Production of bioactive peptides from striped catfish (*Pangasius hypophthalmus*) skin by enzymatic membrane reactor

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ABSTRACT

In order to utilize the waste of fishery industry, the skin of striped catfish fillet processing waste was investigated to produce antioxidant and antihypertensive peptides using an enzymatic membrane reactor system.

Firstly, the striped catfish skin from fillet processing industry that still contains amount of fish muscle was extracted protein using an autoclave. The extracted protein showed a high yield of 74.8 %. The effect of hydrolysis time on degree of hydrolysis and bioactive activities of hydrolysate was studied using Alcalase 2.4 L 20 units g^{-1} protein at pH 8.0 and 50 ^{o}C. The results showed that degree of hydrolysis increased with the increase of hydrolysis time. Degree of hydrolysis played an important role in bioactive activities of hydrolysate. The 5-h hydrolysate (DH= 40.2 %) exhibited the highest bioactive activities including DPPH radical scavenging activity (41.3 μM TE/mg), ABTS radical scavenging activity (2062 μM TE/mg protein), lipid peroxidation inhibition activity which was higher than that of 200 ppm α-tocopherol and 1000 ppm ascorbic acid, and ACE inhibitory activity (IC_{50} = 831 μg/mL).

Secondly, for selection of membrane size of ultrafiltration, the effects of different molecular weight cut-offs (MWCOs) including 10, 5, and 1 kDa membranes on permeate flux and antioxidant, ACE inhibitory activity of peptides were studied. The operating condition in batch mode was transmembrane pressure (TMP) of 1.5 bars, and cross flow velocity (CFV) of 1.5 m s^{-1}. The results showed that the ultrafiltration using 5 kDa and 1 kDa was successful for separation antioxidant and ACE inhibitory activity peptides since ultrafiltration of the hydrolysates resulted in a significant increase in its bioactive activity in the permeate

fractions with DPPH radical scavenging activity (45.2 μM TE/mg), ABTS radical scavenging activity (2474.7 μM TE/mg protein), and ACE inhibitory activity (IC_{50} = 8.3 μg/mL). Based on gel filtration chromatography results, ACE inhibitory peptides had molecular weight ranging of 307 Da to 429 Da while antioxidant peptides had molecular weight in the range of 829.5 Da to 1355 Da. Furthermore, ACE inhibitory and antioxidant activity of peptides showed negligible changes in the simulated gastrointestinal digestion.

Finally, the continuous enzymatic membrane reactor system was employed to produce bioactive peptides from striped catfish skin protein. Hollow fiber membranes with molecular weight cut-offs of 5 and 1 kDa were equipped with stirred reactor tank to produce antioxidant and ACE inhibitory peptides, respectively. The 1% (w/v) extracted protein solution was hydrolysed by Alcalase 2.4 L 20 units g^{-1} protein, at pH 8.0 and 50 ^{o}C and separation of peptides was performed at constant conditions; transmembrane pressure (TMP = 0.5 bar), cross flow velocity (CFV = 2 m s^{-1}). The results showed that the continuous enzymatic membrane reactor system using 5 and 1 kDa membranes was successful for production of antioxidant and ACE inhibitor peptides since it gave high conversion, productivity of target peptides and stable operation. At the maximum activity, DPPH and ABTS radical scavenging activity conversion were 224% and 154%, respectively; the productivities were 58.6 mg DPPH radical scavenging peptides/unit enzyme and 40.2 mg ABTS radical scavenging peptides/unit enzyme; ACE inhibitory activity conversion and productivity were 20.5% and 0.53 mg ACE inhibitory peptides/unit enzyme.

The present study showed that antioxidant, ACE inhibitory peptides could be obtained from the skin by-products of striped catfish fillet processing industry by continuous enzymatic membrane reactor system.

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LIST OF ABBREVIATIONS

A	= Absorbance
ABTS	= 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
ACE	= Angiotensin-I converting enzyme
AOAC	= Association of Official Agricultural Chemists
CEMR	= Continuous enzymatic membrane reactor
Da	= Dalton
DH	= Degree of hydrolysis
DI	= Deionized
DPPH	= 2,2-Diphenyl-1-picrylhydrazyl
DTT	= DL-Dithiothreitol
EMR	= Enzymatic membrane reactor
E: S	= Enzyme: substrate
h	= Hours
HHL	= Hippuryl-L-Histidyl-L-Leucine
HT	= Hydrolysis time
J	= Flux
kDa	= Kilodaltons
L	= Liter
M	= Molar
MF	= Microfiltration
Mg	= Milligram
min	= Minutes
mL	= Milliliter
mM	= Millimolar
MW	= Molecular weight
NF	= Nanofiltration
$O_2^{\bullet-}$	= Superoxide anion
$OH^{\bullet}$	= Hydroxyl radical
OPA	= O-phthalaldehyde
R_j	= Retention coefficient

LIST OF ABBREVIATIONS (Continued)

R_m, R	= Membrane resistance
RO	= Reverse osmosis
SDS	= Sodium dodecyl sulfate
Std.	= Standard deviation
TMP	= Transmembrane pressure
TE	= Trolox equivalent
Trolox	= 6-hydroxy-2,5,7,8-tetramethylchromance-2-carboxylic acid
µL	= Microliter
µM	= Micromolar
U	= Units
UF	= Ultrafiltration
w/v	= Weight by volume
w/w	= Weight by weight

CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Striped catfish (*Pangasius hypophthalmus*) is a large freshwater fish. It is an important species in freshwater aquaculture in Thailand, Vietnam, Malaysia, Indonesia and China. In the fillet processing, it generates considerable quantities of by-products, including abdominal organs, head, bone and skin, that in total represents about 65% of the fish by weight (Thuy *et al*., 2007). The utilization of these by-products is interesting because they may be used as potential materials for producing collagen, gelatin or biologically active peptides. From previous studies, catfish skin can be used as raw materials for production of gelatin (See *et al*., 2010; Mahmoodani *et al*., 2011) and collagen (Singh *et al*., 2011).

Enzymatic hydrolysis has grown considerably as an alternative to produce bioactive peptides. These peptides can be separated from protein hydrolysate by various methods. However, ultrafiltration has been used successfully in protein separation for commercial scale (Afonso and Borquez, 2002). It is also possible that the combination of ultrafiltration and enzyme reactor, so call "enzymatic membrane reactor", to develop a continuous process for producing bioactive peptides. The advantages of such a system include improved utilization of the enzyme since it is continuously recycled, the permeate is free of suspended matter, product properties (such as molecular weight distribution and clarity) are more consistent and uniform resulting in products of high quality, and the process is continuous, leading to higher productivity.

To our knowledge, the production of antioxidative, ACE inhibitory peptides from striped catfish skin using enzymatic membrane reactor (EMR) has not been reported. In the present study, the striped catfish skin from fillet processing that still contains amount of fish muscle was studied to produce peptides with antioxidant, ACE inhibitory activities using EMR.

1.2 Review of literature

1.2.1 The striped catfish and structure of fish skin

The striped catfish is a species of shark catfish (Pangasiidae family). It is a large freshwater fish and is not a shark. This fish has English names such as iridescent shark, pangasius catfish, striped catfish, sutchi catfish, freshwater catfish, tra catfish or locally referred to as "Ca tra" (in Vietnamese), "Pla sawai" (in Thai) and "Patin" (in Malaysian), and the science name is *Pangasius hypophthalmus.* This fish is native to the rivers of South-East Asia, including the Chao Phraya River in Thailand and the Mekong River in Cambodia and Vietnam. This freshwater fish normally lives in a tropical climate and prefers water with a pH of 6.5–7.5 and a temperature range of 22–26 ^{o}C. Striped catfish is characterized by a laterally compressed body, a short dorsal with one or two spines, a well-developed adipose, a long anal fin, strong pectoral spines, and two pairs of barbels. The position of the mouth is described as being terminal. There are six branched dorsal fin rays and the pelvic fins have 8-9 soft rays. The gill rakes are described as being normally developed, with small gill rakes being interspersed with larger ones. It has the swim-bladder with a single chamber extending posteriorly above anal fin (Figure 1). Over their native range, striped catfish is divided into two distinct populations: stocks in the Mekong River in Cambodia and Vietnam belong to one population (southern stock); and stocks above Khone falls in Lao and Thailand form a separate population (northern stock). The southern stock is subject to more intensive fishing than the northern stock, and is larger in size (Nguyen, 2008). Striped catfish skin left over from fillet processing accounts for 5-8% of the fish weight and this skin is a good raw material for production of collagen, gelatin, and bioactive peptides.

Figure 1. Striped catfish (*Pangasius hypophthalmus*).
Source: www.minhquysf.com

The skin is the outer covering of the body. It is the largest organ of the integumentary system made up of multiple layers of epithelial tissues, and guards the underlying muscles, bones, ligaments and internal organs. Skin has elastic and soft film tissues to cover the whole body. Skin construction is quite complex with three main layer such as epidermis, epidermal grand, dermis. They combine together closely. The epidermis in fish is relatively thin, and is the outermost layer of the skin, acting as the body's major barrier against an inhospitable environment. Unicellular mucous glands are abundant in the epidermis. The dermis is composed of dense fibrous connective tissue and is attached to the underlying muscles by a layer of loose connective tissue. The scales of fish are located within the dermis (Ceroke, 1990). Epidermal glands consist of grain cells and a lot of mucous fluid which includes glycoprotein mucous fluid and other fluid such as gelatin. The dermis consists of dense fibrous connective tissue containing a number of blood vessels. This tissue has an arrangement of protein fibres, cells and non-living intercellular substance. Collagen fibres consist of bundles. All of them contain in endoplasmic reticulum. The bundles textile together that made fish skins is more elastic. The scales of fish are located within the dermis (Ceroke, 1990).

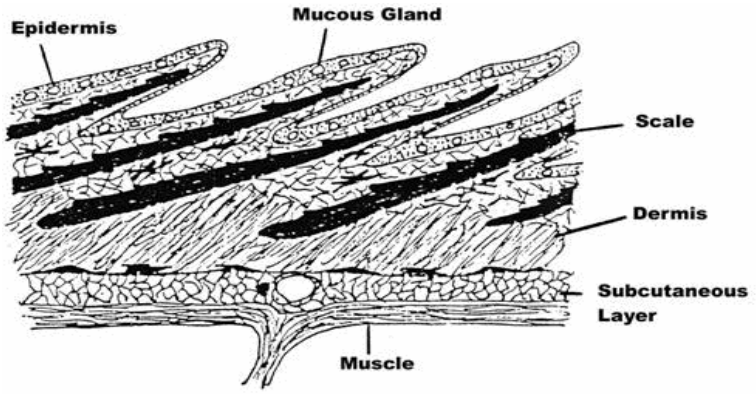

Figure 2. Cross sections through perch skin.
Source: Ceroke (1990)

1.2.2 Fish gelatin and gelatin hydrolysis

Fish gelatin, partially hydrolyzed or denatured collagen, has received attention as the alternative of mammalian and avian gelatin. It can be extracted from

the by-products rich in collagen such as fish skin, bone, scale, or the skin of some invertebrate, and so on. Composition and properties of fish gelatin can be governed by the sources of raw materials. Processing parameters such as pretreatment, extraction temperature, bleaching, drying, and so on influence the chemical and functional properties of gelatin (Benjakul *et al*., 2012). The rising interest in the utilization of skin by-products from fisheries processing for production of gelatin is one of the main reasons why exploring different species and optimizing the extracting conditions of gelatin has attracted the attention of researchers in the last decade. Extraction temperature and time are two important factors affecting the gelation of gelatin. Generally, bloom strength of gelatin gel decreases as the extraction temperature and time increase. During the collagen to gelatin transition, many non-covalent bonds are broken along with some covalent inter- and intra-molecular bonds and a few peptide bonds are cleaved (Benjakul *et al*., 2012). Work by Yang and coworkers (2008) showed that the cobia retorted skin gelatin hydrolysate produced by a retorting treatment (121 ^{o}C for 30 min) and its derivatives (enzyme treatments and enzyme treatments through centrifugal ultrafiltration filter with 3 kDa MWCO membrane) exhibited strong DPPH free-radical scavenging activity and lipid peroxidation inhibition. They also reported that the retorting treatment results in sufficient denaturation of soluble collagen and the strong thermohydrolysis provides an effective method for the production of skin gelatin hydrolysates.

At the same time, a large number of studies have dealt with the enzymatic hydrolysis of gelatin for the production of bioactive peptides. Protein hydrolysates are generally obtained by enzymatic proteolysis. A number of commercial proteases have been used for the production of hydrolysates and peptides. Protease specificity affects size, amount, amino acid composition of peptides and their amino acid sequences, which in turn influences the biological activity of the hydrolysates (Chen *et al*., 1995; Wu *et al*., 2003; Jeon *et al*., 1999). Considering the fact that microbial-derived proteases are low-cost and safe, and the product yields are very high, the use of these enzymes to hydrolyze food proteins is valuable (Phelan *et al*., 2009). Alcalase, which is a commercial protease from a microbial source, has been used in numerous studies dealing with gelatin, collagen, and protein hydrolysis

because of its broad specificity as well as the high degree of hydrolysis that can be achieved in a relatively short time under moderate conditions (Diniz and Martin, 1996; Benjakul and Morrissey, 1997). Alcalase (EC 3.4.21.14) is an alkaline bacterial protease produced from *Bacillus licheniformis.* It has been proven to be one of the best enzymes used in the preparation of protein hydrolysate (Guérard *et al*., 2001). It is endopeptidase which is able to hydrolysyse proteins with broad specificity for peptide bonds and prefers a large uncharged residue. It was more active at alkaline pH and remained active to pH 6.0. The activity was high in pH range of 6.5-8.5 but showed considerable loss of activity at pH 10.5 (Adler-Nissen, 1986).

Several fish skin gelatins were hydrolysed with proteolitic enzyme and hydrolysates have potent antioxidant, antihypertensive activity such as Alaska pollack skin (Byun and Kim, 2001), hoki skin (Mendis *et al*., 2005a), cobia skin (Yang *et al*., 2008), tuna skin (Gómez-Guillén *et al*., 2010), pangasius catfish (Mahmoodani *et al*., 2012). Besides exploring diverse types of bioactivities, of an antimicrobial, antioxidant or antihypertensive nature, studies have also focused on the effect of oral intake in both animal and human models, revealing the excellent absorption and metabolism of Hyp-containing peptides.

Amino acid compositions of gelatin extracted from pangasius catfish skin and its hydrolysates were studied by Mahmoodani *et al*. (2012) (Table 1 and Table 2). The amino acid composition of the gelatin from pangasius catfish skin was nearly identical with that of the hydrolysates. They are rich in Glycine (31.23%), Proline (11.98%), Alanine (9.45%), Glutamine (7.83%), and Hydroxyproline (6.31%). The amino acid compositions were similar to those of gelatin and gelatin hydrolysates from Alaska pollack skin (Kim *et al*., 2001). Peptides from gelatin hydrolysate of Alaska pollack skin were composed of 13 and 16 amino acid residues; and both peptides contained a Glycine residue at the *C*-terminus and the repeating motif Gly-Pro-Hyp showed high antioxidative activity (Kim *et al*., 2001). Proteolytic digestion of Alaska pollack skin gelatin also exhibited a high ACE inhibitory activity. The peptides were composed of Gly-Pro-Leu and Gly-Pro-Met that showed IC_{50} values of 2.6 and 17.13 μM, respectively (Byun and Kim, 2001). Byun and Kim. (2002) studied structure and activity of ACE inhibitory peptides derived from Alaskan pollack skin.

The results showed that among 6 types of tripeptides and 2 types of dipeptides, which consisted of Glycine, Proline, and Leucine were examined, the highest ACE inhibitory activity was observed for Leu-Gly-Pro (IC_{50} = 0.72 μM). The peptide had a Leucine residue at the *N*-terminal and a Proline residue at the *C*-terminal.

Table 1. Amino acid composition (%) of gelatin extracted from pangasius catfish skin.

Amino acids	Content (%)
Asparagine (Asp)	5.28 ± 0.13
Serine (Ser)	3.79 ± 0.07
Glutamine (Glu)	7.83 ± 0.10
Glycine (Gly)	31.23 ± 0.17
Histidine (His)	0.71 ± 0.04
Arginine (Arg)	5.08 ± 0.17
Threonine (Thr)	3.40 ± 0.14
Alanine (Ala)	9.45 ± 0.11
Proline (Pro)	11.98 ± 0.12
Tyrosine (Tyr)	0.61 ± 0.04
Valine (Val)	2.76 ± 0.09
Methionine (Met)	2.63 ± 0.18
Lysine (Lys)	3.45 ± 0.17
Isoleucine (Ile)	1.64 ± 0.05
Leucine (Leu)	2.74 ± 0.06
Phenylalanine (Phe)	1.17 ± 0.02
Cysteine (Cys)	0.02 ± 0.01
Tryptophan (Trp)	0.01 ± 0.01
Hydroxyproline (Hyp)	6.31 ± 0.09

Source: Mahmoodani *et al*. (2012)

Table 2. Amino acid composition (%) of pangasius catfish skin gelatin hydrolysate and gelatin hydrolysate permeated 1 kDa MWCO membrane.

Amino acids	Content (%) [a]	Content (%) [b]
Asparagine (Asp)	4.34	3.43
Serine (Ser)	3.24	2.18
Glutamine (Glu)	5.61	4.43
Glycine (Gly)	32.33	38.22
Histidine (His)	0.61	0.54
Arginine (Arg)	6.14	5.22
Threonine (Thr)	3.08	2.13
Alanine (Ala)	9.01	8.15
Proline (Pro)	12.82	14.7
Tyrosine (Tyr)	0.72	1.45
Valine (Val)	2.84	2.17
Methionine (Met)	4.74	6.51
Lysine (Lys)	2.65	1.25
Isoleucine (Ile)	1.25	1.33
Leucine (Leu)	2.60	1.62
Phenylalanine (Phe)	1.98	2.47
Cysteine (Cys)	0.01	0.03
Tryptophan (Trp)	0.03	0.09
Hydroxyproline (Hyp)	6.00	4.13

Source: Mahmoodani *et al*. (2012)

[a] Amino acid composition (%) of gelatin hydrolysate.

[b] Amino acid composition (%) of gelatin hydrolysate permeated 1 kDa MWCO membrane.

1.2.3 Bioactive activity of fish-derived peptides

1.2.3.1 Antioxidant activity of fish-derived peptides

Bioactive peptides refer to specific protein fragments that have a positive impact on body function or condition and which ultimately may influence health beyond their basic role as nutrient sources (Hartmann and Meisel, 2007). These

peptides are usually short in length (2-20 amino acids residues) and their activity is based on size, amino acid composition and sequence (Kitts and Weiler, 2003; Korhonen and Pihlanto, 2006). Moreover, the potential to elicit biological responses depends on the ability of the peptides to cross the intestinal epithelium and enter the blood circulation or to bind directly to specific epithelial cell surface receptor sites (Korhonen and Pihlanto, 2006). Many studies have reported that fish skin protein can be used to produce bioactive peptides with functional and biological properties including ACE inhibitory, antimicrobial, antioxidant and Ca-binding activities. A number of studies have demonstrated that peptides derived from different fish skin protein hydrolysates act as potent antioxidants (Table 3).

