}\cdot m^{-2}$. Peptides of smaller molecular weight than 10 kDa would have run out the gel and thus not featured in either **Fig 4.11** or **Table 4.2**. Although the increasing flux led to the decreasing degree of hydrolysis due to the short contact time between immobilized enzymes and substrates, the azo-casein substrate was hydrolyzed at the flux of 122 $L\cdot h^{-1}\cdot m^{-2}$, leading to a decrease from 35% to 21% at 34 kDa and an increase from 3% to 10% at 10 kDa in comparison to the positive control (**Table 4.2**). An observable fading between 26 – 34 kDa and appearance of peptides between 17 – 26 of lane 7 and 8 compared with the positive control (lane 3) in **Fig. 4.11**, can confirm this semi-quantitative result.

Table 4.2. The molecular weight distribution of peptides in hydrolysates of azo-casein from a single layer reactor at different flux

Marker (kDa)	Positive control	Flux (L·h^{-1}·m^{-2})				
		6	12	31	61	122
34	35%	–	–	–	7%	21%
26	8%	–	–	–	–	10%
17	–	–	–	–	–	6%
10	2%	60%	47%	38%	21%	10%

The SDS-PAGE image in **Fig. 4.12**, was observed from the hydrolysates of azo-casein with multilayer AGPM reactors at 6 L·h^{-1}·m^{-2}, together with that of the hydrolysate of SMPs hydrolysis with a five-layer AGPM reactor at the same flux. Lanes 1 – 7 show that all the original azo-casein in the feed was hydrolyzed to a certain extent no matter how many layers were used. However, hydrolysates of molecular weight less than 10 kDa were undetectable in this experiment due to the limitation of the SDS PAGE use in this study. Nevertheless, around 60% was already the band of 10 kDa when using a single-layer AGPM reactor, indicating that the overwhelming peptides in these hydrolysates were of 10 kDa or less molecular weight.

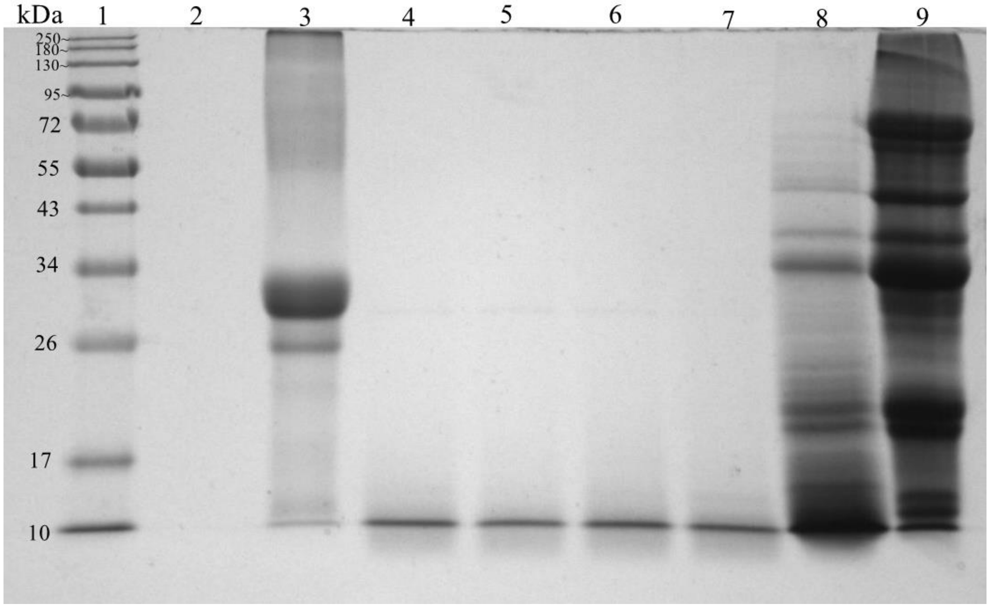

Fig. 4.12. SDS-PAGE images of hydrolysates from multilayer AGPM reactor with azo-casein or SMPs: Lane 1, molecular weight marker; Lane 2, negative control (PBS at pH 8.5); Lane 3, positive control for lane 4 – 8 (0.5% azo-casein at pH 8.5); Lanes 4 – 7, hydrolysates at 6 L·h^{-1}·m^{-2} with 2, 3, 4, 5 layers of PAN-BM, respectively; Lane 8, hydrolysates of 1.4% SMPs at pH 8.5 using a five-layer AGPM reactor; and Lane 9, positive control for lane 8 (1.4% SMPs at pH 8.5)

