UNIVERSITI PUTRA MALAYSIA

OPTIMIZATION AND ENZYMATIC HYDROLYSIS OF TILAPIA BY-PRODUCT AND FRACTIONATION OF PROTEIN HYDROLYSATE USING MEMBRANE ULTRAFILTRATION

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By

JUMARDI BIN ROSLAN

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Chairman : Associate Professor Siti Mazlina Mustapa Kamal, PhD
Faculty : Engineering

Large amount of by-products is generated during tilapia processing, including skins, bones, frames and tails. The conversion of fish by-products through enzymatic hydrolysis is the most promising alternative in order to produce a valuable products such as fish protein hydrolysate (FPH) which rich in an essential nutrients and bioactive peptides that offer physiological functions, such as antihypertensive activity. FPH consists of peptide mixtures with various sizes, there is a need for separate them using ultrafiltration (UF) membrane in order to improve yield and obtain a specific size of peptide which closely related to the high potent of antihypertensive activity. Therefore, this research is focused on the production of a small-sized bioactive peptide from tilapia by-product which responsible for angiotensin I-converting enzyme (ACE) inhibitory activity through enzymatic hydrolysis and membrane fractionation process.

This study has three main objectives (1) to optimize the parameters for improvement of enzymatic hydrolysis of tilapia by-products using response surface methodology in order to achieve high degree of hydrolysis (DH), (2) to characterize the protein hydrolysates from enzymatic hydrolysis of tilapia by-product emphasizing on ACE inhibitory activity, chemical composition and functional properties, and (3) to evaluate the performances of the single and multilayer membranes for the fractionation of tilapia's by-product protein hydrolysate, in order to enrich the peptide with a high ACE inhibitory activity.

The optimization of enzymatic hydrolysis using alcalase for preparing the tilapia muscle's (TM) and by-product (TB) protein hydrolysates were performed through a response surface methodology (RSM). The *O*-phtaldialdehyde (OPA) method was employed to measure the degree of hydrolysis (DH). The optimum enzymatic hydrolysis conditions for the TM was obtained at pH 7.5, temperature of 50℃, substrate concentration of 2.5% (w/v) and enzyme concentration of 95.6 (AU/kg protein) with DH value is 25.41%. For TB, the highest DH was achieved at 20.42%

with the optimum conditions at pH 7.5, temperature at 60°C, substrate concentration of 15% (w/v) and 60.2 (AU/kg protein) of enzyme concentration.

Under these optimum conditions, the TM and TB protein hydrolysates were further hydrolyzed for 30-720 minutes to investigate the highest ACE-inhibitory activity could be achieved. The highest ACE-inhibitory activities was achieved at 1 hour of hydrolysis and selected for the next analysis such as peptide size distribution, chemical compositions, physical appearance and functional properties. It was found that both samples have various sizes of low molecular peptides ranging from 1.06 to 26.6 kDa. TM and TB protein hydrolysates have shown a good nutritional value with respect to high protein contents (36.55 and 65.64%, respectively) and essential amino acids such as lysine, leucine and threonine. The high amount of hydrophobic amino acids in both the TM and TB protein hydrolysates might contribute to high ACE-inhibitory activities. TM and TB protein hydrolysates were rich in mineral elements such sodium, phosphorus and potassium, indicating a potential of samples to be an alternative sources of mineral. From scanning electron microscopy result, smooth microstructures in aggregation packed flake-like structures formed with the broken structures in irregular and cracked particles, representing shorter peptide chain length were observed for both samples. The findings also demonstrated that TM and TB protein hydrolysate have high nitrogen solubility (>80% at pH 2-9), and possessed good water-holding capacity, and oil holding capacity.