Table 3. Antioxidative peptides from fish skin protein hydrolysis.

Fish skin	Enzyme	Peptide sequence	MW	Antioxidant activity	References
Cobia skin	Bromelain Papain Pancreatin Trypsin		< 700 Da	DPPH scavenging activity, inhibition of linoleic acid peroxidation	Yang *et al*. (2008)
Alaska pollack skin	Neutrase Protamex Pepsin Flavourzyme Papain Alcalase	2-8 amino acids	180-1000 Da	DPPH IC_{50}=2.5 mg/ml, reducing power (0.14 at 1 mg/ml), reduced glutathione (53.8% at 1 mg/ml)	Jia *et al*. (2009)
Sole skin	Alcalase		< 6.5 kDa	Chelating ability (84.7% at 0.2 mg/ml), FRAP (4 μM), ABTS (16 mg VCEAC/g)	Giménez *et al*. (2009)
Tuna skin	Alcalase		< 10 kDa	FRAP (11.55 EC1), ABTS (17.57 mg VCEAC/g)	Gómez *et al*. (2010)
Nile tilapia skin	Alcalase Pronase E Pepsin Trypsin	Asp-Pro-Ala-Leu-Ala-Thr-Glu-Pro-Asp-Pro-Met-Pro-Phe	1382 Da	Against free radical-induced cellular and DNA damage in murine microglial cells	Vo *et al*. (2011)

Some of these antioxidant peptides have exhibited varying capacities to scavenge free radicals (Nalinanon *et al*., 2011; Yang *et al*., 2008); some peptides are capable of chelating metal ions, which acts as pro-oxidant (Jia *et al*., 2010; Giménez *et al*., 2009); several studies have indicated that peptides derived from fish proteins exhibited greater antioxidative properties than α-tocopherol in the linoleic acid oxidation and the lecithin liposome systems (Kim *et al*., 2007; Je *et al*., 2005; Phanturat *et al*., 2010).

1.2.3.2 ACE inhibitory activity of fish-derived peptides

Peptides ranging from 2 to 20 amino acids, which inhibit ACE activity, have been produced by the enzymatic hydrolysis of casein and whey proteins. Other proteins include zein, gelatin, yeast, corn, and fish. Associated advantages of these peptides include treatment of cardiovascular diseases, cancer, diabetes, osteoporosis, hypertension, gastrointestinal disorders, and renal function. These peptides have a potential as antihypertensive components in functional foods or nutraceuticals (Li *et al*., 2004; Vercruysse *et al*., 2005). Many peptides derived from fish proteins that have been performed ACE inhibitory activity such as peptides from sardine, bonito, tuna, and salmon. Biologically active peptides can be released from their parent protein by enzymatic hydrolysis during gastrointestinal digestion, fermentation or maturation during food processing or proteolysis by food-grade enzymes derived from different origins (animal, microorganisms or plants) (Phelan *et al*., 2009). However, the most common way to produce bioactive peptides is through enzymatic hydrolysis. A large number of studies have demonstrated the release of ACE-inhibitory peptides by hydrolysis with gastrointestinal enzymes such as pepsin, trypsin, and chymotrypsin (Vermeirssen *et al*., 2004; Pihlanto-Leppala *et al*., 2000; Roufik *et al*., 2006; Lourenço *et al*., 2007). In addition to live microorganisms, proteolytic enzymes from bacterial and fungal sources have been also used to generate bioactive peptides from various proteins. The use of commercially available microbial-derived food-grade proteinases to hydrolyse food proteins is advantageous as these enzymes are low-cost and safe, and the product yields are very high (Mao *et al*., 2007). Several ACE inhibitory peptides obtained by enzymatic hydrolysis of fish skin protein were shown in Table 4. In some cases, enzymatically hydrolysed fish skin gelatins have shown

better biological activities compared to the peptides derived from fish muscle proteins to act as antioxidants and antihypertensive agents (Kim and Mendis, 2006).

Table 4. ACE inhibitory peptides from fish skin protein hydrolysis.

Fish skin	Enzyme	Peptide sequence	MW	Bioactive activity	References
Nile tilapia skin	Alcalase Pronase E Pepsin Trypsin	Asp-Pro-Ala-Leu-Ala-Thr-Glu-Pro-Asp-Pro-Met-Pro-Phe	1382 Da	Antioxidant, antihypertension	Vo *et al*. (2011)
Pacific cod skin	Neutrase Pepsin Papain Trypsin Alcalase Chymotrypsin	Thr-Cys-Ser-Pro Thr-Gly-Gly-Gly-Asn-Val	388 Da 485.5 Da	Antioxidant, antihypertension	Ngo *et al*. (2011)
Pangasius catfish skin	Alcalase		< 1 kDa	Antihypertension IC_{50} = 3.2 µg/ml	Mahmoodani *et al*. (2012)
Atlatic salmon skin	Alcalase Papain	Ala-Pro Val-Arg	< 1 kDa	Antihypertension IC_{50} = 0.06 mg/ml; IC_{50} = 0.332 mg/ml	Gu *et al*. (2011)
Skate skin	Alcalase Chymotrypsin Neutrase Trypsin Pepsin	Pro-Gly-Pro-Leu-Gly-Leu-Thr-Gly-Pro Gln-Leu-Gly-Phe-Leu-Gly-Pro-Arg	975.38 Da 874.45 Da	Antihypertension IC_{50} = 95 µM IC_{50} = 148 µM	Lee *et al*. (2011)
Alaska pollack skin	Alcalase, Pronase E Collagenase	Gly-Pro-Leu Gly-Pro-Met	0.9-1.9 kDa	Antihypertension IC_{50} = 2.6 µM IC_{50} = 17.13 µM	Byun and Kim (2001)

The majority of ACE inhibitory peptides are relatively short sequences containing from 2 to 12 amino acids (Natesh *et al*., 2003). Of many ACE-inhibitory peptides identified from different food sources, structure–activity studies indicated that *C*-terminal tripeptide residues play a predominant role in competitive binding to

the active site of ACE. It has been reported that this enzyme prefers substrates or inhibitors containing hydrophobic (aromatic or branched side chains) amino acid residues at each of the three *C*-terminal positions. The most effective ACE-inhibitory peptides identified containing Tyr, Phe, Trp or Pro at the *C*-terminal (Ledesma *et al*., 2011). Ruiz *et al*. (2004) and Alemán *et al.* (2011) have suggested that amino acid Leucine may contribute significantly to increase ACE-inhibitory potential.

1.2.3.3 Multi-functional peptides

Recent researches revealed that some protein hydrolysates contain peptides possessing several different biological activities. For example, peptide fractions from Alcalase enzymatic hydrolysis of tuna and squid gelatins presented both antioxidant and antimicrobial properties (Gómez-Guillén *et al*., 2010) or an antihypertensive and antioxidant peptide was obtained from Nile tilapia gelatin by Alcalase enzymatic hydrolysis in the study of Vo *et al*. (2011). These protein hydrolysates with different bioactivities may be therefore used as multifunctional ingredients in functional foods to control various human diseases. The ACE inhibitory activity is the most studied biological activity for the fish-derived peptides. Studies conducted with mildly hypertensive subjects, have demonstrated a great potential in reduction of blood pressure following daily ingestion of fish-derived ACE inhibitors (Meisel, 2005).

1.2.4 Separation of bioactive peptides by ultrafiltration

1.2.4.1 Basic concepts of ultrafiltration

Though the term ‘‘membrane technology’’ refers to a number of separation processes with different characteristics, the general principle is based on the selective permeability of the membrane to allow the target substances to penetrate through the membrane while unwanted substances are normally rejected by the membrane. Based on the size of the substances to be separated and the resultant characteristics needed for the membrane, membrane techniques are generally classified into reverse osmosis, nanofiltration, ultrafiltration, microfiltration…

Ultrafiltration (UF) is a variety of membrane filtration in which hydrostatic pressure forces a liquid against a semipermeable membrane. Suspended solids and solutes of high molecular weight are retained while water and low molecular weight solutes pass through the membrane. UF is the principle to select the size of sample and the cross flow filtration. The sample feed is divided to two phases, sample filters the membrane is called permeate and sample rejects by membrane which is called retentate. UF is one of the most used in waste water treatment or the industry systems such as food and biotechnology industry for purifying and concentrating macromolecular (10^3 - 10^6 Da) solutions, especially protein solutions. This process promotes the separation of molecules in solution on the basis of size; a pressure gradient is the driving force of the process. Commonly, the UF is used in separation of protein compounds include colloidal particles, biomolecules, polymers, and some sugars. Now, UF has been widely used as preferred method for protein separation. Protein fractionation is rapidly becoming more selective through improvements in membrane and module design. Membrane separation techniques offer advantages of lower cost and ease to scale-up for commercial production. However, the lack of membrane selectivity and its fouling due to protein absorption during filtration have severely restricted UF applications (Baker, 2000).

Advances in material science and membrane manufacturing technology have led to membrane based filtration techniques becoming a mainstream technology since the early 1990s. With a number of advantages such as high efficiency, simple equipment, convenient operation and low energy consumption, membrane technology has become one of the most important industrial separation techniques (Cheryan, 1998).

UF membranes typically have pore sizes in the range from 10 to 1000 Å and are capable of retaining species in the molecular weight range of 300-500,000 Daltons. Typically rejected species include sugars, polymers and colloidal particles, biopeptides (Kulkarni *et al*., 1992). Commercial applications of UF are numerous and found in many different files. For instance, UF has been used for clarification of fruit juices (Girard and Fukunoto, 2000), recovery of protein (Zwijnenberg *et al*., 2002) and gelatin solutions (Dutre and Gragardh, 1995).

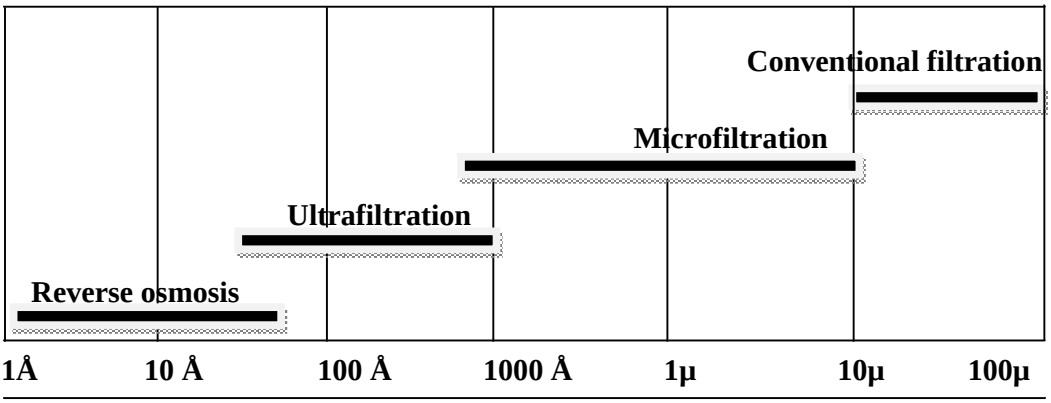

Figure 3. Separation capacity of membrane process.

Source: Richard and Alexander (1995)

Filtration can be defined as a method of separating particulate matter in a continuous liquid using the permeable barrier. The permeation flux of UF is the flow rate of permeate per membrane area, when sample is water the permeation flux (J) can be described by Darcy's law as follow (Baker, 2000):

$$J = \frac{TMP}{\mu_m R_m} \qquad (1\text{-}1)$$

The other type of samples such as sugar, fruit juice, protein, the permeate flux follow by the equation as

$$J = \frac{TMP - \Delta_\pi}{\mu_p R} \qquad (1\text{-}2)$$

Where J is the permeation flux ($m^3\ m^{-2}\ s^{-1}$)

TMP is the transmembrane pressure (Pa)

R, R_m are the membrane resistance (m^{-1})

Δ_π is the osmotic pressure difference between permeate and the feed

μ_p, μ_m are the viscosity of permeate (Pa.s)

The TMP is known as a transmembrane pressure across the membrane and can be calculated as

$$TMP = \frac{P_{in} + P_{out}}{2} - P_p \qquad (1\text{-}3)$$

Where P_{in} is the pressure into membrane

P_{out} is the pressure at the outlet of the membrane

P_p is the permeate pressure. The permeate pressure is very close to 0, so it is often ignored.

The flux, based on Darcy's law is

$$J = \frac{TMP}{\mu R_T} \quad (1\text{-}4)$$

Where R_T is total hydraulic resistance (m^{-1}), comprising the membrane resistance (R_m) and the fouling resistance (R_f)

$$R_T = R_m + R_f \quad (1\text{-}5)$$

R_f can be broken down to reversible fouling resistance (R_{rf}) and irreversible fouling resistance (R_{if}).

$$R_f = R_{rf} + R_{if} \quad (1\text{-}6)$$

1.2.4.2 Factors affect the performance of membrane process

The key hydrodynamic factors in membrane processing are temperature, transmembrane pressure and cross flow velocity. The permeate flux increases with the TMP but the relation between them is only linear when the feed is pure water. If the feed is another product, the flux becomes independent of the pressure and mass transfer controlled when the pressure increase above the level, the concentration polarization layer reaches a limiting concentration (Charoenphun, 2013).

Cross flow velocity (CFV)

CFV is a key factor influencing membrane flux and fouling, especially for reduction of concentration polarization or reversible fouling resistance. In general, increasing cross flow velocity could decrease fouling of feed because high shear rates generated at the membrane surface tend to shear off deposit material and thus reduce the hydraulic resistance of the fouling layer. Fouling may be more severe when a solution filtered at low feed flow rate (Torres *et al*., 2002).

Transmembrane pressure (TMP)

TMP is a key operating parameter for pressure driven membrane processes. During membrane filtration, two distinct operation regions exist regarding to permeate flux. The region in which the permeate flux increase with increasing TMP is called the pressure-dependent region. Under conditions where the permeate flux

does not increase with increasing TMP, it is called the pressure independent region (Grandison and Lewis, 1996).

Feed property

The properties of the feed material also significantly affect the performance of the membrane process through their influence on membrane fouling. Some of the important feed properties are feed concentration, presence and properties of feed particles, pH and ionic strength of the feed and electric charge of the particles in the feed. Generally, an increase in the feed concentration results in a decline in the flux. This is due to higher fouling of the membrane in the presence of a higher concentration of foulness.

Temperature

It is known that increasing feed temperature increases the permeate flux in UF due to a decrease in permeate viscosity, which enhances permeate flow, and increases diffusivity, which enhances dispersion of the polarized layer.

1.2.4.3 Concentration polarization and fouling

Concentration polarization (CP) and fouling are important processes and affect membrane, especially UF. Concentration polarization and fouling result in flux decrease. However, the process affects retention, rejection factors and membrane performance. The CP refers to accumulate solute on membrane surface. The solute concentration at the membrane increase until, in the steady state, the rate of solute diffusion away from the membrane equals the rate of convective transport toward it. CP may reduce the efficiency of the separation process by lowering the solvent flux due to the increase of osmotic pressure (which depends on the solute concentration only at the membrane), by increasing solute concentration in the filtrate (if solute rejection by the membrane is incomplete), or both of them (Deen *et al*., 1974). The importance of CP depends on the membrane separation process. Membrane performance in UF and electrodialysis process is seriously affected by CP. On the other hand, CP affects membrane performance in reverse osmosis, but it is usually well controlled in industrial systems. Membrane fouling is defined as the process in

which solute or particles deposit onto the membrane surface or into membrane pores such that membrane performance is deteriorated. It presents major obstacle for the wide spread use of this technology. Formation of a strong matrix of fouling layer with the solute during continuous filtration process will result in reversible fouling being transformed into irreversible fouling layer. Irreversible fouling is normally caused by strong attachment of protein. Flux and retention coefficient are the indicators of membrane fouling (Baker, 2000).

Protein fouling may be due to one or more of the following mechanisms: (1) Formation of a dynamic membrane (surface layer) on the front face of the membrane. In UF, fouling occurs predominantly on the membrane surface where the dynamic membrane controls membrane behavior. (2) Fouling within the membrane structure: The protein deposits within the membrane pore as well as on the surface. In UF, the amount of protein deposited within the membrane pores is small compared with that on the membrane surface. (3) Fouling at the pore entrance: The deposition of materials on the surface of the membrane must have in some way obstructed the pore entrances. The loss of effective membrane porosity is dependent upon the size of the depositing molecules and the pore size (Marshall *et al*., 1993). Fouling of membrane during practical application for protein separation resulted from its adsorption on membrane surface. It significantly increases hydraulic resistance to flow, which reduced filtration flux rate and induced unfavorable effect on efficiency and economics of protein recovery processes (Marshall *et al*., 1993). Protein adsorption results in the formation of a surface layer, internal membrane fouling and fouling at the pore entrance. The structure and charge of protein, composition, hydrophobicity and surface potential of the membrane surface play important role in protein adsorption on the membrane (Kuzmenko *et al*., 2005).

There are three factors contributing to membrane fouling during protein separation including feed properties (concentration, pH, and ionic strength), membrane material (pore size and physical, chemical properties), and processing variables (TMP, temperature, and CFV). The role of feed properties: An increase in the feed concentration during UF generally results in a decrease in the permeate flux but has little effect on the membrane retention characteristics, except when the component size changes with concentration. Where surface fouling occurs, increasing

concentration has little effect on irreversible membrane fouling but causes an increase in the rate of membrane fouling. At high concentration, surface fouling is like to dominate (Nigam *et al*., 2008). In addition, proteins are complex molecules and their aggregation or interaction with the membrane surface, which is influenced by the pH and ionic strength, is not clearly understood (Charoenphun, 2013). The role of the membrane material: Most UF membranes have a wide pore size distribution. Membrane selectivity is poor with membranes of a wide pore size distribution. Thus, the permeate flow, component retentions and membrane selectivity change as the membrane fouls with time. Furthermore, the charges on membrane are strongly dependent upon the membrane material, the pH and the ionic strength of the feed solution. The operation with a membrane of similar charge to the protein can enhance the permeate flux if concentration polarization is minimized. With membranes of a large pore size, greater protein selectivity may be possible. Likewise, a series of investigations prove that proteins are generally less easily adsorbed to hydrophilic membranes than hydrophobic membranes. However, where concentration polarization and total protein deposition are high, the effect of hydrophobicity is masked by the effects of concentration polarization (Huisman *et al*., 2000). The role of processing variables: In UF, an increase rate of fouling results in an increase the final component retention. The greater applied pressure (greater initial flux) has the tendency to promote fouling. Drastic fouling may occur at high flux due to its greater throughput for the same fouling duration and the amount of foulant material brought towards the membrane surface is proportional to the throughput. More importantly, greater permeate flux may result in severe concentration polarization and increase drag force acting on the foulant molecules towards the membrane surface, both of which can lead to severe fouling. In turn of temperature, increasing the temperature generally results in an increase in the permeate flux due to the dual effect of lowering the permeate viscosity, which assists flow rate, and of increasing diffusivity, which assists the dispersion of the polarized layer. Fouling on the membrane surface may be reduced due to the increase in diffusivity and a lessening of concentration polarization. On the other hand, the removal of the surface layer may lead to greater internal fouling (Yan-Jun *et al*., 2000). The effect of CFV, increasing the CFV generally results in an improvement in permeate flux. The membrane resistance is

reduced when the velocity is increased. In addition, increasing the velocity reduces membrane fouling as well as the polarization layer. Further evidence shows that CFV enhanced back transport and reduced concentration polarization at greater CFV. Alternatively, the greater shear force along a membrane surface might also help to sweep foulant away from the membrane (She *et al*., 2009).