4.3.8. Hydrolysis of SMPs with AGPMs

The peptide profiles of the hydrolysates of SMPs obtained with a continuous five-layer AGPMs reactor at 6 L·h^{-1}·m^{-2} are shown in **Fig. 4.12** as well (Lane 8 and 9). The positive control, in this case, is Lane 9 in the SDS-PAGE, which is the 1.4% SMPs solution at pH 8.5. This lane shows that SMPs are composed of proteins with a wide range of molecular weights. The bands of Lane 8 indicate that most large molecular weight proteins in SMPs were hydrolyzed to a certain extent and a significant portion of the product peptides were of 10 kDa. It is understood that all peptides smaller than 10 kDa were undetectable in the SDS-PAGE.

The results of semi-quantitative analysis by UltraQuant are present in **Table 4.3**. 14% protein of the SMPs was in the range of 130 – 250 kDa where zero percent of protein remained after hydrolysis using a five-layer AGPMs reactor. Meanwhile, protein at the band of 10 kDa increased remarkably from 3 to 51% after hydrolysis, implying most high-molecular-weight SMPs were hydrolyzed to low-molecular-weight peptides. BPA-2 was used to measure the SMPs solution before and after hydrolysis. This assay indicated that the protein concentration of SMPs dropped from 14.0 ± 0.09 mg/mL to 4.4 ± 0.04 mg/mL after going though the five-layer AGPMs reactor. This result suggests that around 69% (n = 3, RSD = 1.0%) of SMPs were hydrolyzed to be peptides whose molecular weights are smaller than the lower limit of the BPA measurement which is 3000 – 5000 Da [48].

Table 4.3. The molecular weight distribution of peptides in hydrolysates of SMPs from a five-layer reactor at 6 L·h^{-1}·m^{-2}

Molecular weight (kDa)	Before hydrolysis	After hydrolysis
130 – 250	14%	0%
26 – 95	33%	11%
17	32%	23%
10	3%	51%

4.4. Conclusion

In this study, BMs were successfully developed by immobilizing a commercially important protease, Alcalase, on to electrospun PAN nanofibrous membrane (EPNM) modified in two different ways. The first modification approach was to coat the EPNM with PDA, onto which Alcalase was covalently bonded. The second approach was to graft the PDA coating with GA as a spacer, which in turn covalently links to Alcalase to provide additional flexibility and to reduce steric hindrance. The Alcalase-bearing BM with GA spacer exhibited enhanced performance. Results demonstrated better pH and thermal stability of BMs than the free Alcalase and their excellent reusability. Tests on the hydrolysis of model substrate azo-casein showed that the extent of hydrolysis increased with the decrease of feed flux when a monolayer BM reactor was used. Results also demonstrated with multilayer BM reactors that the extent of hydrolysis increasing with the number of layers at constant feed flux. The established continuous proteolytic system showed that large molecular weight soybean milk proteins could be hydrolyzed to low molecular weight peptides in a five-layer BM reactor at a flux of 6 $L \cdot h^{-1} \cdot m^{-2}$.

4.5. References

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Chapter 5:

Conclusions and Recommendations

5.1. Conclusions

In this thesis, two different biocatalytic nanofibrous membranes have been prepared with PAN as the matrix polymer for electrospinning and with two different modification approaches to immobilize Alcalase covalently for protein hydrolysis in a dead-end continuous reactor. The following are concluded based on the synthesis of these two modification approaches.

(1) Alkali modification, which is safe and simple, was successfully used to modify EPNMs; then the carbodiimide method involving EDC and NHS was used to activate the carboxyl groups pendent on the alkali-treated PAN nanofibrous membranes for Alcalase immobilization (Chapter 3).