The fractionation of TB protein hydrolysate with the dead-end ultrafiltration (UF) membrane was investigated through single and multilayer membrane using a regenerated cellulose membrane with 10 and 5 kDa molecular weight cut off (MWCO). The performance of the fractionation using the single membrane (10 and 5 kDa) and multilayer membranes (10/5 and 5/5 kDa) were investigated through the effects of stirring speed (0-600 rpm), pH (3, 5, 7, 8 and 9) and salt concentration (NaCl; 0 M, 0.2 M, 0.4 M, and 0.6 M) on the flux and peptide transmission. The best fractionation process were found at the stirring speed of 600 rpm and pH 8 for both single and multilayer membranes which is based on the highest permeate flux and peptide transmission obtained. The permeate produced from each membrane were evaluated their ACE-inhibitory activity. For single membrane, it was found that 5 kDa membrane (71.83%) has higher ACE inhibitory activity compared to 10 kDa membrane (64.32%). Both permeates from multilayer membrane exhibited higher ACE inhibitory activity as compared to single membrane. It is proven that there is a relationship between peptide size and ACE inhibitory activity.

Through a selectivity analysis using Fast Pressure Liquid Chromatography (FPLC), the most permeate produced were composed of peptides lower than 1500 Da. It was found that, fractionation using 10 kDa membrane produced more peptide with large size as compared to other membranes. The percentage of peptides with size less than 500 Da increased as the smaller membrane pore size used. More peptides with small size which is less than 500 Da were obtained for 5/5 kDa multilayer membrane indicating that the peptide selectivity of membrane can be improved through multilayer membrane. Overall, the conversion of tilapia by-products into fish protein hydrolysate has shown a great potential to be used in nutraceutical and pharmaceutical products which is based on the high ACE inihibitory activity obtained from enzymatic hydrolysis and membrane fractionation process.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGOPTIMUMAN DAN HIDROLISIS BERENZIM BAGI BAHAN SAMPINGAN TILAPIA DAN PEMISAHAN PROTEIN HIDROLISAT MENGGUNAKAN MEMBRAN ULTRATURASAN

Oleh

JUMARDI ROSLAN

Mac 2016

Pengerusi : Profesor Madya Siti Mazlina Mustapa Kamal, PhD
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Banyak bahan sampingan telah dihasilkan ketika pemprosesan ikan tilapia termasuk kulit, tulang, rangka dan ekor. Pertukaran bahan sampingan ini melalui hidrolisis berenzim menjadi alternatif yang sangat baik bagi menghasilkan produk bernilai tinggi seperti hidrolisat protein ikan yang kaya dengan nutrien penting dan bioaktif peptida yang memberi fungsi fisiologi seperti aktiviti antihipertensi. Hidrolisat protein ikan terdiri daripada campuran peptida yang perlbagai saiz, terdapat keperluan untuk memisahkannya menggunakan membran ultraturasan bagi memperbaiki hasil dan memperoleh peptida bersaiz khusus yang sangat berkait rapat dengan aktiviti antihipertensi yang tinggi. Oleh itu, kajian ini difokuskan kepada penghasilan bioaktif peptida bersaiz kecil daripada bahan sampingan tilapia yang berperanan kepada perencatan aktiviti enzim pertukaran angiotensin (ACE) melalui proses hidrolisis berenzim dan pemisahan membran.

Kajian ini mempunyai tiga objektif utama iaitu (i) untuk mengoptimumkan parameter operasi hidrolisis berenzim ke atas otot dan bahan sampingan tilapia bagi mencapai darjah hidrolisis yang tinggi, (ii) untuk mencirikan hidrolisat protein tilapia dari segi perencatan aktiviti ACE, komposisi kimia dan sifat kefungsian, dan (iii) untuk menilai prestasi membran tunggal dan multilapisan dalam memisahkan hidrolisat protein daripada bahan sampingan tilapia dan menggunakan sistem membran bagi memperkayakan peptida yang mempunyai perencatan aktiviti ACE yang tinggi.