1.2.5 Enzymatic membrane reactor (EMR) concept

1.2.5.1 Performance of EMR

The combination of membrane separation and enzymatic reactor is called enzymatic membrane reactor. It is well known that most of the enzymatic processes currently in industrial use are carried out in batch reactors. The basic feature of a membrane reactor is the separation of enzyme, products and substrates by a semi-permeable membrane that creates a selective physical/chemical barrier. Permeable substrates and products can selectively be separated from the reaction mixture by the action of a driving force (chemical potential, pressure, electric field) present across the membrane that causes the movement (diffusion, convection, electrophoresis migration) of solutes. On the other hand, the enzyme is retained within the system by the membrane, allowing the establishment of a continuous operation with substrate feed and product withdrawal. The whole system can be set up by assembling and interconnecting vessels and membrane modules (Cobal *et al*., 2001).

Conversion

The conversion in EMR is used to illustrate the amount of protein recovered in permeate. Total nitrogen contents in the substrate and permeate are used to calculate the conversion (X) and it is expressed as follow (Cui *et al*., 2011).

$$X\,(\%) = \frac{N_{permeate}}{N_{unhydrolysed\ substrate}} \times 100 \qquad (1\text{-}7)$$

Where $N_{permeate}$ is the total nitrogen in the permeate (g)

$N_{unhydrolysed\ substrate}$ is the total nitrogen in substrate (g)

Productivity

Productivity is defined as the mass of hydrolysate produced per unit mass of enzyme (mg N/mg enzyme) or per unit volume of permeate (mg N/mL permeate). For the batch reactor, productivity (P_{batch}) is expressed as follow (Cui *et al*., 2011).

$$P_{batch} = \frac{X S_0}{E} \quad (1\text{-}8)$$

Where X is the conversion (%)

S_0 is the total nitrogen in the substrate (mg/mL)

E is the amount of enzyme (mg/mL)

For the EMR, productivity (P_{EMR}) is expressed as

$$P_{EMR} = \frac{N J_t t}{E V} = \frac{X S_0 J_t t}{E V} \quad (1\text{-}9)$$

Where N is the average product output (mg N/mL) in a time period t (min)

J_t is the flow rate (mL/min)

E is the amount of enzyme (mg/mL)

V is the substrate volume (mL)

Capacity

The capacity of EMR (C_{EMR}) is defined as the mass of hydrolysate produced per unit mass of enzyme per unit time (mg N/mg enzyme/min) and it is expressed as follow (Cui *et al*., 2011).

$$C_{EMR} = \frac{N J}{E V} = \frac{X S_0 J}{E V} \quad (1\text{-}10)$$

1.2.5.2 Operating parameters for EMR

1.2.5.2.1 Enzyme to substrate ratio

The specificity of protease acting on protein varies with enzyme to substrate ratio in a manner that the enzyme appears to be able to hydrolyze fewer peptide bonds when the enzyme to substrate ratio is low. On the other hand, when enzyme to substrate ratio is increased the rate of splitting certain peptide bonds appears to increase more than proportionally to enzyme to substrate ratio (Adler-Nissen, 1986). The enzyme to substrate ratio also affects to the rate of reaction. For example, if there are more enzyme molecules than substrate molecules then the

substrate would be limiting factor, as there would not be enough substrate to continue forming products. When all the active sites are in use, the optimum rate will have been achieved. Similarly, the substrate concentration affects to the rate of reaction that the limiting factor is the lack of active sites. The rate of this reaction will be slow as there are not enough active sites (Clegg *et al*., 1996).

1.2.5.2.2 Transmembrane pressure (TMP)

The increase of TMP has both positive and negative effects on the permeate flux. As pressure increases further, the concentration polarization layer reaches a limiting concentration and the flux becomes independent of pressure and becomes mass-transfer controlled. However, increasing pressure above a critical point may result in low flux (Cheryan, 1998).

1.2.5.2.3 Cross flow velocity (CFV)

The CFV is a very important factor influencing membrane flux and fouling. High shear rates generated at the membrane surface tend to shear off deposited material and thus reduce the hydraulic resistance of the fouling layer (Cheryan, 1998). The feasibility of producing protein hydrolysates using an UF reactor system was evaluated by Deesile and Cheryan (1981). They studied the effect of CFV on reactor product output. The result shown approach to steady-state was more rapid at higher flux, but reactor output simultaneously decreased. This is due to a decrease in residence or contract time at higher CFV.

1.2.5.3 Production of bioactive peptides using EMR

Membrane separation is a useful technique to extract, concentrate, separate or fractionate the compounds. The use of enzymatic membrane reactor to integrate a reaction vessel with a membrane separation unit is emerging as a beneficial method for producing peptides. These bioactive compounds from membrane reactor technology show diverse biological activities such as antihypertensive, antioxidant and Calcium bioavailability properties (Table 5).

Table 5. Biological activities of bioactive peptides from membrane bioreactor.

Protein source	Bioactivity	References
Alaska Pollack skin gelatin	Antioxidant	Kim *et al*. (2001)
Hoki frame protein	Antioxidant	Je *et al*. (2005a)
Alaska Pollack frame protein	Antioxidant	Je *et al*. (2005b)
Jumbo squid skin gelatin	Antioxidant	Mendis *et al*. (2005b)
Alaska Pollack frame protein	Antihypertensive	Je *et al*. (2004)
Alaska Pollack back bone	Calcium bioavailability	Jung *et al.* (2006)

An enzymatic membrane reactor system compose of two main systems: a bioreactor unit that is responsible for biodegradation of raw materials and the membrane unit that is responsible for the separation of desired bioactive molecules according to molecular size. Various enzymes can be used depending on the desired final products. UF membrane based separations of enzymatic hydrolysates can achieve the removal of peptides from non-hydrolyzed proteins and proteolytic enzymes. Peptides with specific molecular weight distributions can be obtained by using membranes with appropriate MWCO.

1.3 Research objectives

1. To investigate antioxidant and ACE inhibitory activity of the hydrolysate from skin by-product of striped catfish (*Pangasius hypophthalmus*) fillet processing.
2. To enrich antioxidant and ACE inhibitory peptides from the hydrolysate with ultrafiltration.
3. To produce antioxidant and ACE inhibitory peptides from striped catfish skin protein using continuous enzymatic membrane reactor.

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CHAPTER 2

ANTIOXIDATIVE AND ACE-INHIBITORY ACTIVITIES OF PEPTIDES DERIVED FROM SKIN BY-PRODUCT OF STRIPED CATFISH (*Pangasius hypophthalmus*) FILLET PROCESSING *

2.1 Introduction

High blood pressure is a major risk factor associated with cardiovascular disease, the biggest cause of death (Howell and Kasase, 2010). Hypertension is commonly treated with antihypertensive or blood pressure lowering drugs, such as captopril, benazepril, enalapril, fosinopril, lisinopril, moexipril, perindopril, quinapril, ramipril, and trandolapril (Miyoshi *et al*., 1991). These drugs are angiotensin I converting enzyme (ACE) inhibitors. As many synthetic drugs like ACE inhibitors have side effects, peptides from food sources provide an attractive alternative (Howell and Kasase, 2010). Recent researches have reported discoveries of peptides, which are isolated and characterized from a number of fish skin by-products such as Nile tilapia skin (Vo *et al*., 2011), Pacific cod skin (Ngo *et al*., 2011), Atlantic salmon skin (Gu *et al*., 2011), Skate skin (Lee *et al*., 2011) that inhibited ACE and can be used as nutraceuticals and functional food ingredients.

There is mounting evidence that links uncontrolled generation of reactive oxygen species (ROS) from free radical reactions to a variety of diseases, such as cancer, atherosclerosis, coronary heart disease, diabetes, neurological malfunctioning, and weakening of the immune system. It has been shown that antioxidants and free radical scavengers are crucial in the prevention of pathologies such as cancer, heart diseases, biological damage in living tissues, and neurodegenerative diseases in which reactive oxygen species or free radicals are implicated (Pena-Ramos and Xiong, 2002).

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On the other hand, ROS negatively impact in the quality and nutrition of food products (Pena-Ramos and Xiong, 2001). Lipid oxidation is of significant concern to the food industry and consumers because it leads to the development of undesirable off-flavors, odors, and potentially toxic reaction products (Pena-Ramos *et al*., 2004). However, lipid oxidation can be effectively prevented by using antioxidants. Thus, antioxidants with a strong capability to inhibit oxidation both in food and in the human body are of high demand. Many synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are used as food additives to prevent deterioration. Although these synthetic antioxidants show stronger antioxidant activities than those of natural antioxidants such as α-tocopherol and ascorbic acid, the use of these chemical compounds has begun to be restricted because of their induction of DNA damage and their toxicity (Kong and Xiong, 2006). Therefore, the search for natural antioxidants as alternatives to synthetic ones is of great interest among researchers. A number of studies have shown that peptides and protein hydrolysates of fish by-products possess significant antioxidant activity such as Alaska pollack skin (Kim *et al*., 2001; Jia *et al*., 2010), Alaska pollack frame protein (Je *et al*., 2005), hoki frame protein (Kim *et al*., 2007), cobia skin gelatin (Yang *et al*., 2008), tuna backbone protein (Je *et al*., 2007).

2.1.1 Review of literature

Fish processing by-products are potential sources of collagen and gelatin. Moreover, fish skin gelatin can be modified into biologically active peptides by protease treatments and such purified peptides exhibited a potential to act as antioxidants. In recent years, there has been an increasing demand to produce protein hydrolysates containing peptides with specific physiological properties, which could be marketed as functional food ingredients. Some fish skin hydrolysates have been found to have noticeable antioxidant, ACE inhibitory activity and it has been suggested that they might be a candidate to be a natural antioxidant, ACE inhibitor. For example, Yang *et al*. (2009) studied the process for the production of tilapia retorted skin gelatin hydrolysates with optimized antioxidative properties.The results showed that tilapia retorted skin gelatin hydrolysate inhibited lipid peroxidation (75.0%) and exhibited noticeable DPPH free-radical scavenging (80.3%). The

production of antioxidant peptide fractions from tuna skin gelatin using Alcalase was studied by Gómez-Guillén *et al*. (2010). Results of anti-oxidative activity, in terms of ferric reducing antioxidant power (FRAP) and radical scavenging (ABTS) capacities of fractionated hydrolysates with molecular weight of 1 to 10 kDa were 9.12 EC1 and 17.07 mg VCEAC/g protein, respectively. Lee and coworkers (2011) studied the ACE inhibitory activity of skate skin protein hydrolysates and its corresponding fractions. The purified peptides from skate skin had an IC_{50} value of 95 μM and 148 μM and their amino acid sequences were dentified to be Pro-Gly-Pro-Leu-Gly-Leu-Thr-Gly-Pro (975.38 Da) and Gln-Leu-Gly-Pro-Arg (874.45 Da), respectively. Vo *et al*. (2011) studied production of an ACE inhibitory and antioxidant peptide derived from enzymatic digestion of Nile tilapia gelatin. The purified peptide was Asp-Pro-Ala-Leu-Ala-Thr-Glu-Pro-Asp-Pro-Met-Pro-Phe (1382 Da) that showed the highest ACE inhibitory activity of 52% at 500 μg/mL concentration.

2.1.2 Objective

The objective of this study was to produce antioxidant, ACE inhibitory peptides from striped catfish skin by-products by enzymatic hydrolysis using Alcalase.

2.2 Materials and methods

2.2.1 Materials

Samples of striped catfish skin were obtained from the fish fillet processing and stored at -20 ^{o}C. Alcalase from *Bacillus licheniformis* 2.4 L, o-phthalaldehyde (OPA), DL-dithiothreitol (DTT), ACE from rabbit lung, 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), linoleic acid, ascorbic acid, α-tocopherol and other chemicals were purchased from Sigma–Aldrich Chemical Company.

2.2.2 Proximate analysis

The striped catfish skin was thawed and washed with running tap water and then cut into pieces (1 x 1 cm). The cleaned fish skin was drained using

cheesecloth. After that proximate composition was determined. The moisture, ash and lipid content of the raw fish skin were determined according to AOAC (1999); and protein content was determined by Kjeldahl method (AOAC, 1999) (Appendix 1-4).

2.2.3 Extraction of protein from striped catfish skin using autoclave

The cleaned skins were added with distilled water (1:2, w/v) and the protein was extracted by retorting in an autoclave (SX-500 Tomy, Tokyo, Japan) at 121 ^{o}C for 30 min. After retorting, the extracted solution was filtered through a metal sieve (100 mesh, InterNet, Inc., Minneapolis, MN, USA) to remove skin residues. The filtrated solution was manually defatted after lipid and protein separated at refrigerator temperature. The solution was centrifuged at 3000 xg for 20 min at 25 ^{o}C to remove insoluble residues. The samples were kept in plastic bottle at – 20 ^{o}C. The moisture, lipid content of the extracted solution was determined according to AOAC (1999) and protein content was determined by Kjeldahl method (AOAC, 1999). The yield of extraction process was calculated as the ratio of protein content in the extracted solution to its initial concentration in the skin using the following equation.

$$\text{Yield (\%)} = \frac{\text{Protein content in the extracted solution}}{\text{Protein content in the skin}} \times 100 \qquad (2\text{-}1)$$

2.2.4 Protease activity assay

Protease activity was assayed by Anson (Anson, 1938) method as modified by Thangam and Rajkumar (2002) with slight modification, using Casein as the substrate. The reaction mixture, containing 5 mL of 0.65% casein in 50 mM potassium phosphate buffer, pH 7.5 and 5 mL of suitably diluted enzyme, was incubated at 37 ^{o}C for 10 mins. The reaction was terminated by the addition of an equal volume of 110 mM trichloroacetic acid and filtered through Whatman No. 1 filter paper. To 2 mL of filtrate, 5 mL of 500 mM Na_2CO_3 solution and 1 mL of 5-fold-diluted Folin-Ciocalteau reagent were added and mixed thoroughly. The color developed after 30 mins of incubation at 37 ^{o}C was measured at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μmol Tyrosine per minute at pH 7.5 at 37 ^{o}C (Color by Folin-Ciocalteau reagent) (Appendix 5).

2.2.5 Enzymatic hydrolysis of extracted solution from striped catfish skin

The protein solution was hydrolysed by Alcalase 2.4 L (EC 3.4.21.14) at pH 8.0 and 50 ^{o}C in a 4-L reactor for 6 h. The extracted protein solution was diluted to obtain a protein concentration of 1% (w/v) by 0.1 M sodium phosphate buffer, pH 8.0. The enzyme to substrate ratio was fixed at 20 unit g^{-1} protein. The pH of the mixture was measured by a pH meter (Eutech, Cyber Scan pH 110, Singapore) and manually adjusted to pH 8.0 during the hydrolysis by 6 N NaOH and 6 N HCl. Aliquots of hydrolysate were collected every 60 mins during the hydrolysis. The sample aliquots were heated in boiling water (95 ^{o}C) for 10 mins to inactivate Alcalase. They were kept in plastic bottles at – 20 ^{o}C for analyses.

2.2.6 Protein determination

Protein determination was carried out using Lowry' s method (Lowry *et al*., 1951). Briefly, the 1 mL of samples were mixed with 5 mL of alkaline-copper reagent (2% Na_2CO_3 in 0.1 N NaOH: 1% potassium sodium tartrate: 0.5% $CuSO_4$ in the ratio of 100: 1: 1) at room temperature for 10 mins. The mixture was then added and mixed immediately with 0.5 mL of diluted Folin-Ciocalteau reagent (Folin-phenol: distilled water in the ratio of 1:1). The absorbance at 750 nm was determined using spectrophotometer (Thermo Scientific GENESYS 20, Tokyo, Japan). The calibration curve was prepared using bovine serum albumin as standard (Appendix 6).

2.2.7 Estimation of degree of hydrolysis

The degree of hydrolysis (DH%), which is defined as the percentage of peptide bonds cleaved, was calculated based on the determination of free amino groups by the OPA method as modified by Wanasundara *et al*. (2002). Briefly, the OPA reagent was prepared daily; it consisted of 6 mM OPA (first dissolved in 97% ethanol, v/v) and 5.7 mM DL-dithiothreitol in 0.1 M sodium tetraborate decahydrate containing 1% (w/v) sodium dodecyl sulphate (SDS). Samples (0.4 mL) from the hydrolysis reaction were mixed with 3 mL of OPA reagent and incubated for 2 mins at room temperature before measuring the absorbance at 340 nm using a spectrophotometer (GENESYS 10S UV-VIS Thermo Scientific, Tokyo, Japan). The

amount of free amino groups was calculated as serine-NH_2 moieties (Appendix 7). Total free amino groups in the proteins were determined by acid hydrolysis of the protein (6 N HCl at 110 ^oC for 24 h). The degree of hydrolysis (DH%) was calculated as the ratio of released free amino groups due to enzymatic hydrolysis to total free amino groups released due to complete acid hydrolysis and expressed as a percentage value as Eq. 2-2.

$$\text{DH } (\%) = \frac{[NH_2]_{T_x} - [NH_2]_{T_0}}{[NH_2]_{T_{Total}} - [NH_2]_{T_0}} \times 100 \qquad (2\text{-}2)$$

Where $[NH_2]_{T_x}$ is the number of α-NH_2 groups in the supernatant after x min of protease catalyzed hydrolysis for each experiment point.

$[NH_2]_{T_0}$ is the number of α-NH_2 groups at 0 min of hydrolysis.

$[NH_2]_{T_{Total}}$ is the number of α-NH_2 groups resulting from acid hydrolysis (complete hydrolysis of protein is assumed).