However, relatively low activity retention of Alcalase-functionalized PAN biocatalytic nanofibrous membranes (PAN-BMs) obtained is likely due to the short covalent bond between Alcalase and membrane (**Fig. 5.1a**) and may cause low-efficiency protein hydrolysis.

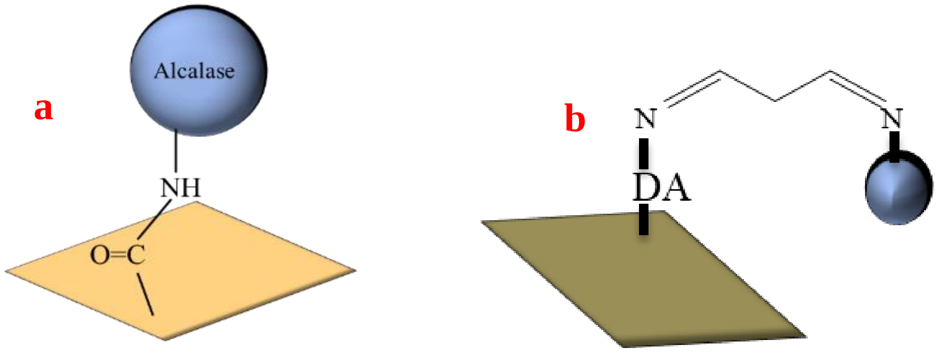

Fig. 5.1. The covalent bonds between Alcalase and BMs: (a) PAN-BMs; (b) AGPMs

(2) EPNMs can be modified by PDA coating followed by GA grafting for enzyme immobilization based on the characterization of SEM and the presence of functional

groups in FT-IR spectra. Grafted GA played a role as the elongating "bridge" by covalently bonding with PDA and enzyme with Schiff-base formation and/or Michael addition (**Fig. 5.1b**). Higher activity retention of Alcalase-bearing-GA-grafting-PDA-coating PAN nanofibrous membranes (AGPMs) was observed than PAN-BMs.

(3) Both PAN-BMs and AGPMs were more stable in a boarder range of pH than free enzyme. Immobilized Alcalase on AGPMs performed better stability in the alkali range likely due to the microenvironments of immobilized Alcalase as PDA is negatively charged for the abundant amino groups/hydroxyl groups, which likely protect Alcalase from denaturation/inactivation in high bulk pH.

(4) Both PAN-BMs and AGPMs performed improved thermal stability. The covalent bonds between enzymes and membranes can reduce enzyme mobility whereas increase their rigidification. The PAN-BMs thus have better thermal stability than AGPMs. On the other hand, the short covalent bond of PAN-BMs also increases the steric hindrance and inhibition of product release due to the less space for the change of enzyme conformation and exposure of the active side, resulting in lower activity retention.

(5) Both PAN-BMs and AGPMs can be reused, which is suitable for the industrial purpose of cost reduction. However, AGPMs showed a bit less reusability. The modification approach was based on PDA coating, which is depended on the adhesion of PDA on EPNMs. The adhesion may be weakened after reusing AGPMs under relatively high temperature and the leaching of PDA layer which is with immobilized enzyme may thus occur.

(6) Decreasing flux for the BM reactor with either single-layer PAN-BM or AGPM can increase the extent of hydrolysis of azo-casein. The higher extent of hydrolysis was observed with the use of AGPMs than PAN-BMs at all tested flux, even in the higher flux that we tested, which may contribute to the higher activity retention of AGPMs. An intuitionistic comparison is labelled in **Fig. 5.2** for indicating the different extent of hydrolysates hydrolyzed by different BMs.