Pengoptimuman hidrolisis berenzim menggunakan alkalase bagi menyediakan hidrolisat protein daripada otot (TM) dan bahan sampingan (TB) tilapia dilakukan melalui kaedah permukaan sambutan (RSM). Kaedah *O*-phtaldialdehyde (OPA) digunakan untuk mengukur darjah hidrolisis (DH). Keadaan optimum hidrolisis berenzim bagi TM diperolehi pada pH 7.5, suhu 50℃, kepekatan substrat 2.5% (w/v) and kepekatan enzim 95.6 (AU/kg protein) dengan nilai DH 25.41%. Manakala TB, DH tertinggi dicapai pada 20.42% dengan keadaan optimum pH 7.5, suhu 60℃, kepekatan substrat 15% (w/v) dan 60.2 (AU/kg protein)kepekatan enzim.

Pada keadaan optimum ini, TM dan TB dihidrolisis seterusnya selama 30-720 min untuk menentukan perencatan aktiviti ACE tertinggi yang boleh dicapai. Perencatan aktiviti ACE tertinggi dicapai pada hidrolisis selama 1 jam dan dipilih untuk analisis seterusnya seperti taburan saiz peptida, komposisi kimia, penampilan fizikal dan sifat kefungsian. Didapati, kedua-dua hidrolisat protein TM dan TB yang dihasilkan menunjukkan pelbagai saiz peptida dengan berat molekul rendah antara julat 1.06 to 26.6 kDa. Hidrolisat protein TM dan TB menunjukkan nilai pemakanan yang tinggi berdasarkan kepada kandungan protein (masing-masing, 36.55 and 65.64%) dan jumlah asid amino perlu yang tinggi seperti lisina, leusina dan threonine. Jumlah asid amino hidrofobik yang tinggi pada kedua-dua hidrolisat protein TM dan TB mungkin menyumbang kepada perencatan aktiviti ACE yang tinggi. Hidrolisat TM dan TB kaya dengan unsur mineral seperti natrium, fosforus dan kalium, yang menunjukkan sampel boleh menjadi sumber mineral alternatif. Daripada keputusan mikroskop elektron imbasan (SEM), struktur mikro yang halus seperti bentuk gumpalan padat bertih dengan struktur partikel hancur yang tidak seragam, menggambarkan rantaian peptida yang pendek dilihat bagi kedua-dua sampel. Penemuan juga menunjukkan bahawa hidrolisat TM dan TB mempunyai kelarutan nitrogen yang tinggi (>80% pada pH 2-9) dan mempunyai kapasiti pemegang air dan minyak yang baik.

Pemisahan hidrolisat protein TB dengan ultraturasan hujung-mati telah ditentukan melalui membrane tunggal dan multilapisan menggunakan membrane selulosa terjana dengan pemotong berat molekul 10 dan 5 kDa. Prestasi pemisahan menggunakan membran tunggal (10 dan 5 kDa) dan membrane multilapisan (10/5 dan 5/5 kDa) ditentukan dengan melihat kesan parameter operasi dan fizikokimia termasuk kelajuan putaran (0-600 rpm), pH (3, 5, 7, 8, dan 9), dan kepekatan garam (NaCl; 0 M, 0.2 M, 0.4 M, and 0.6 M) ke atas fluks dan pemindahan peptida. Proses pemisahan yang terbaik didapati pada kelajuan putaran 600 rpm dan pH 8 bagi kedua-dua membran tunggal dan lapisan berdasarkan kepada nilai fluks dan pemindahan peptida yang tinggi diperolehi. Hasil turasan daripada setiap membran seterusnya diuji perencatan aktiviti ACE. Bagi membrane tunggal, didapati membran 5 kDa menunjukkan perencatan aktiviti ACE lebih tinggi (71.83%) berbanding membran 10 kDa (64.32%). Perencatan aktiviti ACE didapati lebih tinggi pada kedua-dua membran multilapisan berbanding dengan membrana tunggal. multilapisan 10/5 kDa, menunjukkan perencatan aktiviti ACE sangat dipengaruhi oleh konfigurasi dan saiz liang membran. Ini membuktikan terdapat hubungan antara saiz peptide dan perencatan aktiviti ACE.