2.2.8 Determination of DPPH radical scavenging activity

The DPPH radical scavenging activity was determined by the method of Binsan *et al*. (2008) with some modifications. To the 1.5 mL sample (1 mg/mL), 1.5 mL of 0.15 mM DPPH in 50% ethanol was added. The mixture was mixed well using a vortex mixer (Scientific Industries, Inc, Bohemia, NY, USA) and allowed to stand at room temperature in the darkness for 30 min. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve was prepared using Trolox in the range of 0-60 μM (Appendix 8). The antioxidant capacity was expressed as μmol Trolox equivalent (μM TE)/mg protein.

2.2.9 Determination of ABTS radical scavenging activity

The ABTS radical scavenging activity of the peptides was determined by the method of Binsan *et al*. (2008) with some modifications. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the darkness. The solution was then diluted by mixing ABTS solution with methanol in order to obtain an absorbance

of 1.1 ± 0.02 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. 150 µL samples (0.2 mg/mL) were mixed with 2850 µL of ABTS solution and the mixture was left at room temperature for 2 h in the darkness. The absorbance was then measured at 734 nm using a spectrophotometer. The control was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve of Trolox ranging from 0 to 600 µM was prepared (Appendix 9). The antioxidant capacity was expressed as µmol Trolox equivalent (µM TE)/mg protein.

2.2.10 Determination of antioxidative activity in a linoleic oxidation system

The antioxidative activity in a linoleic acid model system was determined according to the method of Yang *et al*. (2009) with some modifications. Briefly, a 2.5 mL aliquot of 2 mg/mL linoleic acid emulsion (in 95% ethanol) was mixed with 0.5 mL protein hydrolysate (0.2 mg/mL) and 2 mL of 0.2 M, pH 7.0 sodium phosphate buffers. The mixture was incubated at 37 ^{o}C in darkness for 7 days. To analyze lipid peroxidation inhibition, a 0.1 mL aliquot of the mixture was combined with 4.7 mL ethanol (75%), 0.1 mL ammonium thiocyanate (30%), and 0.1 mL ferrous chloride (20 mM) in 3.5% HCl. After stirring the mixture for 3 min, the absorbance of the colored solution was read at 500 nm. The control was prepared in the same manner except that no protein hydrolysate or antioxidants were added. Antioxidative activities of 200 ppm α-tocopherol and 1000 ppm ascorbic acid were used as a reference for comparative purposes. The percentage of lipid peroxidation inhibition was expressed as Eq. 2-3.

$$\text{Inhibition percent } (\%) = [1 - (\frac{A_1}{A_0})] \times 100 \qquad (2\text{-}3)$$

Where A_0 is the absorbance of the blank (phosphate buffer without hydrolysate).

A_1 is the absorbance in the presence of the hydrolysate sample.

2.2.11 Determination of angiotensin-I converting enzyme inhibitory activity

The inhibition of ACE was determined by the method of Cushman and Cheung (1971) described by Lee *et al*. (2010) with some modifications. The reaction

mixture contained 8.3 mM Hippuryl-L-Histidyl-L-Leucine (Hip-His-Leu) in 0.5 M NaCl and 5 mU ACE in 50 mM sodium borate buffer (pH 8.3). A sample (50 µL) was added to above reaction mixture (50 µL) and mixed with 8.3 mM HHL (150 µL) containing 0.5 M NaCl. After incubation at 37 ^{o}C for 1 h, the further reaction was stopped by the addition of 0.1 M HCl (250 µL). The resulting hippuric acid was extracted by the addition of 1.5 mL of ethyl acetate. After centrifugation (800 xg, 15 mins), 1 ml of the upper layer was transferred into a glass tube and evaporated at room temperature for 2 h in a vacuum. The hippuric acid was redissolved in 3 mL of distilled water, and absorbance was measured at 228 nm using a spectrophotometer (GENESYS 10S UV-VIS Thermo Scientific, Tokyo, Japan). The control and blank were prepared in the same manner, except that 50 µL of buffer was used instead of the sample. The ACE inhibitory activity was expressed as IC_{50} value (µg/mL). The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity. The percentage of inhibition level was calculated as follows:

$$\text{Inhibition level (\%)} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}} - A_{\text{Blank}}} \times 100 \qquad (2\text{-}4)$$

Where $A_{Control}$ is the absorbance of control

A_{Sample} is the absorbance of the sample

A_{Blank} is the absorbance of the blank

2.2.12 Statistical analysis

All experiments were performed in triplicate. All data were subjected to analyse of variance (ANOVA) and the differences between means were evaluated by Duncan's Multiple Range Test (Duncan, 1995).

2.3 Results and discussion

2.3.1 Proximate compositions of striped catfish skin and extracted protein solution

Proximate compositions of striped catfish skin and extracted skin protein solution are shown in Table 6.

Table 6. Proximate compositions of striped catfish skin and extracted protein solution.

Composition (% w/w)	Skin	Extracted protein solution
Moisture	64.6 ± 0.57	85.6 ± 1.43
Protein	28.0 ± 0.33	11.9 ± 0.19
Lipid	6.5 ± 0.16	2.4 ± 0.12
Ash	0.4 ± 0.04	-

Each value is expressed as the mean ± standard deviation (n=3).

The crude protein content in the striped catfish skin from fillet processing industry (28%) was higher than that of some other fish skins from previous studies such as red tilapia (25.4%), catfish (19.9%), snakehead (19.3%), pangasius catfish (19%). The protein content of raw skin material from the fillet processing industry is higher than protein content from others because it still contains large amount of fish muscle. The lipid content in the skin (6.5%) was high. Therefore, the extracted solution was removed lipid before enzymatic hydrolysis to reduce the fat content.

2.3.2 Yield of extracted protein

To convert insoluble native collagen to gelatin, a treatment is required to break noncovalent bonds and disorganize the protein structure, producing adequate swelling and cleavage of intra- and intermolecular bonds, leading to subsequent collagen solubilization (Stainsby, 1987). Cheow *et al*. (2007) reported that hot water extraction causes thermo-hydrolysis and subsequent solubilization of swollen gelatin. Different yield values for gelatin extracted from other fish skins were reported in open literature. Some of these were for pangasius catfish (10.8%), catfish (27.8%), snakehead (16.6%), and red tilapia (11.6%) (See *et al*., 2010); black tilapia (5.4%), red tilapia (7.8%) (Jamilah and Harvinder, 2002); cobia (46.9%) (Yang *et al*., 2008); megrim (7.4%), Dover sole (8.3%), cod (7.2%), hake (6.5%) (Gómez-Guillén *et al*., 2002); and shortfin (7.3%), bigeye snapper (9.4%), brownstripe red snapper (9.4%) (Jongjareonrak *et al*., 2006). The yield of retorted skin protein solution obtained from

extraction process was 74.8%. It was found that the yield value of extracted protein from the skin of striped catfish by-products was greater than those of other fish skins.

2.3.3 Effect of hydrolysis time on degree of hydrolysis (DH)

The extracted protein hydrolysate from retorting process was hydrolysed with Alcalase 2.4 L (20 units g^{-1} protein, pH 8, and 50 ^{o}C) for 6 h in a 4L reactor. The degree of hydrolysis is generally used as a proteolysis monitoring parameter, and it is the most widely used indicator for comparison among different protein hydrolysates (Guérard *et al*., 2002). The effect of hydrolysis time on extracted protein hydrolysis determined as DH (%) using the OPA method is shown in Figure 4. There was a sharp increase of DH in the first 60 min (DH = 25.9 ± 0.22 %) and the DH rate increased significantly as hydrolysis time increased ($p<0.05$), and reached a peak of 42.5 ± 0.84 % after 6 h of hydrolysis. This result was in accordance with Gómez-Guillén *et al*., (2010), who reported that the tuna skin gelatin hydrolysate with Alcalase exhibited a DH of 47.52%; Lee *et al*., (2011) reported that the DH of the skate skin hydrolysate with Alcalase was 65.9%; and the hydrolysis of the sole and squid skin gelatins with Alcalase resulted in 35% and 50% DH, respectively (Giménez *et al*., 2009).

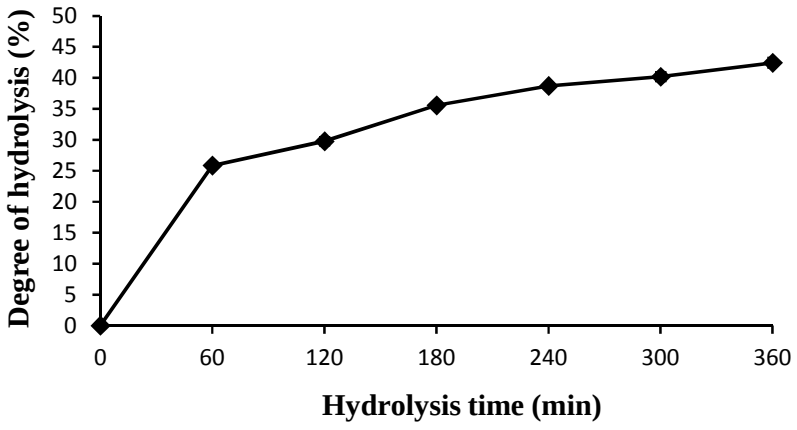

Figure 4. Effect of hydrolysis time on DH of extracted protein hydrolysate by Alcalase. Each value is expressed as the mean ± standard deviation (n=3).

2.3.4 Effect of hydrolysis time on antioxidant activities of hydrolysate

2.3.4.1 DPPH radical-scavenging activity

DPPH radical-scavenging activity is generally used to determine hydrogen-donating ability of protein hydrolysates (Wu *et al*., 2003; Thiansilakul *et al*., 2007). The DPPH free radical can remain stable even at room temperature and shows maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance such as an antioxidant, the radical is scavenged, as visualized by changing its color from purple to yellow, and the absorbance is reduced (Shimada *et al*., 1992). However, DPPH can only be dissolved in organic media, especially in ethanol, this being an important limitation when prediction the role of hydrophilic antioxidants. Figure 5 shows that DPPH radical-scavenging activitiy tended to increase as the hydrolysis time increased ($p<0.05$) and it varied from 34.5 ± 0.72 to 41.3 ± 1.23 μM TE/mg. This indicated that the hydrolysate possessed the ability to donate the hydrogen atom to free radicals, in which the propagation process could be retarded (Faithong *et al*., 2010). The DPPH radical scavenging activity of hydrolysates after 5 h of hydrolysis appeared to remain stable and then declined when the hydrolysis time increased that may be because the lengths of peptides were shortened and lessened the antioxidant activity, similar to that reported by Xia *et al*. (2012) and Charoenphun (2013).

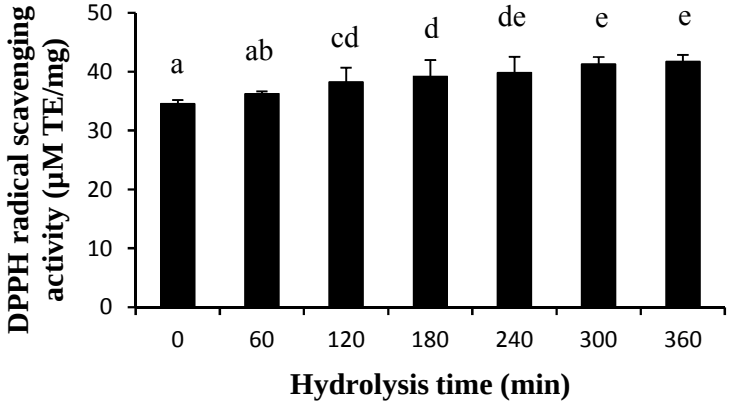

Figure 5. Effect of hydrolysis time on DPPH radical scavenging capacity of hydrolysate. Different letters on the bars indicate significant differences ($p<0.05$).

The DPPH radical scavenging capacity of striped catfish skin hydrolysate in this study was higher than those of protein hydrolysates from bigeye snapper skin (10 - 14 μM TE/mg protein) (Phanturat *et al*., 2010) and ornate threadfin bream muscle (8.58 μM TE/g) (Nalinanon *et al*., 2011).

2.3.4.2 ABTS radical-scavenging activity

The ABTS assay is based on an electron transfer reaction. During the reaction, the blue-green ABTS radical cation, that has absorption maxima at 734 nm, is generated through a reaction between ABTS and potassium persulphate. In the presence of an antioxidant, pre-formed radical cation is reduced to change color of the reaction solution from blue-green to colorless. The extent of decoloration at 734 nm after 2 hours as a function of antioxidant concentration is measured and the Trolox equivalent antioxidant capacity value is obtained by calculating the concentration of antioxidant giving the same percentage inhibition of absorbance of the radical cation as Trolox. This assay can be used in measuring antioxidative capacity of both water-soluble and lipid-soluble pure compounds as well as food extracts (Re *et al.,* 1999). Figure 6 indicates that all hydrolysates showed an increase ABTS radical scavenging activity with increasing hydrolysis time. The highest ABTS radical scavenging activity of hydrolysate was found at 5-h hydrolysate (2062 ± 14.4 μM TE/mg protein) which was higher than those of ornate threadfin bream muscle (159 μM TE/g) (Nalinanon *et al*., 2011) and tilapia muscle (220.4 μg TE/g) (Charoenphun, 2013); and was corresponding to the activity of hydrolysate from bigeye snapper skin (1800-2300 μM TE/mg protein) (Phanturat *et al*., 2010).

The result suggests that hydrolysates could scavenge the radicals and donate an electron to the free radical, leading to the termination of the radical chain reaction and prevention or retardation of propagation. Foh *et al*. (2010) reported that antioxidant capacity of protein hydrolysate is not only dependent on the type of protease used but also on the hydrolysis time and the molecular weight of the produced peptide. Furthermore, low molecular weight peptides generally showed higher antioxidant activity (Qian *et al*., 2008).

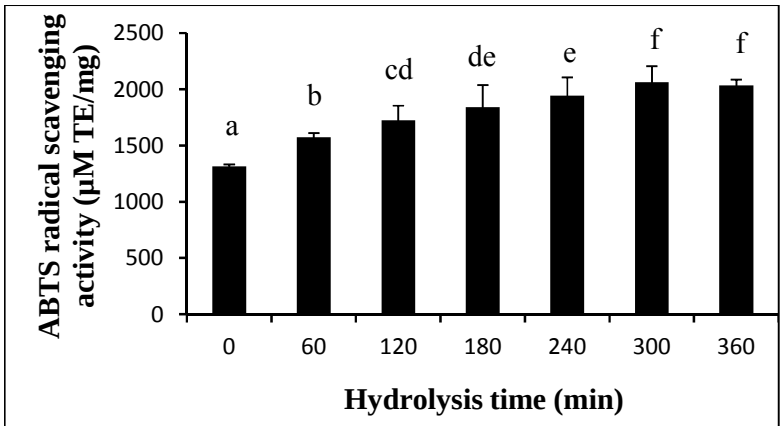

Figure 6. Effect of hydrolysis time on ABTS radical scavenging capacity of hydrolysate. Different letters on the bars indicate significant differences ($p<0.05$).

ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating compounds (scavengers of aqueous phase radicals) and of chain-breaking antioxidants (scavenger of lipid peroxyl radicals) (Binsan *et al*., 2008). $ABTS^{\bullet+}$ can be dissolved in aqueous and organic media, in which the antioxidant activity can be measured due to the hydrophilic and lipophilic nature of compounds in samples. In contrast, $DPPH^{\bullet}$ can only be dissolved in organic media, especially in ethanol. DPPH radical scavenging activity is generally used to determine hydrogen-donating ability of protein hydrolysates (Wu *et al*., 2003; Thiansilakul *et al*., 2007). Both $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ are characterized by excellent reproducibility under certain assay conditions, but they also show significant differences in their response to antioxidants (Wojdylo *et al*., 2007). As shown in Figure 5 and Figure 6, scavenging effect of the hydrolysates on ABTS radical was more efficient than that of DPPH radical as evidenced by higher ABTS radical scavenging activity.

2.3.4.3 Inhibition of lipid peroxidation in the linoleic oxidation system

Linoleic acid, an unsaturated fatty acid, is usually used as a model compound in lipid oxidation and antioxidation-related assays of emulsion system, in which carbon-centered, peroxyl radicals and hydroperoxides are involved in the oxidation process (Burton and Ingold, 1986; Zhu *et al*. 2006). During the assay, the

extent of lipid peroxidation with and without the presence of antioxidant can be measured by using the ferric thiocyanate assay (Kikuzaki and Nakatani, 1993). Lipid hydroperoxides in the reaction media can convert Fe^{2+} of the ferrous thiocyanate to Fe^{3+} in order to produce the red colored ferric-thiocyanate complex that can be measured spectrophotometrically at 500 nm (Mihaljevic *et al*. 1996). During incubation, the system containing the hydrolysate lowered the oxidation as evidenced by lower A_{500} compared with that of the control ($p<0.05$). The control showed a continuous increase in A_{500} throughout the 7 days of incubation. An increase in A_{500} indicates the formation of peroxide in the linoleic acid model system (Yen and Chen, 1995). Some peptides from various fish skins such as Alaska pollack skin (Kim *et al*., 2001), bigeye snapper skin (Phanturat *et al*., 2010), cobia skin (Yang *et al*., 2008), tilapia skin (Yang *et al*., 2009) that showed inhibitory activity in the linoleic acid model system.

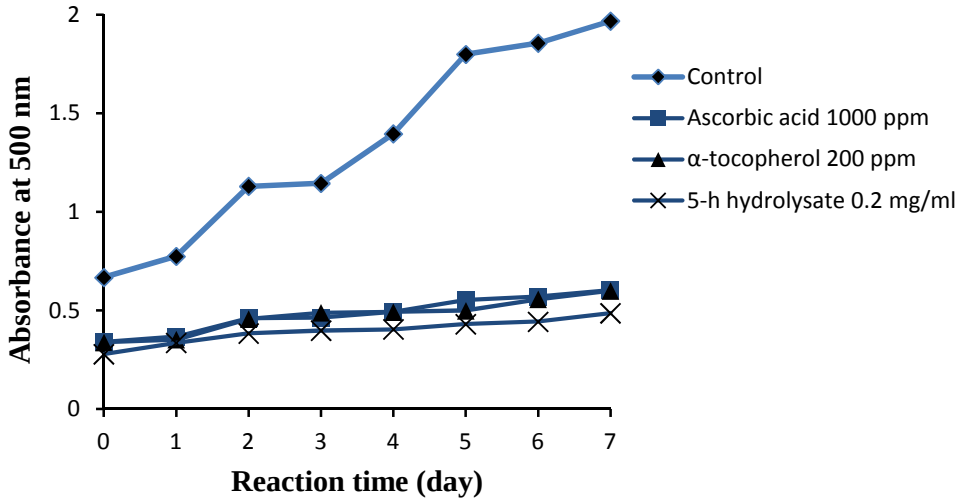

Figure 7. Lipid peroxidation inhibition of 5-h hydrolysate measured in a linoleic acid model at 37 ^{o}C for 7 days. Ascorbic acid and α-tocopherol were used as positive controls. Lower absorbance at 500 nm represents higher lipid peroxidation inhibition.