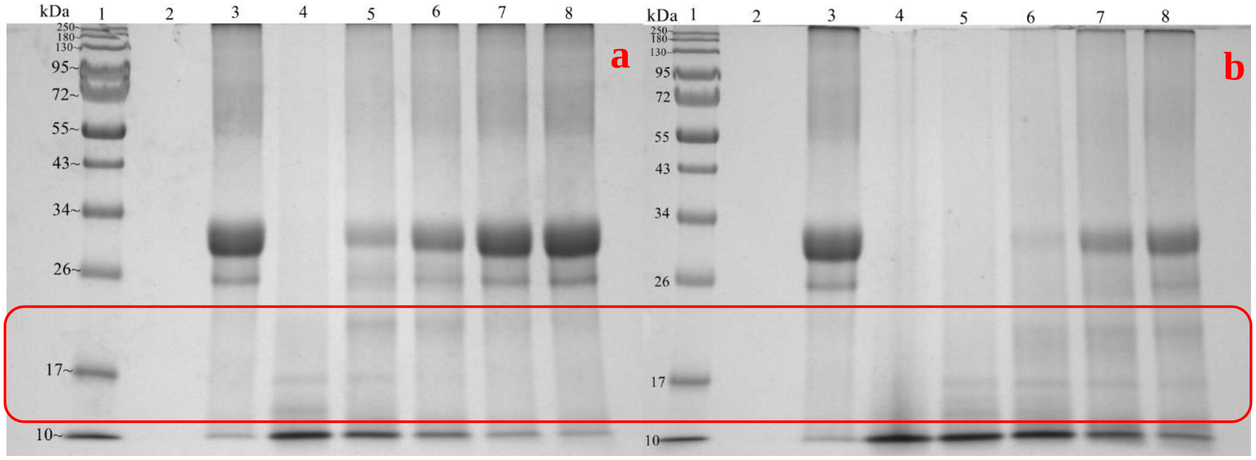

Fig. 5.2. SDS-PAGE images for the comparison of azo-casein hydrolysis with different BMs: (a) PAN-BMs; (b) AGPMs

(7) These BMs may be applied in the production of peptides to hydrolyze high-molecular-weight proteins to small-molecular-weight peptides, even with certain molecular weight (e.g., 17 kDa) in a fine-tune condition, as certain bands of hydrolysates were observed in SDS-PAGE of azo-casein and SMPs hydrolyzed by different BMs.

(8) A membrane reactor with either PAN-BMs or AGPMs can be applied for the continuous hydrolysis of SMPs. And both BMs showed a similar extent of hydrolysis of SMPs

(around 70%) with a five-layer reactor at the flux of 6 L/h·m^2 and similar distribution of hydrolyzed peptide profiles in different molecular weight.

Both BMs performed better pH and thermal stability than free enzymes, which make them potential BMs for industrial applications. Effects of pH, temperature, flux and numbers of membrane layers were investigated in this project, which would be the groundwork for prospected production of peptides by a membrane reactor with EPNMs modified by alkali modification or PDA coating from protein hydrolysis since it has been reported that Alcalase can produce high-value bioactive peptides with antioxidant and emulsifying properties. Moreover, BMs modified by these approaches may also be potentially applied in hydrolyzed peptides production for allergenicity reduction, anti-biofilms for environmental protection and so on.

5.2. Recommendations

This project demonstrated two different modification approaches for Alcalase immobilization, and the great potential of BMs thus created for protein hydrolysis in a continuous reactor. According to our results, the following studies are warranted:

(1) In terms of enzyme activity: BMs with higher activity may be prepared by enhancing the mobility of immobilized enzyme with a spacer of suitable length between enzymes and the membrane. Since the length of DNA can be controlled by the number of base-pairs and DNA directed immobilization has not been studied for enzyme immobilization on nanofibrous membranes, these two modification approaches can be further developed combining DNA directed immobilization to improve the enzyme activity of BMs.

(2) In terms of applications:

(3.1) Further studies, such as tests of peptides solubility, foaming and emulsifying property, antioxidant activity and allergenicity reduction, can be carried out for the further application of the production of peptides by the use of membrane reactor with BMs modified by two modification approaches studied in this thesis.

(3.2) The physical separation function of these BMs has not been explored in this project. Investigation on real-time separation and hydrolysis of low-soluble proteins/mixture (e.g., grounded soybean) can thus be made.