Melalui analisis pemilihan menggunakan kromatografi cecair tekanan pantas (FPLC), kebanyakkan hasil turasan terdiri daripada peptida dengan berat molekul rendah lebih daripada 1500 Da. Didapati bahawa pemisahan menggunakan membran 10 kDa menghasilkan lebih banyak peptide dengan saiz yang lebih besar berbanding dengan membran lain. Peratusan peptida dengan saiz kurang daripada 500 Da meningkat dengan penggunaan liang membran yang lebih kecil. Lebih banyak peptida dengan saiz kurang daripada 500 Da diperolehi pada membran multilapisan 5/5 kDa menunjukkan pemilihan peptida bagi membran dapat diperbaiki melalui membran multilapisan. Secara keseluruhan, penggubahan bahan sampingan tilapia kepada hidrolisat protein ikan menunjukkan potensi yang baik untuk digunakan pada produk nutraseutikal dan farmaseutikal yang berdasarkan kepada perencatan aktiviti ACE yang tinggi diperolehi daripada proses hidrolisis berenzim dan pemisahan membran.

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I certify that a Thesis Examination Committee has met on 22 March 2016 .to conduct the final examination of Jumardi bin Roslan on his thesis entitled "Optimization and enzymatic hydrolysis of tilapia by-product and fractionation of protein hydrolysate using membrane ultrafiltration" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy.

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FPH	fish protein hydrolysate
AA	amino acid
ANOVA	analysis of variance
AOAC	association of official analytical chemists
CCD	central composite design
CCRD	central composite rotatable design
DH	degree of hydrolysis
TNBS	trinitrobenzenesulfonic acid
OPA	*O*-Phtaldialdehyde
ACE	angiotensin I-converting enzyme
UF	ultrafiltration
HCl	hydrochloric acid
FPLC	fast pressure liquid chromatography
NaCl	sodium chloride
SEM	scanning electron microscope
RC	regenerated cellulose
MWCO	molecular weight cut-off
TB	tilapia by-product
NaOH	sodium hydroxide
TM	tilapia muscle
kDa	kilodalton
Da	dalton
RAS	renin-angiotensin system
TCA	trichloroacetic acid

CRFD	completely randomized factorial design
RSM	response surface methodology
RCBD	randomized complete block design
WHC	water-holding capacity
OHC	oil-holding capacity
RO	reverse osmosis
NF	nanofiltration
MF	microfiltration
TMP	transmembrane pressure
PS	polysulfone
PES	polyethersulfone
CA	cellulose acetate
PA	polyamide
PVDF	polyvinylidenefluride
PAN	polyacrylonitrile
BSA	Bovine serum albumin
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
EC	emulsifying capacity

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Tilapia is a cultured freshwater fish, abundant in several parts of the world, and has become the second most cultured fish species after carp (Fitzsimmon, 2004; El Sayed, 2006). Due to its special features such as fast-growing, tolerant to environmental changes and disease, and a source of nutritional benefits, tilapia farming in Asia has increased over the years and became widely available (Murthy *et al*., 2011). Red tilapia (*Oreochromis sp.*) is the most farmed species in Malaysia with yields reaching ~81% of the total tilapia production (33,260 tons) (Ng, 2009; Department of Fisheries, 2011). In order to increase tilapia marketability, several endeavors have been sought to improve the quality of its attributes through isolation of tilapia protein.