As shown in Figure 7, the oxidative activity of linoleic acid was markedly inhibited by the addition of the hydrolysate. The 5-h hydrolysate (DH = 40.2%) at a low protein concentration of 0.2 mg/mL exhibited a higher peroxidation

inhibition compared to that of natural antioxidants, 200 ppm α-tocopherol and 1000 ppm ascorbic acid on the seventh day in a linoleic acid model system. Lipid peroxidation inhibitory capacity of 5-h hydrolysate in this study (75.3% at protein concentration of 0.2 mg/mL) is higher than that of other hydrolysates from cobia skin (60-71% at protein concentration of 10 mg/mL) and tilapia skin (70% at protein concentration of 0.2 mg/mL) (Yang *et al*., 2008; Yang *et al*., 2009). The antioxidative activity of protein hydrolysate was possibly attributed to the ability of peptides to interfere with the propagation cycle of lipid peroxidation, so slowing radical-mediated linoleic acid oxidation (Thiansilakul *et al*., 2007). In the free radical-mediated lipid peroxidation system, antioxidative activity of peptides or proteins is dependent on molecular size and properties such as hydrophobicity and electron transferring ability of the amino acid residues in the sequence (Qian *et al*., 2008). Many antioxidative peptides have hydrophobic amino acid residues such as Valine or Leucine at the N-terminus of the peptides, which are more likely able to interact with the fatty acids (Chen *et al*., 1995; Kim *et al*., 2001).

2.3.5 Effect of hydrolysis time on ACE inhibitory activity of hydrolysate

ACE inhibitory activity of hydrolysate with different hydrolysis time expressed as IC_{50} is shown in Figure 8. IC_{50} value of hydrolysate decreased as hydrolysis time increased ($p<0.05$). ACE inhibitory activity of extracted protein (IC_{50} value of 1556 ± 16.61 μg/mL) increased after hydrolysis (IC_{50} value ranging from 1233 ± 29.31 μg/mL to 831 ± 33.39 μg/mL). It was suggested that peptides with ACE inhibitory activity could be generated during hydrolysis. The ACE inhibitory activity appeared to increase as hydrolysis time increase because the lengths of peptides were shortened and increased ACE inhibitory activity (Je *et al*., 2004). The highest ACE inhibitory activity of striped catfish skin protein hydrolysate (IC_{50} value of 831 ± 33.39 μg/mL) was found at hydrolysis time of 5 h. The highest ACE inhibitory activity of skin hydrolysate in the present study was almost similar with that of blacktip shark gelatin (0.94-1.77 mg/mL) (Kittiphattanabawon *et al*., 2013), salmon skin gelatin (1.17 mg/ml) (Gu *et al*., 2011), and skate skin gelatin (1.89 mg/ml) (Lee *et al*., 2011). Enzyme hydrolysis was performed in order to achieve the desired degree of hydrolysis to obtain biologically active peptides. From previous studies, ACE

inhibitory activity of peptides increased with prolonged incubation with enzyme. However, longer hydrolysis time led to the peptides lost their ability to inhibit ACE (Wu *et al*., 2008; Xu *et al*., 2014). The structure of amino acid for interactions between the substrate and the active site of ACE affected ACE inhibitory activity (Ondetti *et al*, 1977). Cushman and Cheung (1971) reported that peptides containing aromatic at the C-terminal end and the branch-chain aliphatic amino acid at the N-terminal were effective for high ACE inhibitory activity because of the interaction between these amino acids at the active site of ACE.

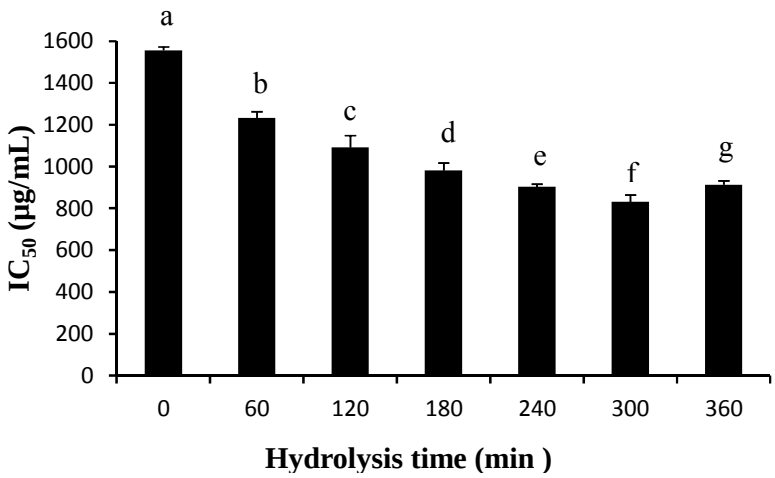

Figure 8. ACE inhibitory activity of striped catfish hydrolysate at various hydrolysis times. Different letters on the bars indicate significant differences ($p<0.05$). The lower IC_{50} value represents the higher ACE inhibitory activity.

2.4 Conclusion and suggestion

The extracted protein showed a high yield of 74.8 %. The degree of hydrolysis increased with the increase of hydrolysis time and reached the highest DH of 42.5 % after 6 h of hydrolysis. The 5-h hydrolysate (DH= 40.2 %) exhibited the highest bioactive activities including DPPH radical scavenging activity (41.3 µM TE/mg), ABTS radical scavenging activity (2062 µM TE/mg protein), lipid peroxidation inhibition activity which was higher than that of 200 ppm α-tocopherol

and 1000 ppm ascorbic acid, and ACE inhibitory activity (IC_{50} = 831 μg/mL). Therefore, the 5-h hydrolysate sample was used as material for studying enrichment of bioactive peptides by ultrafiltration in the next study.

The results also suggested that the hydrolysate from retorting process with peptide content 52.35 mg/g protein possessed high bioactive activities including DPPH radical scavenging activity (34.5 μM TE/mg protein), ABTS radical scavenging activity (1316 μM TE/mg protein), and ACE inhibitory activity (IC_{50} value of 1556 μg/mL).

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CHAPTER 3

ENRICHMENT OF ANTIOXIDATIVE AND ANGIOTENSIN-I CONVERTING ENZYME INHIBITORY PEPTIDES DERIVED FROM HYDROLYSATE OF STRIPED CATFISH SKIN BY ULTRAFILTRATION

3.1 Introduction

The production of bioactive peptides is normally performed using a tree-step procedure including extraction, hydrolysis, and separation. In term of separation, methods used for peptide fractionation and enrichment include ion exchange and gel filtration. However, these methods cannot be employed for mass production (Ward and Swiatek, 2009). Alternatively, using ultrafiltration with low molecular weight cut-off has been found to be useful for separating small peptides from high molecular weight residues (Korhonen and Pihlanto, 2006). It is known that UF has received tremendous importance for concentration, purification, and fractionation of various products in diverse fields such as food, pharmaceutical, and biotechnological industries (Foh *et al*., 2010). Nowadays, ultrafiltration provides one of the best technologies available for enrichment of high quality bioactive peptides derived from various protein sources (Charoenphun, 2013).

Mostly, UF is a gentle method to fractionate, purify, and concentrate of bioactive peptides. Picot *et al*. (2010) reported the effect of UF on antioxidant and ACE inhibitory peptides from fish protein hydrolysate. The molecular weight distribution is high to select the molecular weight cut-off of the membranes but deficient to define a strategy to obtain enriched fractions in bioactive peptides. Obviously, the optimum conditions and increasing the efficiency on production of bioactive peptides with small size by UF had rarely been reported. The performance of UF including permeate flux and selectivity depends on several factors such as feed properties, operating parameters (Chabeaud *et al*., 2009) and property of membrane (Baker, 2000).

3.1.1 Review of literature

Ultrafiltration using membranes with different molecular weight cut-offs is used in the production of antioxidant and ACE inhibitory peptides from fish protein hydrolysates. Some examples of application UF for production of bioactive peptides are shown in Table 7.

Table 7. Production of antioxidant and ACE inhibitory peptides from fish protein hydrolysates using UF.

Material	Membrane (kDa)	Molecular Weight	Activities	References
Alaska pollack skin	10, 5, 1	1.5-4.5 kDa	antioxidant	Kim *et al*. (2001)
Cobia skin	10, 5, 3	700 Da	antioxidant	Yang *et al*. (2008)
Tuna skin	10, 1	< 1 kDa	antioxidant	Gómez-Guillén *et al*. (2010)
Alaska pollack frame protein	30, 10, 5, 1	672 Da	antioxidant	Je *et al*. (2005)
Hoki frame protein	10, 5, 3, 1	1-3 kDa	antioxidant	Kim *et al*. (2007)
Alaska pollack frame protein	30, 10, 5, 3, 1	< 1 kDa	ACE inhibition	Je *et al*. (2004)

3.1.2 Objectives

The objectives of this study were (1) to investigate effects of different molecular weight cut-offs (MWCOs) membrane on permeate flux and bioactive activities of peptides to select the most suitable MWCO for separating antioxidant and ACE inhibitory peptides from the hydrolysate using UF; (2) to fractionate target peptides using Sephadex^{M} column; (3) to study effects of gastrointestinal digestion on bioactive activity of peptides.

3.2 Materials and methods

3.2.1 Materials

Samples of striped catfish skin were obtained from the fish fillet processing and stored at -20 ^{o}C. Alcalase from *Bacillus licheniformis* 2.4 L, o-phthalaldehyde (OPA), DL-dithiothreitol (DTT), ACE from rabbit lung, 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and other chemicals of analytical grade were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). Polysulphone hollow fiber membranes with 10, 5, and 1 kDa MWCO (diameter = 1, 1, and 0.5 mm; area = 0.01, 0.01, and 0.014 m^2) were purchased from GE Healthcare Bio-Science Ltd. (Bangkok, Thailand).

3.2.2 Enzymatic hydrolysis of striped catfish skin protein solution

The 1% (w/v) of extracted protein solution was hydrolysed by 20 units g^{-1} protein of Alcalase 2.4 L at pH 8.0 and 50 ^{o}C in a 4-L reactor for 5 h and then heated at 95 ^{o}C for 10 min to inactivate enzyme. The pH of the mixture was measured by a pH meter (Eutech, Cyber Scan pH 110, Singapore) and manually adjusted to pH 8.0 during the hydrolysis by 6 N NaOH and 6 N HCl. The density was determined by electrical balance (A&D GF-3000, Japan), and viscosity was determined by glass capillary viscometer (Schott instruments, Germany). The density and viscosity of protein hydrolysate were 1035 (kg m^{-3}) and 0.00133 (Pa s), respectively. The 5-h hydrolysate was used for enrichment of peptides using UF.

3.2.3 Ultrafiltration system

The UF system was set up according to Laorko *et al*., (2011). Polysulphone hollow fiber membranes (GE Healthcare Bio-Science Ltd. Bangkok, Thailand) were used. Pressure transducers were used to measure the pressure at the inlet and outlet of the membrane module. The cross flow rate was controlled by a peristaltic pump (Masterflex, USA). The transmembrane pressure (TMP) was controlled using a retentate valve; permeate valve, and variable feed-speed pump. The temperature of the feed was controlled by circulating hot water through a jacket-feed

tank. Permeate was weighed continuously using an electrical balance (A&D GF-3000, Japan) connected to a PC computer to measure its flux (Figure 9).

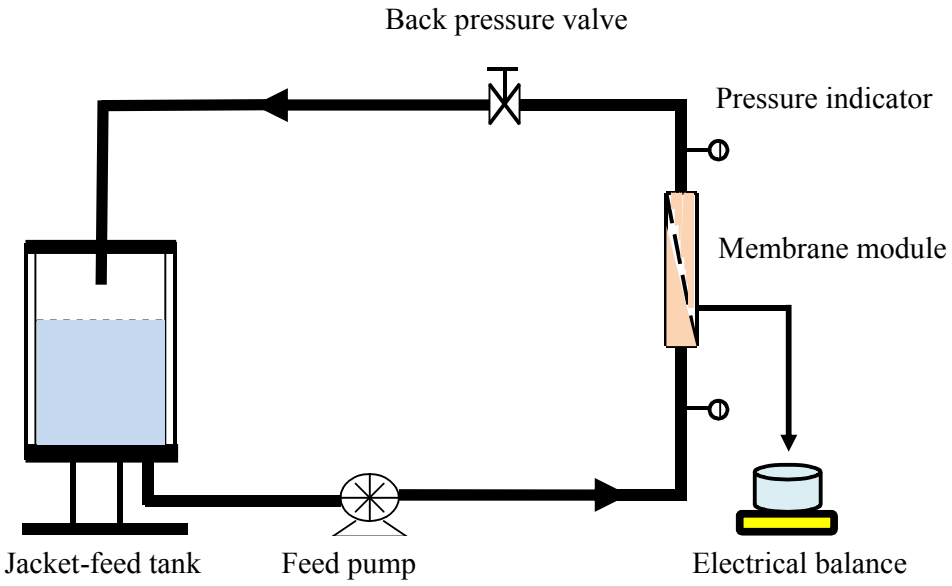

Figure 9. Schematic diagram of ultrafiltration system.

3.2.4 Study effect of MWCO membranes on bioactive peptides separation

Antioxidant and ACE inhibitory peptides were separated from the 5-h protein hydrolysate using three different MWCO membranes (10, 5, and 1 kDa). The operating condition in batch mode was transmembrane pressure (TMP) of 1.5 bars, and cross flow velocity (CFV) of 1.5 m s^{-1}. The protein content and antioxidant, ACE inhibitory of the feed and permeate were analysed. The protein transmission (T_p) was calculated by method of Cui *et al*. (2011) as follow:

$$T_p(\%) = \frac{C_p}{C_f} \times 100 \qquad (3\text{-}1)$$

Where C_p and C_f are protein concentration in permeate and the feed (mg mL^{-1}), respectively.

The percentage of permeate recovery was calculated according to Eq. 3-2.

$$\text{Recovery } (\%) = \frac{\text{Permeate volume}}{\text{Feed volume}} \times 100 \qquad (3\text{-}2)$$

3.2.5 Fractionation of bioactive peptides from hydrolysate

Permeates from UF were dried using freeze dryer (Flexi Dry, Dura Dry, NY, USA). The obtained hydrolysates were fractioned using Sephadex^{M} G-15 and G-25 column. Permeate of 5 kDa MWCO membrane was loaded into a Sephadex^{M} G-25 column (2.5 x 110 cm). The elution was carried out with 50 mM sodium phosphate buffer pH 7.0 at a flow rate of 1.6 mL/min. The 3 mL fractions were collected and their absorbance was read at 220 and 280 nm. A standard distribution was determined by chromatographing independently using the following standards: Blue dextran (2,000 Da), vitamin B12 (1,355 Da), flavin adenine dinucleotide disodium (829.5 Da), and Potassium dichromate (294.18 Da).

Permeate of 1 kDa MWCO membrane was loaded into a Sephadex^{M} G-15 column (1.8 x 50 cm). The elution was carried out with 50 mM sodium phosphate buffer pH 7.0 at a flow rate of 0.3 mL/min. The 3 mL fractions were collected and their absorbance was read at 220 and 280 nm. A standard distribution was determined by chromatographing independently using the following standards: Reduced glutathione (429 Da), Hip-His-Leu (307 Da), and Tyrosine (181.91 Da).

The fractions of Sephadex^{M} G-25 column were determined for their ABTS, DPPH radical scavenging activity and the fractions of Sephadex^{M} G-15 column were determined for their ACE inhibitory activity. All fractions were determined soluble protein content by Lowry method (Lowry *et al*., 1951) (Appendix 6). The fractions exhibiting the highest bioactive activities were pooled, followed by freeze-drying. Lyophilized powders were kept at – 20 ^{o}C until use for determination its stability in GI tract model systems.

3.2.6 Stability of bioactive peptides in gastrointestinal tract model system

In the present study, the stable ability of antioxidant and ACE inhibitory peptides in gastrointestinal tract model system was investigated. Simulated GI digestion using an *in vitro* pepsin–pancreatin hydrolysis was carried out according to the method of Cinq-Mars *et al*. (2008) and You *et al*. (2010) modified as Nalinanon *et al*. (2011). For monitoring DPPH, ABTS radical scavenging and ACE inhibitory activity of hydrolysate during the GI tract model system, the pH of the hydrolysate

was adjusted to pH 2.0 with 6 M HCl. Pepsin (E/S 1: 35, w/w) was added, and mixture was incubated with continuous shaking in a water bath for 1 h at 37 °C. The pH was then adjusted to 5.3 with 0.9 M $NaHCO_3$ solution and further to pH 7.5 with 6 M NaOH. Pancreatin was added (E/S 1: 25, w/w), and mixture was further incubated with continuous shaking in a water bath for 3 h at 37 °C. At the end of the incubation, the enzyme was inactivated at 95 ^{o}C for 10 mins. Then, the GI digestive mixture was cooled to room temperature and centrifuged at 5000 xg for 15 mins. The supernatant was used for DPPH, ABTS radical scavenging and ACE inhibitory activity assays. DPPH, ABTS radical-scavenging activity was expressed as μM TE/mg and ACE inhibitory activity was expressed as IC_{50} (μg mL^{-1}).

3.2.7 Analytical methods

The protein content was analysed by the method of Lowry *et al*. (1951). The samples were used to evaluate antioxidant activity by *in vitro* methods including DPPH radical scavenging activity (Binsan *et al*., 2008), ABTS radical scavenging activity (Binsan *et al*., 2008), and ACE inhibitory activity (Lee *et al*., 2010). All details of the methods were explained in chapter 2.

3.2.8 Statistical analysis

All experiments were performed in triplicate. All data were subjected to analysis of variance (ANOVA) and the differences between means were evaluated by Duncan's Multiple Range Test (Duncan, 1995).

3.3 Results and discussion

There were six sample fractions obtained during ultrafiltration process (permeate and retentate of 10, 5, and 1 kDa MWCO membranes).

3.3.1 Effect of different MWCO membranes on permeate flux and % recovery

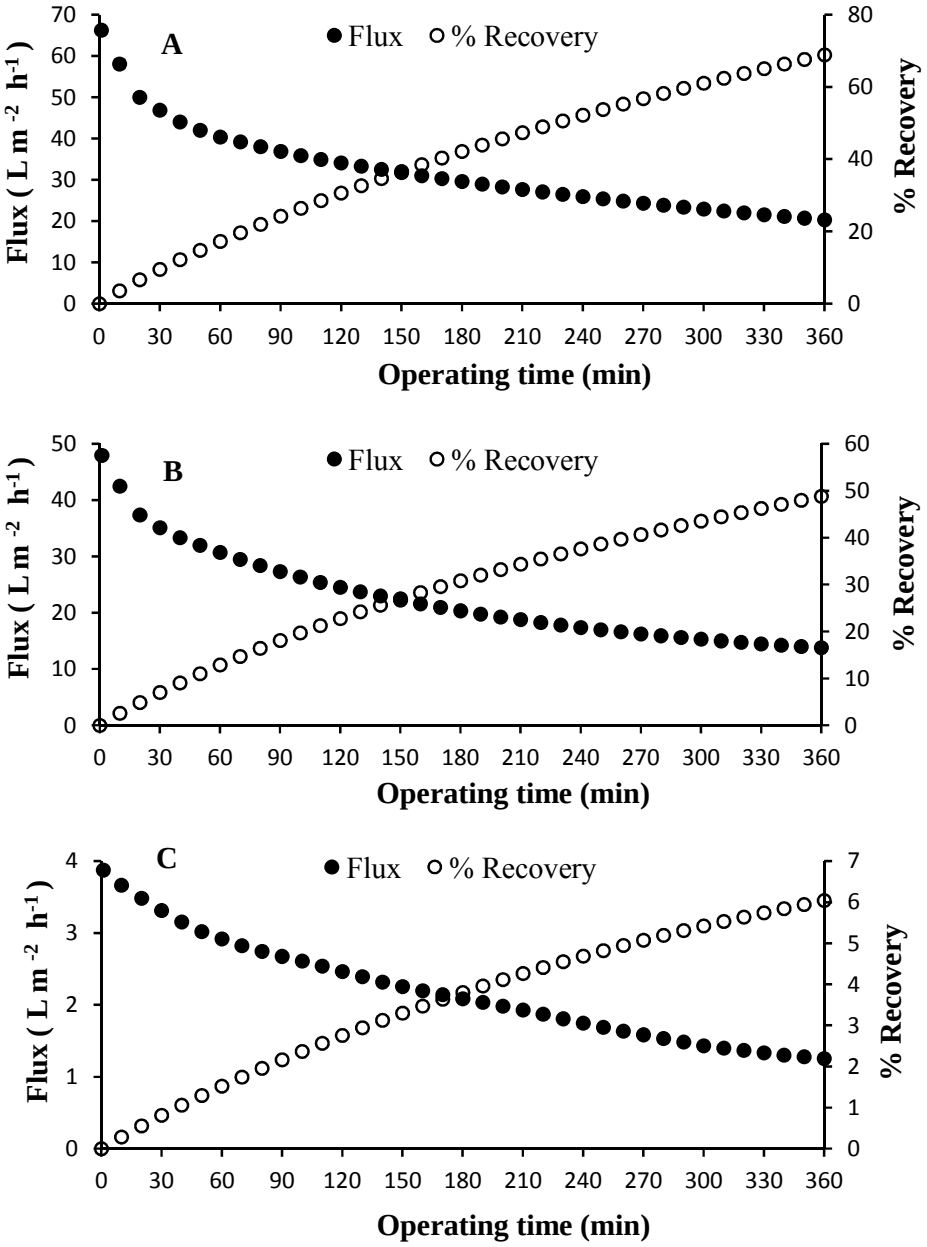

Figure 10. Permeate flux and % recovery during ultrafiltration of the 5-h hydrolysate in batch mode (TMP = 1.5 bars, CFV = 1.5 m s^{-1}, temperature = 50 ^{o}C). 10 kDa MWCO (A), 5 kDa MWCO (B), and 1 kDa MWCO (C) membranes.

Figure 10 shows filtration time versus permeate flux and % recovery of separating by 10, 5, and 1 kDa MWCO membranes during 6 h. In the case of UF 10 kDa membrane, the permeate flux decreased sharply in the first 60 minutes of the filtration and then decreased gradually to 20.3 L m^{-2} h^{-1} at recovery of 68.9 % (operating time = 6 h), average flux value was about 32.2 L m^{-2} h^{-1}. For separation of bioactive peptides using UF 5 kDa, the permeate flux decreased sharply in the first 30 minutes of the filtration and then decreased gradually to 13.8 L m^{-2} h^{-1} at recovery of 48.8 % (operating time = 6 h), average flux value was about 22.9 L m^{-2} h^{-1}. The permeate flux decreased gradually from 3.9 to 1.3 L m^{-2} h^{-1} at recovery of 6.0 % (operating time = 6 h), and the average permeate flux of 2.2 L m^{-2} h^{-1} was obtained in the case of UF 1 kDa. The permeate flux decrease with operating time. It results from the concentration polarization effect, for instance the accumulation of the solute particles on the surface of membrane that offers an extra resistance to the solvent flow through the membrane (Karthik *et al*., 2002). The permeate recovery increases sharply as the operating time increase.

3.3.2 Effect of different MWCO membranes on protein transmission and bioactive activities of peptides

The membranes with different MWCO significantly impacted on the separation of protein and bioactive peptides. Table 8 shows average protein transmission and bioactive activity of retentate and permeate samples. The results of protein content in retentate and permeate from UF processes were shown in appendix 10. The lower T_p of 1 kDa permeate (66.4 %) compared to 5 kDa (82.6 %) and 10 kDa (98.3 %) can be explained by the size of peptides and pore size of membrane. Ultrafiltration of the hydrolysate resulted in a significant increase in its bioactive activity in the permeate fractions ($p<0.05$). The highest antioxidant activity was obtained from permeate of 5 kDa membrane. It suggested that peptides with molecular weights below 5 kDa in permeate play an important role in its antioxidant activity. This result was in accordance with Chang *et al*. (2007), who reported that low molecular weight peptides (1-3 kDa) have higher scavenging activity than high molecular weight counterpart. Results from work by Wu *et al*. (2003) revealed that the peptide with molecular weight of approximately 1400 Da possessed a stronger *in*

vitro antioxidant activity than that of the 900 and 200 Da peptides. Kim *et al*. (2001) reported that peptides from Alaska pollack skin hydrolysate which have molecular ranging from 1.5 to 4.5 kDa showed the highest antioxidant activity. Jeon and coworkers (1999) reported that cod fish protein hydrolysate fractions below 5 kDa most effectively reduced lipid oxidation in a linoleic acid emulsion system. Permeate of MWCO 1 kDa membrane showed the highest ACE inhibitory activity. The results indicated that molecular weight of most ACE inhibitory peptides, which was produced and separated from the hydrolysate, was smaller than 1 kDa. This result was in accordance with Je *et al*. (2004), who reported that Alaska pollack frame protein hydrolysate that having a molecular mass below 1 kDa showed the highest ACE inhibitory activity.

Table 8. Average protein transmission (T_P) and bioactive activity of the retentate and permeate obtained from UF process of 5-h hydrolysate (TMP = 1.5 bars, CFV = 1.5 m s^{-1}, 50 ^oC).

T_P and bioactive activity	5-h hydrolysate	UF fraction		
		10 kDa	5 kDa	1 kDa
T_P (%)	-	98.3 ± 2.59	82.6 ± 1.53	66.4 ± 1.07
ABTS	2062.6 ± 14.40	-	-	-
ABTS in retentate	-	1939.3 ± 27.53	1793.0 ± 77.13	2278.4 ± 82.73
ABTS in permeate	-	2166.4 ± 34.94	2474.7 ± 10.77	2040.5 ± 13.93
DPPH	43.3 ± 1.23	-	-	-
DPPH in retentate	-	37.3 ± 1.21	34.4 ± 3.66	42.6 ± 1.28
DPPH in permeate	-	41.7 ± 3.27	45.2 ± 2.13	38.1 ± 0.91
ACE	831.7 ± 33.39	-	-	-
ACE in retentate	-	1084.2 ± 30.71	1143.3 ± 49.32	2736.5 ± 71.65
ACE in permeate	-	159.7 ± 1.61	125.0 ± 1.09	8.3 ± 0.27

* ABTS: ABTS radical scavenging activity (μM TE/mg)
DPPH: DPPH radical scavenging activity (μM TE/mg)
ACE: ACE inhibitory activity (IC_{50} value = μg/ mL)

Figure 11 shows filtration times versus T_P during ultrafiltration of protein hydrolysate under batch mode. The T_P decreased slightly as operating time increase. The changing of T_P corresponding with changing of permeates flux and operating time because peptides with molecular weight smaller than the pore size of

membrane decreased gradually as operating time increased. From 0 min to 360 min of operating time, the T_P decreased from 99.6 to 92.6 %, 86.7 to 78.2 %, and 70.5 to 64.5 % when hydrolysates were subjected to 10, 5, 1 kDa MWCO membranes, respectively.

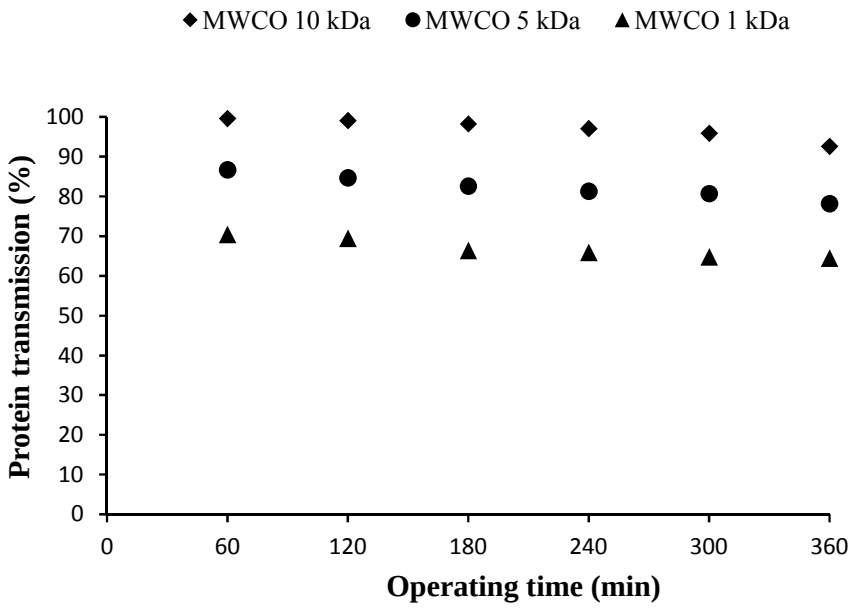

Figure 11. Protein transmission (T_P) during ultrafiltration (MWCO 10, 5, and 1 kDa) of 5-h hydrolysate in batch mode (TMP = 1.5 bars, CFV = 1.5 m s^{-1}, temperature = 50 ^oC).

Likewise, decreasing of T_P leads to the decrease of antioxidant and ACE inhibitory activity of peptides. Figure 12 and Figure 13 show filtration times versus DPPH, ABTS radical scavenging activity of peptides in permeate and retentate during ultrafiltration of protein hydrolysate, respectively. The antioxidant activities of peptides in permeate and retentate fell steadily when the operating time increase.

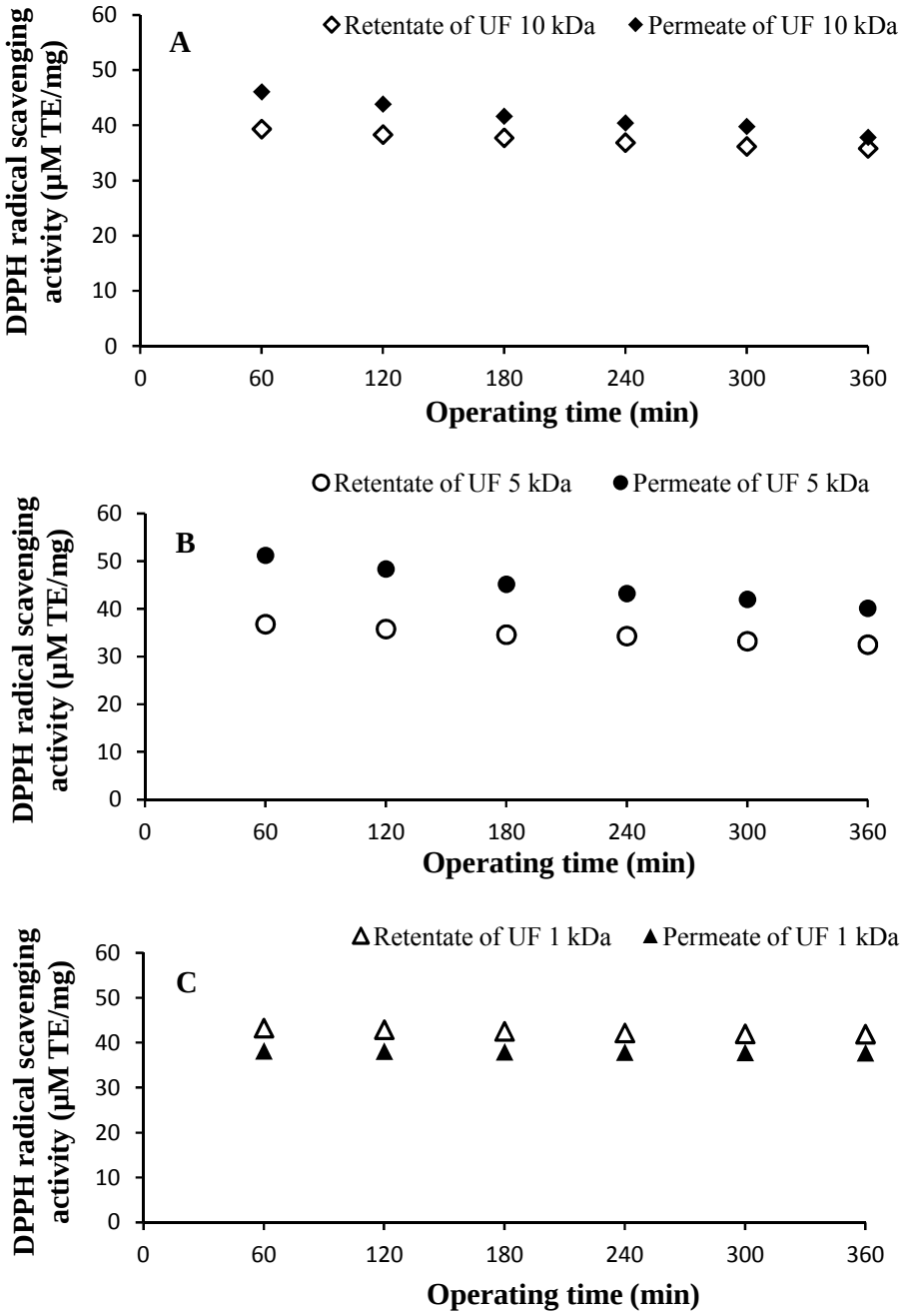

Figure 12. DPPH radical scavenging activity of peptides in permeate and retentate during UF of 5-h hydrolysate in batch mode (TMP = 1.5 bars, CFV = 1.5 m s^{-1}, 50 ^{o}C). 10 kDa MWCO (A), 5 kDa MWCO (B), and 1 kDa MWCO (C) membranes.

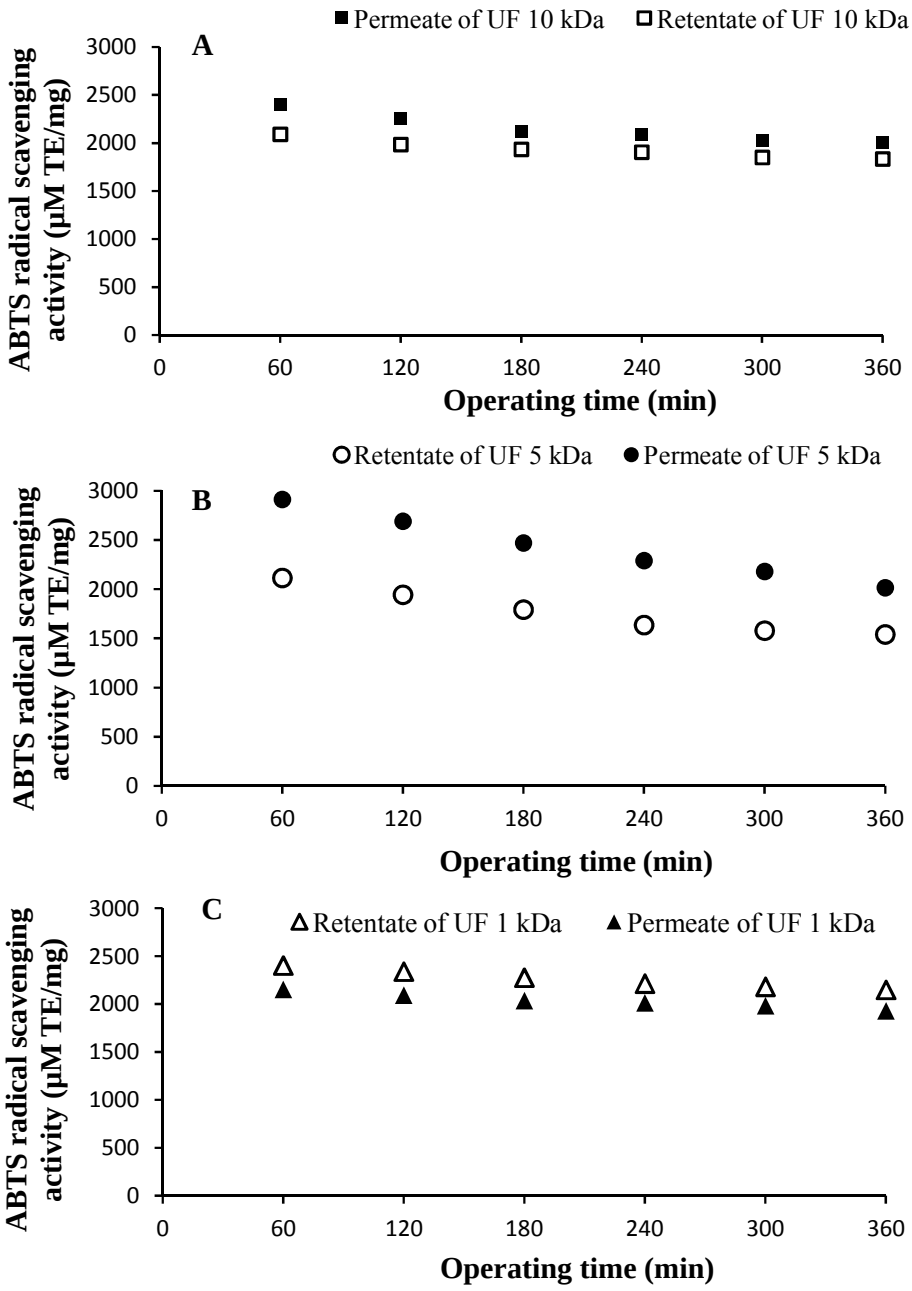

Figure 13. ABTS radical scavenging activity of peptides in permeate and retentate during UF of 5-h hydrolysate in batch mode (TMP = 1.5 bars, CFV = 1.5 m s^{-1}, 50 ^{o}C). 10 kDa MWCO (A), 5 kDa MWCO (B), and 1 kDa MWCO (C) membranes.