Tilapia protein isolates have been shown to have excellent physicochemical characteristics such as good gelling, foaming and emulsifying properties that can be used in the development of processed food products with improved functional properties (Murthy *et al*., 2011; Zhou *et al*., 2006; Rawdkuen *et al*., 2009). In addition to their beneficial physicochemical characteristics, tilapia protein isolates also have high nutrients and are rich sources of bioactive peptides. These bioactive peptides however, are inactive within the sequence of the parent protein (Vercruysse *et al*., 2005). The conversion of tilapia protein isolates into fish protein hydrolysate (FPH), it facilitates the release of bioactive peptides that possess numerous physiological functions such as antithrombotic, anticancer, antimicrobial, antioxidative and antihypertensive which could be used as components in healthcare and pharmaceutical products (Claire and Swaisgood, 2000)

During processing of tilapia fish to obtain tilapia muscles, there are many by-products generated including skins, bones, frames and tails. These by-products are normally disposed without any attempt to make them useful. Several efforts have been made to utilize these by-products by converting it into fish meal for animal feed, fish oil, fertilizer and fish silage (Choudhury & Bublitz, 1996; Choudhury & Gogoi, 1995). However, most of these products possess low economic value. Fish by-products from various fish species contain considerable amount of protein varying from 15-60% (Valdimarson & James, 2001; Fahmi *et al*., 2004; Sathivel *et al*., 2004; Je *et al*., 2004; Jung *et al*., 2006) that is known to possess high nutritional value in terms of essential amino acid (AA) composition (Venugopal *et al*., 1996). The conversion of fish by-products into value added products such as bioactive peptides can be a very promising alternative.

Since 1970s, the production of FPH from fish by-products has received growing attention as the latter contains rich essential nutrients and bioactive peptides (Je *et al*., 2004; Jung *et al*., 2006; Kim *et al*., 2001). These efforts can pave the way for complete utilization of fish waste, which will extend the food supply and benefit human health. Several research groups around the world have been working on producing FPH through enzymatic hydrolysis reaction using selected proteolytic enzymes to cleave specific peptide bonds (Kitts & Weiler, 2003; Raghavan & Kristinsson, 2008; Raghavan *et al*., 2008; Theodore

& Kristinsson, 2007). The enzymatic approach has become the most preferred method for hydrolysis of fish protein due to several advantages including easy monitoring, high specificity, mild reaction conditions, less undesirable products, and high product quality and yield.

Enzyme selection is a crucial factor in the hydrolysis of proteins based on the fact that different enzymes have different specificity, which consequently produce protein hydrolysates with different chemical and functional properties (Kristinsson and Rasco, 2000a; Korhonen & Pihlanto, 2006). There are numerous commercial proteases available in the market that can be used for hydrolyzing fish proteins including alcalase, neutrase, flavourzyme, protamex, pepsin, trypsin, chymotrypsin, papain, bromelain and ficin (Aspmo *et al*., 2005; Guerard *et al*., 2001; Liaset *et al*., 2000; Himonides *et al*., 2011; Lee *et al*., 2010). Alcalase has been shown to be one of the most efficient enzymes for the preparation of fish protein hydrolysate attributed to its ability to attain a high degree of hydrolysis in a relatively short period under mild conditions and can produce fish protein hydrolysate with high nutrient contents and good functional properties (Adler-Nissen, 1986; Shahidi *et al*., 1995; Benjakul and Morrissey, 1997; Kristinsson and Rasco, 2000b; Guerard *et al*., 2001; Wasswa *et al*., 2007; Amiza *et al*., 2011; See *et al*., 2011).

Enzymatic hydrolysis of food protein is usually monitored through degree of hydrolysis (DH), which is the percentage of peptide bonds cleaved during the process. DH can be monitored using several methods including the pH-stat, osmometry, soluble nitrogen content, trichloroacetic acid, formol titration, trinitrobenzenesulfonic acid (TNBS) and *O*-phthaldialdehyde (OPA) methods. In order to produce fish protein hydrolysates with high DH and desired properties, the control over the enzymatic process, particularly enzyme specificity and hydrolysis conditions, is crucial.

FPH generated through enzymatic hydrolysis consists of peptide mixtures with various sizes. Peptide sizes have relationship with their functional properties, which an appropriate molecular size can improve the functional properties of end products. Researchers have reported that small peptides (1-5 kDa) from fish protein hydrolysates have strong influence on Angiotensin I-converting enzyme (ACE) inhibition activity (Je *et al*., 2004; Theodore & Kristinsson, 2007; Bougatef *et al*., 2008; Qian *et al*., 2007; Raghavan & Kristinsson, 2009). ACE plays an important role in the regulation of blood pressure and hypertension (Raghavan & Kristinsson, 2009) and the inhibition of ACE activity (>50%) is a good target for antihypertension treatment.

Hypertension is one of the most common chronic health problems and is a major risk factor for atherosclerosis, stroke, myocardial infarction and end-stage renal disease (Itou *et al*., 2007; Jung *et al*., 2006). The mechanism of blood pressure regulation by ACE involve conversion of an inactive form of the decapeptide (angiotensin I) to the octapeptide (angiotensin II), a potent vasoconstrictor, and bradykinin inactivator, which has a depressor action (Jung *et al*., 2006), thus increases blood pressure and leads to heart attack. Due to the high demand for the natural health product for the treatment of this chronic disease, there is a need to produce healthcare product from natural resources such as FPH, which can be used as an alternative to synthetic drug such as captopril that has negative effects on health.

Production of peptides with the desired molecular sizes for the treatment of hypertension is a challenging process and almost impossible to obtain by simply controlling the DH during hydrolysis. Therefore, better understanding of the separation process is required,

especially in the large scale production of these specific peptides. The conventional method for peptides purification usually involve column chromatography because of its high selectivity, but this method requires high cost to scale up. In this regards, ultrafiltration membrane system is a cheaper alternative and the most convenient equipment for that purpose due to its ability to separate FPH effectively, easily controlled and can enrich a small peptide fraction with high biological activity (Cheryan, 1986; Mochizuki & Zydney, 1992; Jeon *et al*., 1999; Je *et al*., 2005; Raghavan & Kristinsson, 2009).

1.2 Problem statements

It is well known that various tilapia species have been cultured around the world such as red tilapia (*Oreochromis niloticus*), black tilapia (*Oreochromis mosambicus*) and blue tilapia (*Oreochromis aurea*). However, red tilapia farming is most preferred due to its special features such as fast growing and high yield of flesh compared to black and blue tilapia leading to the selection of red tilapia as a raw material in this study. Although a few studies have reported on the hydrolysis of tilapia muscle (Abdul-Hamid *et al*., 2002; Raghavan and Kristinsson, 2009; Dekkers *et al*., 2011; Foh *et al*., 2011; Shamloo *et al*., 2012) using different enzymes and hydrolysis conditions, until recently, there has been no research reported on using tilapia by-products especially from its frame, bone, tail and head for the production of FPH. Only studies on the production of FPH from tilapia skin (Yang *et al*., 2009) and tilapia scale (Ngo *et al*., 2010) have been reported. Most of these studies do not take into consideration the effect of process parameters, which is essential information required in controlling the hydrolysis of tilapia. Knowledge of hydrolysis reaction is important not only for the optimization process of enzymatic protein hydrolysis but also to understand the mechanisms affecting the process yield including the limitations of the reaction catalyzed by the selected enzyme. Due to lack of information on controlling the hydrolysis process for tilapia, this research has attempted to use commercial protease, alcalase in producing FPH from tilapia muscle and by-products with the *O*-phtaldialdehyde (OPA) method was selected for monitoring the degree of hydrolysis (DH) in the optimization process, which serves as an initial exploration by comparing the results with that of conventional pH-Stat method (Adler-Nissen, 1986). Determination of DH during hydrolysis of proteins (raw material) is crucial because this process requires control and optimization to obtain reproducible of fish protein hydrolysate with desired characteristics.

Generally, biological activities and functional properties of FPH depend on their molecular size and structure and specific amino acids which mainly related to the DH value. High DH value can usually be achieved through extensive enzymatic hydrolysis, leading to the recovery a mixture of peptides with a small size, thus providing a good biological activity. However, the information regarding changes in biological activity during extensive enzymatic hydrolysis of tilapia by-products is limited. Therefore, there is a need to investigate the effect of extensive enzymatic hydrolysis of TM and TB on biological activity particularly antihypertensive activity which is main focus of the study and then further analysis of their characteristics in order to find out the potential application of these products.