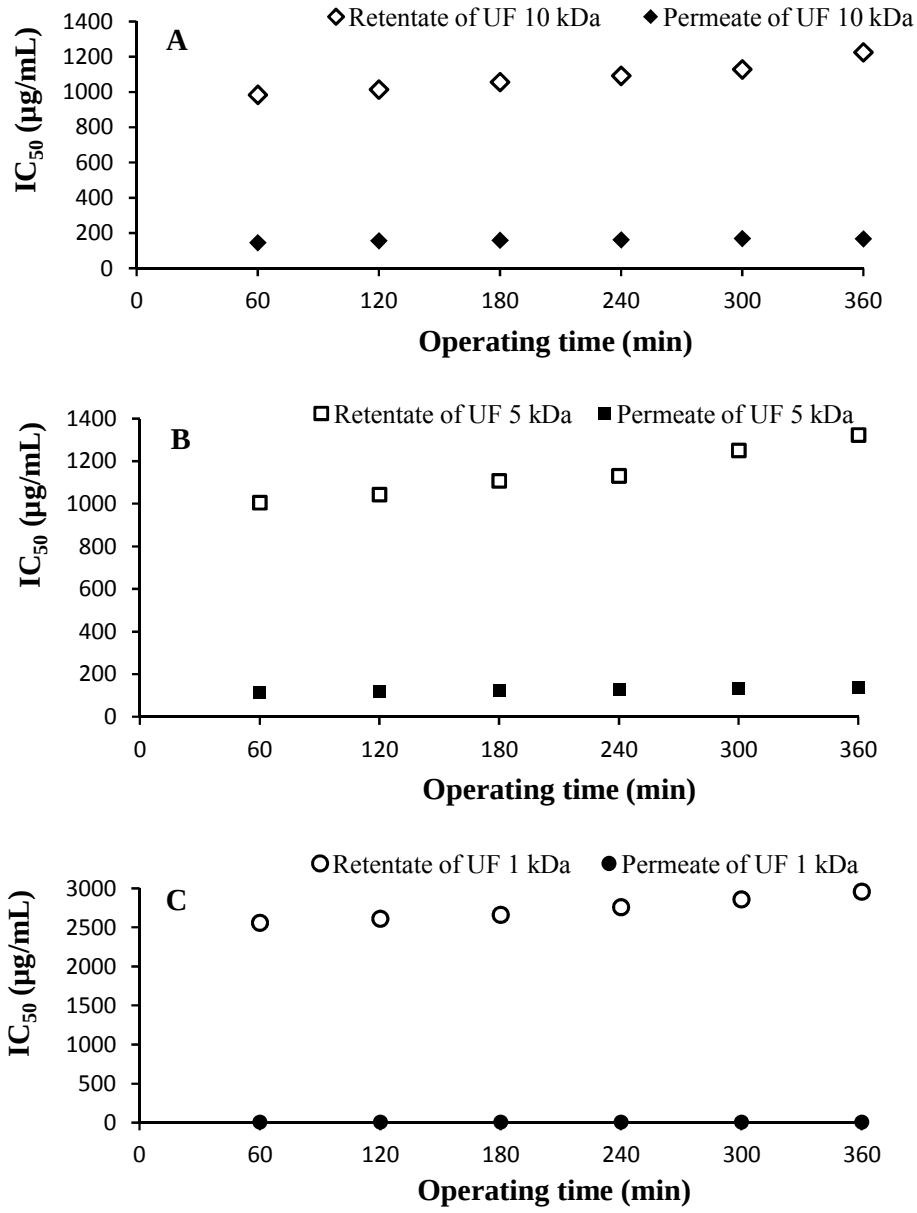

Figure 14. ACE inhibitory activity of peptides in permeate and retentate during ultrafiltration of 5-h hydrolysate in batch mode (TMP = 1.5 bars, CFV = 1.5 m s^{-1}, temperature = 50 ^{o}C). 10 kDa MWCO (A), 5 kDa MWCO (B), and 1 kDa MWCO (C) membranes. The lower IC_{50} value represents the higher ACE inhibitory activity.

Figure 14 shows filtration time versus ACE inhibitory activity of peptides in permeate and retentate during ultrafiltration of protein hydrolysate. In

general, the ACE inhibitory activity of peptides in permeates and retentates fell steadily when the operating time increase (IC_{50} value increased steadily). The ACE inhibitory activity of peptides in permeates was always higher than that in the retentate because low molecular weight of peptides in permeates exhibited high ACE inhibitory activity. The ACE inhibitory activity (IC_{50} average value) of permeates of MWCO 10, 5, and 1 kDa membranes were 159.7, 125.0, and 8.3 μg mL^{-1}, respectively.

3.3.3 Fractionation of bioactive peptides from hydrolysate

The chromatograms of hydrolysates subjected to Sephadex^{M} G-25 and G-15 column are shown in Figure 15 and Figure 16. Amarowicz and Shahidi (1997) reported that the optical density at 220 nm (A_{220}) indicates the peptide bonds and the optical density at 280 nm (A_{280}) represents peptides, proteins or amino acids with aromatic rings. The results of protein content in peaked fractions were shown in appendix 11. Figure 15 shows the chromatogram of the hydrolysate from permeates of UF 5 kDa MWCO membrane which was fractionated using Sephadex^{M} G-25 gel filtration chromatography. There were many peaks of A_{220}, indicating the presence of high and low molecular weight proteins or peptides in the hydrolysate. Many peaks of A_{280} were found in the hydrolysate, reflecting the presence of proteins, peptides or amino acids with aromatic rings (Nalinanon *et al*., 2011). A distinct peak of A_{280} was observed at the fraction 55, reflecting the presence of proteins or peptides mainly containing aromatic amino acids. The highest antioxidant activity as indicated by DPPH and ABTS radical scavenging activities was found at fraction 55 (233.8 μM TE mg^{-1} and 6584.1 μMT E mg^{-1}, respectively) (Table 9), which contained peptides with molecular weights in the range of 829.5 Da to 1355 Da. This result was in accordance with Nalinanon *et al*. (2011), who reported that antioxidative peptides from hydrolysate of ornate threadfin bream muscle with molecular weight of approximately 1.3 kDa exhibited the highest ABTS, DPPH radical-scavenging activity. The DPPH radical scavenging activity of the peptides increased after fractionation, which ranged from 4.88 to 5.81 fold higher than hydrolysate ($p<0.05$) while ABTS radical scavenging activity of the peptides increased 2.95 to 3.14 fold. Fractions number 54-73, showing the highest DPPH, ABTS radical scavenging activity, were pooled and

used for determination its stability in gastrointestinal tract model system. The result suggests that peptides without or low antioxidant activity were removed during fractionation while peptides with high antioxidant activity were concentrated.

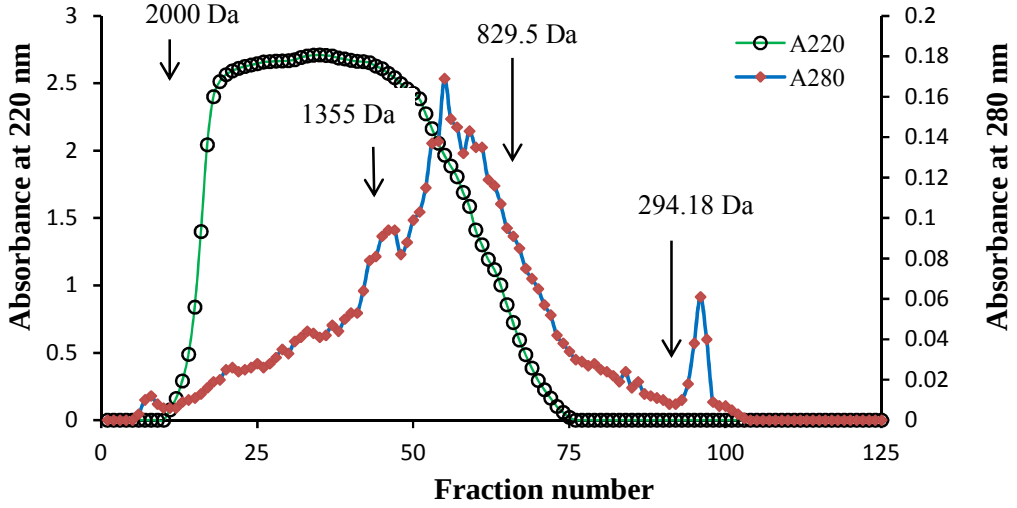

Figure 15. Elution profile of striped catfish skin hydrolysate (from UF 5 kDa MWCO membrane) separated by size exclusion chromatography on Sephadex^{M} G-25. The blue dextran (MW = 2,000 kDa), vitamin B12 (MW = 1355 Da), flavin adenine dinucleotide (MW = 829.5 Da), Potassium dichromate (MW = 294.18 Da), were used to calibrate the standard molecular weights.

Table 9. Antioxidant activities of peaked fractions from Sephadex^{M} G-25 column.

Fraction No.	DPPH radical scavenging activity	ABTS radical scavenging activity
47	112.9 ± 0.92 μM TE mg^{-1}	3022.7 ± 26.24 μM TE mg^{-1}
55	233.8 ± 1.31 μM TE mg^{-1}	6584.1 ± 17.16 μM TE mg^{-1}
59	195.0 ± 1.59 μM TE mg^{-1}	6248.3 ± 10.21 μM TE mg^{-1}
96	119.4 ± 0.94 μM TE mg^{-1}	3683.6 ± 29.63 μM TE mg^{-1}

Figure 16 shows the chromatogram of the hydrolysate from permeates of UF 1 kDa MWCO membrane which was fractionated using Sephadex^{M} G-15 gel filtration chromatography. A peak of A_{220} was observed in fraction 16, reflecting the presence of peptides bonds and a distinct peak of A_{280} was found in the same fraction

indicated the presence of proteins or peptides containing aromatic amino acids. The highest ACE inhibitory activity was obtained at fractions 15 to 18 that having molecular weights 307 Da to 429 Da. Similar findings were also observed from previous works by Je *et al*. (2004); Raghavan and Kristinsson (2009), who reported that peptides with molecular masses below 1 kDa showed the highest ACE inhibitory activity. The peaked fractions showed the highest ACE inhibitory activity (IC_{50} value ranging from 1.22 to 5.88 µg mL^{-1}) (Table 10), which ranged from 141.45 to 681.72 fold higher than hydrolysate (IC_{50} value 831.7 µg mL^{-1}). Fractions 15-18, showing the highest ACE inhibitory activity, were pooled and used for determination its stability in gastrointestinal tract model system. The result suggests that peptides without or low ACE inhibitory activity was removed during fractionation while peptides with high ACE inhibitory activity were concentrated.

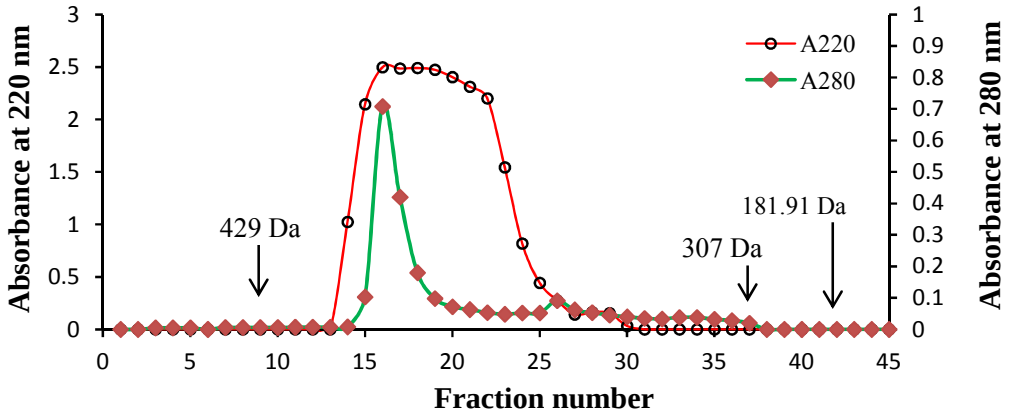

Figure 16. Elution profile of striped catfish skin hydrolysate (from UF 1 kDa MWCO membrane) separated by size exclusion chromatography on Sephadex^{M} G-15. Reduced glutathione (MW = 429 Da), Hip-His-Leu (MW = 307 Da), Tyrosine (MW = 181.91 Da), were used to calibrate the standard molecular weights.

Table 10. ACE inhibitory activity of peaked fractions from Sephadex^{M} G-15 column.

Fraction No.	ACE inhibitory activity (IC_{50})
15	4.98 ± 0.03 µg mL^{-1}
16	1.22 ± 0.01 µg mL^{-1}
22	5.88 ± 0.06 µg mL^{-1}

3.3.4 Stability of bioactive peptides in gastrointestinal tract model system

Although antioxidant, ACE inhibitor peptides exhibited potent antioxidant, ACE inhibitory activity *in vitro*, it has been reported that some of them failed to show antioxidant, ACE inhibitory effects *in vivo*, which is possibly due to hydrolysis by gastrointestinal enzymes (Wu *et al*., 2008). An *in vitro* pepsin-pancreatin hydrolysis simulating GI digestion was used to illustrate its impact on DPPH, ABTS radical scavenging activity of antioxidant peptides (Sephadex^{M} G-25 fractions) and ACE inhibitory peptides (Sephadex^{M} G-15 fractions). The results suggested that fractions showed negligible changes on DPPH, ABTS radical scavenging and ACE inhibitory activities (Table 11). These bioactive peptides could be stable in real digestion system after ingestion in both stomach and intestine which have high proteolytic activity under acidic and alkaline pH, respectively. Results from present study recommended that potent antioxidant, ACE inhibitory peptides derived from the skin of striped catfish fillet processing could possibly maintain their activity after orally administered. Several reports have also concluded that small peptides have low susceptibility to hydrolysis by gastric proteases (Grimble *et al*., 1987; Wu and Ding, 2002).

Table 11. Antioxidant, ACE inhibitory activity of lyophilized powders after digestion by GI proteases.

Bioactive activity	Before GI digestion	After GI digestion
ACE inhibitory (IC_{50})	1.34 ± 0.04 μg mL^{-1}	1.42 ± 0.06 μg mL^{-1}
ABTS radical scavenging	6292.7 ± 24.11 μM TE mg^{-1}	6230.3 ± 19.32 μM TE mg^{-1}
DPPH radical scavenging	222.8 ± 2.05 μM TE mg^{-1}	212.7 ± 1.94 μM TE mg^{-1}

3.4 Conclusion and suggestion

The ultrafiltration using 5 kDa and 1 kDa was successful for separation antioxidant and ACE inhibitory activity peptides since ultrafiltration of the hydrolysate resulted in a significant increase in its bioactive activity in the permeate fractions with DPPH radical scavenging activity (45.2 μM TE/mg), ABTS radical scavenging activity (2474.7 μM TE/mg protein), and ACE inhibitory activity (IC_{50} =

8.3 μg/mL). Based on gel filtration chromatography results, ACE inhibitory peptides had molecular weight ranging of 307 Da to 429 Da while antioxidant peptides had molecular weight in the range of 829.5 Da to 1355 Da. Furthermore, ACE inhibitory and antioxidant activities of peptides showed little changes during the simulated gastrointestinal digestion. Therefore, 5 kDa and 1 kDa MWCO membranes were selected to study production of antioxidant and ACE inhibitory peptides by using continuous enzymatic membrane reactor, respectively in the next study.

3. 5 References

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CHAPTER 4

PRODUCTION OF ANTIOXIDANT AND ANGIOTENSIN-I CONVERTING ENZYME INHIBITORY PEPTIDES FROM THE SKIN OF STRIPED CATFISH FILLET PROCESSING WASTE USING CONTINUOUS ENZYMATIC MEMBRANE REACTOR

4.1 Introduction

Membrane separation is a useful technique to extract, concentrate, separate or fractionate the compounds. The use of enzymatic membrane reactor to integrate a reaction vessel with a membrane separation unit is emerging as a beneficial method for producing peptides. These bioactive compounds from membrane reactor technology show diverse biological activities such as antihypertensive, antimicrobial, and antioxidant properties (Kim and Senevirathne, 2011).

The functional foods and nutraceuticals market is growing at a rapid pace. Membrane processing offers several advantages over conventional methods for separation, fractionation, and recovery of those bioactive components. Very few substances in nature are pure form. Most require some type of separation before resulting form can be consumed directly, or used as ingredients in various processed food and nutraceutical products. Separation processes are an integral part of today's agricultural, food, and bioproduct industries. This is especially true for functional food and the nutraceuticals industry where specific bioactive components are targeted for their health benefits. The overall objective in nutraceuticals processing is to develop a fully integrated processing system to minimize degradation of bioactivity throughout raw material handling, processing, packaging, storage, and retail shelf life of the final supplement and food products (Akin *et al*., 2012). One of key problems in the processing of biomaterials and foods, and indeed in all chemical processes is the purification of specific components by separating them from a complex, multi-component matrix. The ability to separate efficiently without losing activity of the bioactive compounds, and at low energy, capital, and labor costs has been the

deciding factor in the success of some newly-developed nutraceutical products/ingredients. Industrial membrane technology is one successful approach as a low cost separation technique for the concentration and purification of bioactives from various feed streams (Akin *et al*., 2012).

Enzymatic hydrolysis is very costly in conventional batch-type operations because large quantities of enzyme are required and the enzyme can not be reused (Adler-Nissen, 1986). Other disadvantages of the batch process have also been reported such as time consumption, low yield, low productivity and inconsistent quality (Deeslie and Cheryan, 1988). In contrast, the development application of an ultrafiltration membrane reactor for the hydrolysis of proteins has been applied to overcome those problems (Deeslie and Cheryan, 1981). Several studies have concluded that production of protein hydrolysates in a continuous membrane reactor results in higher productivities (Chiang *et al*., 1999) and more uniform products than batch-type reactors (Chiang *et al*., 1995; Deeslie and Cheryan, 1981).

4.1.1 Review of literature

The development of membrane technology has led to a new type of bioreactor in which continuous reaction occurs simultaneously with separation of the product from the reaction mixture. A continuous enzymatic membrane reactor (CEMR) contains a membrane with appropriate pore size and physicochemical properties that is equipped with stirred reactor tank. In continuous enzymatic membrane reactor, the substrate from a feed tank is continuously fed to the reactor in order to compensate for permeate and maintain constant volume in the reactor while the products are continuously withdrawn in permeate. The size of the product molecules in permeate can be controlled by proper selection of the pore size of the membrane. The membrane reactor concept can be applied to any enzymatic reaction with the aim of developing continuous process with high productivities. Chiang *et al.* (2006) concluded that production of protein hydrolysates in a continuous membrane reactor results in higher productivities and more uniform products than batch-type reactors. Depending on the design of the membrane bioreactor, membranes can be selected and used to separate specific peptides from fish skin, scale and muscle (Byun

and Kim, 2001; Jeon *et al*., 2001). The CEMR has been successfully used in the production of bioactive peptides from various proteins such as Alaska pollack skin (Byun and Kim, 2001), chitosan (Jeon *et al*., 2001), and tilapia protein (Charoenphun *et al*., 2012).