FPH consists of peptide mixtures with various sizes and it is necessary to separate them using ultrafiltration (UF) membrane to obtain a specific size of peptide as well as to improve their biological activities. Most membrane studies have used UF as a simple tool for separating FPH, whereby peptides with specific sizes have been successfully obtained

(Jeon *et al*., 1999; Je *et al*., 2005; Raghavan & Kristinsson, 2009; Ranathunga *et al*., 2006; Kim *et al*., 2007). However, production of these peptides in large quantities using UF membrane remains a major constraint. This is because fractionation of peptide may not have a simple and straightforward relationship with membrane pore size. As a result, high throughput of the product cannot be achieved, subsequently limiting the application of membranes for peptide fractionation.

In peptide fractionation, membrane fouling is considered as a major problem that leads to poor separation of FPH and consequently reduces peptide yield. This phenomenon is mainly driven by the effect of concentration polarization, protein adsorption within the porous structure of the membrane, the formation of protein deposits on the upper surface of the membrane, and protein-protein interactions in the bulk solution and/or membrane. Effective separation of peptides can be achieved by selecting the appropriate membrane and controlling the solution and operating conditions. So far, no complete and extensive studies have been done to determine the best fractionation conditions for separation of peptides from tilapia FPH. Therefore, this study aims to investigate the effect of solution conditions such as pH and salt concentration, as well as the stirring speed on the fractionation of tilapia protein hydrolysate, with the ultimate target to control membrane fouling and thus can increase the yield of permeates.

Another problem that commonly arises from the application of UF membranes in fractionation of proteins is low selectivity of the membrane. Poor selectivity in protein fractionation is usually related to the imperfect pore size distribution of the available commercial membranes, thus limiting significantly the resolving power of the membranes (Md. Yunos and Field, 2008). In order to obtain high resolution protein-protein fractionation and selective transmission of solute through membranes, it is desirable to have reasonable transmission of a particular size of peptides and rejection of other species to achieve good separation. Therefore, a new technique in using UF membranes has been introduced specifically to overcome the broad membrane pore size distribution, namely the multilayer membrane. The potential of a multilayer membrane UF to completely reject unwanted species has been demonstrated in literature (Boyd and Zydney, 1997; Feins and Sirkar, 2004; Feins and Sirkar, 2005; Md Yunos & Field, 2006; 2008; Field *et al*., 2009), but most studies using multilayer membranes are only limited to the fractionation of binary protein mixture. One of the advantages in using multilayer membranes is that membranes are stacked together, by which they are housed in one device with an appropriate sandwich arrangement, thus enhancing the rejection characteristics as well as selectivity compared to a single membrane (Boyd & Zydney, 1997; Feins & Sirkar, 2005; Md Yunos & Field, 2008). By stacking membranes, it is possible to achieve essentially a completely pure product (Feins and Sirkar, 2004). In addition, it can also become an alternative to the use of two-stage membrane separation, which involves several unit operations that make it costly. Through the multilayer membrane technique, a single operation can be achieved with relatively less cost.

Thus, this part is more focusing on the fractionation of FPH with the aim to identify factors limiting peptide fractionation as well as gaining better understanding of peptide transmission by applying single and multilayer membrane process, and finally to determine the best membrane configuration that could give high biological activities.

1.3 Research hypotheses

Based on reviews of previous studies, several hypotheses could be made (1) the hydrolysis of tilapia muscle and tilapia by-product using a commercial protease such as alcalase can achieve a high degree of hydrolysis in a relatively short period and can thus produce small peptide sizes with good nutritional and functional properties (Adler-Nissen, 1986; Benjakul and Morrissey, 1997; Amiza *et al*., 2011; See *et al*., 2011; Kristinsson and Rasco, 2000b; Wasswa *et al*., 2007). (2) Application of UF membranes in fractionating FPH can enrich specific peptides of small sizes and also improve the biological activity of FPH (Chabeaud *et al*., 2009; Vandanjon *et al*., 2009; Bourseau *et al*., 2009; Saidi *et al*., 2013; 2104). (3) By adopting this new technique, multilayer membranes can improve selectivity of the peptides (by measuring permeate concentration using chromatography technique) aside from enhancing biological activity such as antihypertensive activity.

1.4 Objectives

Based on the problem statement as discussed above, the objectives of this study are:

1. To optimize the parameters for improvement of enzymatic hydrolysis of tilapia by products using response surface methodology.
2. To characterize the protein hydrolysates from enzymatic hydrolysis of tilapia by-product emphasizing on ACE inhibition activity, chemical and amino acids composition and functional properties.
3. To evaluate the performances of single and multilayer membrane ultrafiltration for fractionating of tilapia by-product protein hydrolysate, and utilizing membrane to enrich peptide with high ACE inhibitory activity.

1.5 Scope of study

This study is aimed to produce fish protein hydrolysates with high ACE inhibitory activity from TM and TB through the enzymatic hydrolysis approach followed by fractionation using ultrafiltration membranes for the recovery of small-sized peptides (< 10 kDa).

Enzymatic hydrolysis of tilapia muscle and tilapia by-product is performed using commercial proetease, alcalase, whereby focus is converged on the screening and optimization processes at various hydrolysis parameters such as pH, temperature (ºC), substrate concentration (w/v, %) and enzyme concentration (w/w, %). Hydrolysis of tilapia muscle and tilapia by-product are monitored through the degree of hydrolysis (DH) using *O*-phtaldialdehyde (OPA) method. Response surface methodology (RSM) was then employed to determine an optimum degree of hydrolysis for both samples.

TM and TB protein hydrolysates produced are characterized in terms of biological activity, specifically ACE inhibitory activity; chemical composition; physical and functional properties. Chemical composition analyses consist of proximate analysis (moisture, protein, ash, and oil content), amino acid composition and mineral content. Physical properties of the FPH which include color and morphology are analyzed by a Konica Minolta color reader, Scanning Electron Microscope (SEM), respectively. Functional properties of the FPH are then characterized in terms of nitrogen solubility, water-holding capacity (WHC), oil-holding capacity (OHC), and emulsifying capacity (EC).

TB hydrolysate with the highest ACE inhibitory activity is further used for fractionation using a stirred cell ultrafiltration membrane in order to obtain high yield of peptides with a small-sized and improved biological activity. Two different sizes of flat sheet regenerated cellulose (RC) membrane type are used with molecular weight cut-off (MWCO) of 5 and 10 kDa. Membrane performances are evaluated in terms of flux and peptide transmission and examined under three different parameters, which are stirring speed (0, 300, 600 rpm), pH (3, 5, 7, 8, 9) and salt concentration (NaCl: 0 M, 0.2 M, 0.4, M, 0.6 M) via single and multilayer (two membranes combined together) membrane configuration. Fractionation using the single (5 and 10 kDa) membrane is studied at different pressures at 1.0, 1.5, 2.0, 2.5, and 3.0 bar. As for the multilayer membrane, the pressures is varied at 2.0, 2.5 and, 3.0 bar. Permeate obtained at optimum conditions during the fractionation of TB hydrolysate from different membrane configurations are further analyzed using Fast Protein Liquid Chromatography (FPLC) in order to identify which peptide components are contained in the permeate. Finally, the ACE inhibitory activity for each permeate is measured.

1.6 Significance of the study

In this research, a fraction of peptides with high ACE inhibitory activity from tilapia by-products hydrolyates can be recovered via enzymatic hydrolysis and fractionation using UF membrane either through single or multilayer membrane configurations. A better insight on hydrolysis mechanism can contribute to a higher product yield. In addition, factors affecting peptide fractionation can be identified and thus a better understanding of the flux and peptide transmissions through single and multilayer membrane can be achieved.

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