4.1.2 Objective

The objective of this study was to investigate the use of CEMR for production of antioxidant and ACE inhibitory peptides from striped catfish skin.

4.2 Materials and methods

4.2.1 Materials

Samples of striped catfish skin were obtained from the fish fillet processing and stored at -20 ^{o}C. The protein solution (1%, w/v) was prepared as the method in chapter 2. Alcalase from *Bacillus licheniformis* 2.4 L, o-phthalaldehyde (OPA), DL-dithiothreitol (DTT), ACE from rabbit lung, 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and other chemicals of analytical grade were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). Polysulphone hollow fiber membranes with 5 and 1 kDa MWCO, fiber diameter of 1 and 0.5 mm, having area of 0.01 and 0.014 m^2, respectively were purchased from GE Healthcare Bio-Science Ltd. (Bangkok, Thailand).

4.2.2 Production of antioxidant and ACE inhibitory peptides from the skin of striped catfish fillet processing waste using CEMR

The schematic diagram of CEMR system is shown in Figure 17. In the system, 1300 mL protein solution (1%, w/v) was loaded to the bioreactor, and then it was hydrolysed at pH 8.0 and 50 ^{o}C. The pH of the mixture was measured by a pH meter and manually adjusted to pH 8.0 during the hydrolysis by 6 N NaOH and 6 N HCl. After the temperature and pH had stabilized, Alcalase 2.4 L 20 units g^{-1} protein was added to the reactor tank. Cross-flow ultrafiltration was carried out with a hollow fiber membrane connected to reactor. The operating condition was transmembrane

pressure (TMP) of 0.5 bars, and cross flow velocity (CFV) of 2 m s^{-1}. Membranes with 5 and 1 kDa MWCO polysulphone membranes (GE Healthcare Bio-Science Ltd. Bangkok, Thailand) were used for producing protein hydrolysate with antioxidant and ACE inhibitory activity, respectively.

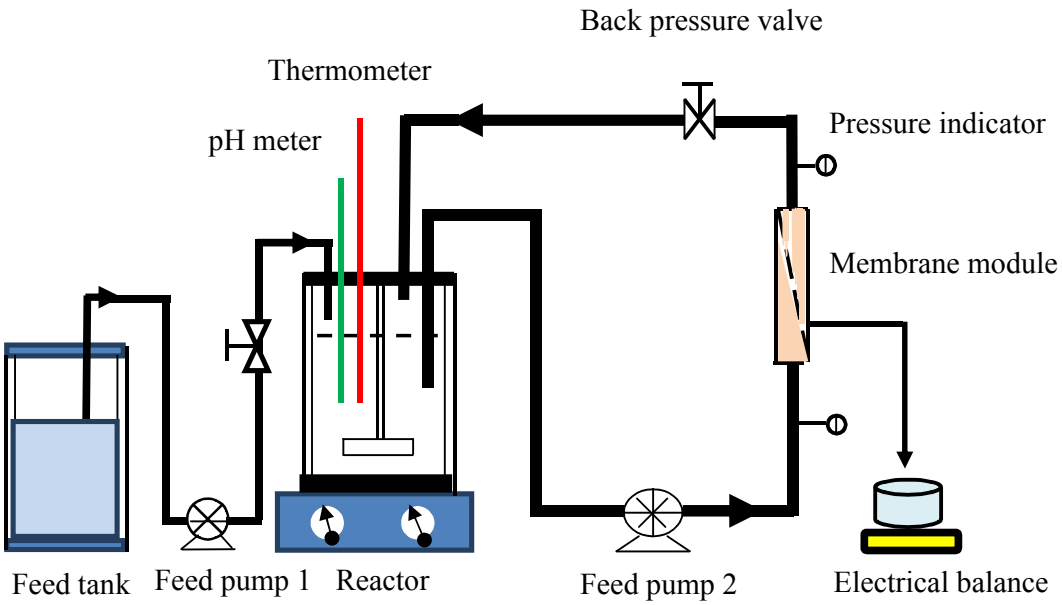

Figure 17. Schematic diagram of continuous enzymatic membrane reactor system.

Pressure transducers were used to measure the pressure at the inlet and the outlet of the membrane module. The cross flow rate was controlled by peristaltic pump 2 (Masterflex, USA). The TMP was controlled using a back pressure valve and variable speed-feed pump. The temperature and pH of mixture in the reactor were measured using thermometer and pH meter. The temperature of the feed was control by circulating water through a feed tank. Protein solution from feed tank was added continuously into the reactor equal to the rate of permeate drain out from the reactor by peristaltic pump 1 (Masterflex, USA). The cross flow rate of the feed was controlled by peristaltic pump 1 and a back pressure valve. Permeate was collected and weigh continuously using an electrical balance (A & D GF-3000, Japan; accuracy 0.001 g) connected to a PC computer to measure its flux. The hydrolysed samples from permeate were collected every 1 h and heated at 95 ^{o}C for 10 min to inactivate enzyme.

4.2.3 Analytical methods

The protein content was determined by the method of Lowry *et al*. (1951). The protease activity was determined by method of Anson (Anson, 1938) described by Thangam and Rajkumar (2002). The degree of hydrolysis of the sample was determined by the OPA method as modified by Wanasundara *et al*. (2002). The samples were used to evaluate bioactive activity by *in vitro* methods including DPPH radical scavenging activity (Binsan *et al*., 2008), ABTS radical scavenging activity (Binsan *et al*., 2008), and ACE inhibitory activity (Lee *et al*., 2010). All details of the methods were explained in chapter 2. The protein transmission (T_P) was calculated by method of Cui *et al*. (2011) as follow:

$$T_p(\%) = \frac{C_p}{C_f} \times 100 \qquad (4\text{-}1)$$

Where C_p and C_f are protein concentrations in permeate and feed (mg mL^{-1}) at hydrolysis time, respectively.

The protein conversion at operating time was expressed according to Cui *et al*. (2011) as follow:

$$\text{Protein conversion } (\%) = \frac{C_p \times V_p}{C_f \times V_f} \times 100 \qquad (4\text{-}2)$$

Where C_p, C_f is protein concentration in permeate and feed (mg mL^{-1}) at hydrolysis time, respectively.

V_p, V_f is permeate and feed volume (mL) at hydrolysis time, respectively.

Productivity of protein is defined as the protein hydrolysed per unit of enzyme (mg $unit^{-1}$ enzyme). The productivity of protein was expressed according to Cui *et al*. (2011) as follow:

$$\text{Productivity of protein} = \frac{\text{Protein conversion}_t \times S_{0,t} \times F_t \times t}{\text{Enzyme concentration} \times \text{Substrate volume}_t} \qquad (4\text{-}3)$$

Where

Protein conversion_t is the protein conversion at hydrolysis time t.

$S_{0,\,t}$ is the total protein concentration in the reactor at hydrolysis time t (mg mL^{-1}).

F_t is the flow rate of permeate at hydrolysis time *t* (mL min^{-1}).

T is a hydrolysis time (min).

Enzyme concentration is unit of enzyme mL^{-1}.

Substrate $volume_t$ is the volume of substrate in the reactor at hydrolysis time t (mL).

The bioactive activity conversion (%) was analysed according to Charoenphun (2013) as follow:

$$\text{Bioactive activity conversion (\%)} = \frac{C_p \times V_p \times A_p}{C_f \times V_f \times A_f} \times 100 \qquad (4\text{-}4)$$

Where

C_p and C_f are protein concentration in permeate and feed (mg mL^{-1}) at hydrolysis time, respectively.

V_p and V_f are the permeate and feed volume (mL) at hydrolysis time, respectively.

A_p and A_f are the bioactive activity of peptides in permeate and feed at hydrolysis time, respectively.

Bioactive productivity was analysed according to Charoenphun (2013) as follow:

$$\text{Bioactive productivity} = \frac{\text{Bioactive activity conversion}_t \times S_{0,t} \times F_t \times t}{\text{Enzyme concentration} \times \text{Substrate volume}_t} \qquad (4\text{-}5)$$

Where

Bioactive $activity\ conversion_t$ is the bioactive activity conversion at hydrolysis time t.

$S_{0,\,t}$ is the total protein concentration in the reactor at hydrolysis time t (mg mL^{-1}).

F_t is the flow rate of permeate at hydrolysis time t (mL min^{-1}).

T is a hydrolysis time (min).

Enzyme concentration is unit of enzyme mL^{-1}.

Substrate $volume_t$ is the volume of substrate in the reactor at hydrolysis time t (mL).

4.2.4 Statistical analysis

All experiments were performed in triplicate. All data were subjected to analysis of variance (ANOVA) and the differences between means were evaluated by Duncan's Multiple Range Test (Duncan, 1995).

4.3 Results and discussion

4.3.1 Effect of operating time on permeate flux during CEMR system

The substrate feeding pattern is a critical variable that possibly influences the conversion rate and the process yield (Alam *et al*., 2011). Charoenphun (2013) reported that the substrate feeding pattern of tilapia protein, that the substrate was continuously added into the reactor equal to the permeate drain out from the reactor, obtained low occurrence of membrane fouling because it had high performance working of enzyme-substrate interaction and gave the highest antioxidant peptide activity because the binding of enzyme and substrate was suitable for producing bioactive peptides. This substrate feeding pattern also gave a more constant permeate flux. However, further increase in the substrate feeding led to decrease of permeate flux with time due to accumulation of unreacted protein (Kim *et al*., 1993; Charoenphun, 2013). In the present study, the substrate was continuously added into the reactor equal to the rate of permeate drain out from the reactor.

Figure 18 shows filtration time versus permeate flux of 5, and 1 kDa MWCO membranes in the CEMR. The permeate flux increases slightly during operating time. This can be explained that the substrate-enzyme interaction was high which resulted in a high flux and low fouling because the protein was converted to peptides continuously that reduced large molecule and effected to constant membrane fouling. The membrane reactor system could maintain a steady production of the hydrolysates for over 8 h. This may suggest that during the process the amount of short peptides and amino acids increases linearly and the process efficiency of the membrane reactor may be resulting from the high substrate retention at short times in reactor. In terms of 1 kDa CEMR system, the period time for permeate flux to be likely stable is longer than that of 5 kDa system and the flux was much smaller than that of 5 kDa system. It may be because 1 kDa CEMR system needs a long hydrolysis time to convert protein to small peptides (< 1 kDa) and amino acids, and the amount of the permeate is very small in comparision with 5 kDa system.

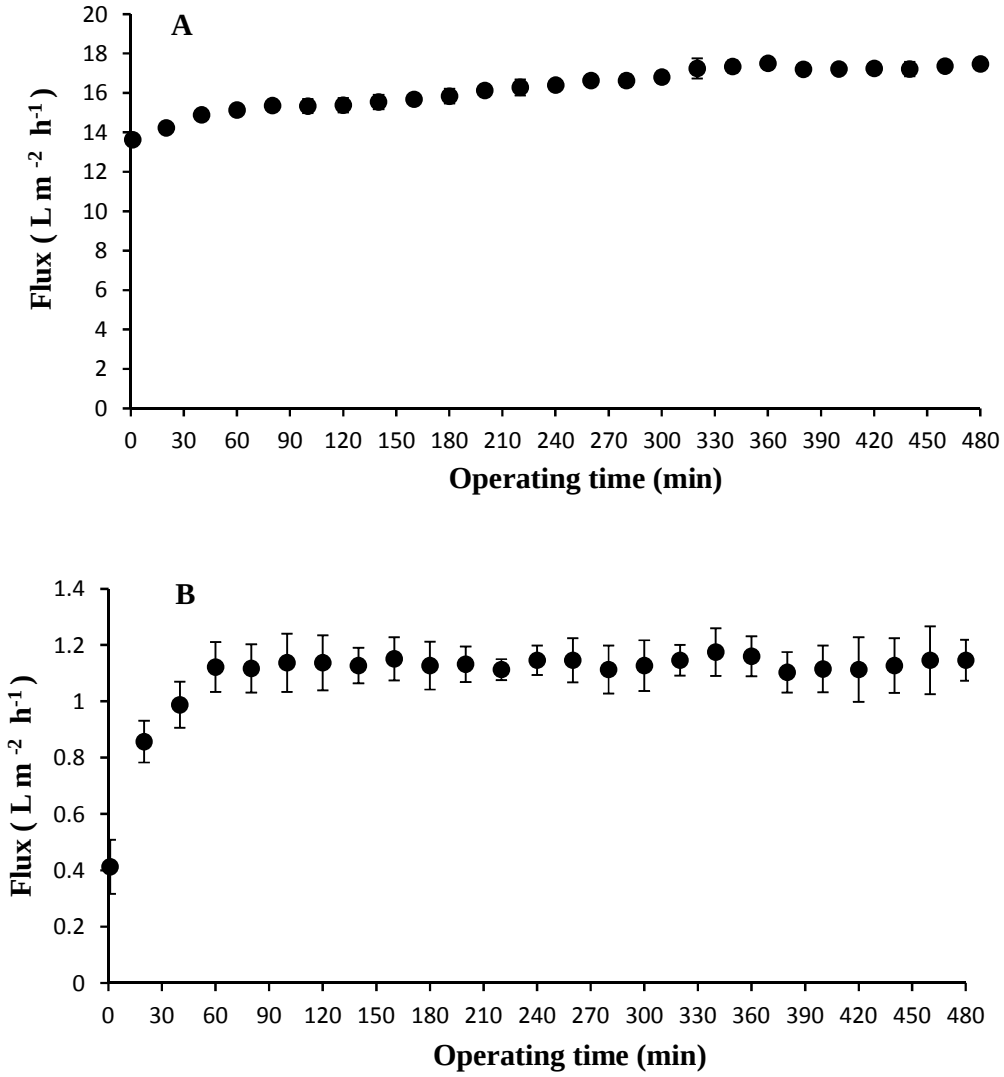

Figure 18. Effect of operating time on permeates flux during CEMR system by 5 kDa MWCO (A) and 1 kDa MWCO (B) membranes.

4.3.2 Effect of operating time on enzyme activity during CEMR system

The significant benefit of a CEMR is the reuse of enzymes. Consequently, the amount of bioactive peptides produced per enzyme loading can be remarkably enhanced by continuously substrate feeding into the bioreactor. The results showed that enzyme activity of the feed in the reactor decreased gradually as operation time increase (Figure 19).

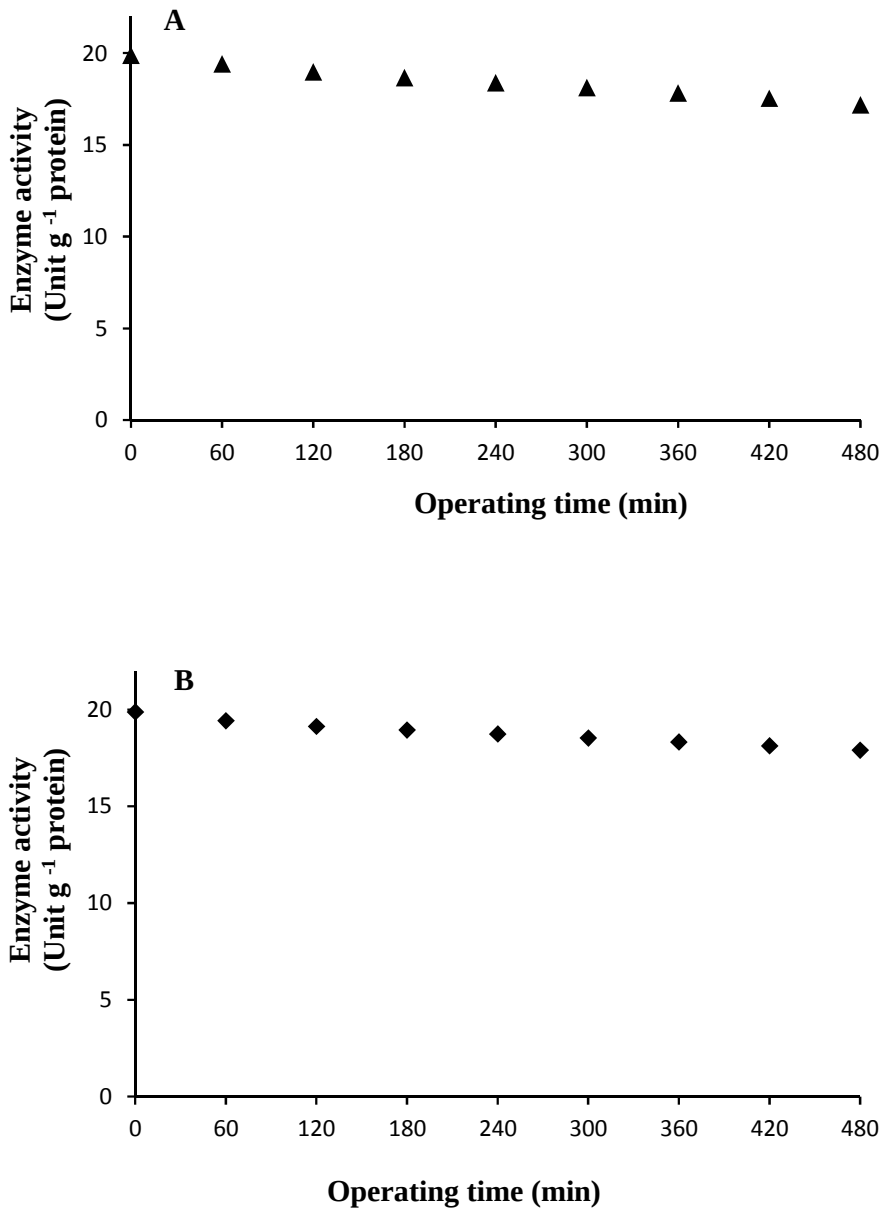

Figure 19. Effect of operating time on enzyme activity during CEMR system by 5 kDa MWCO (A) and 1 kDa MWCO (B) membranes.

Work by Lyagin *et al*., (2012) about membrane reactor system for parallel continuous screening and characterization of biocatalysts suggested that the enzyme activity decreased due to enzyme depletion and enzyme absorption. Mannheim and Cheryan (1990) explained that the decrease in enzymatic membrane reactor performance could be due to several factors including thermal inactivation,

adsorption or poisoning of the enzyme through the contact with membrane, mechanical shearing forces, enzyme inhibitory due to product and hydrolysed substrate accumulation.

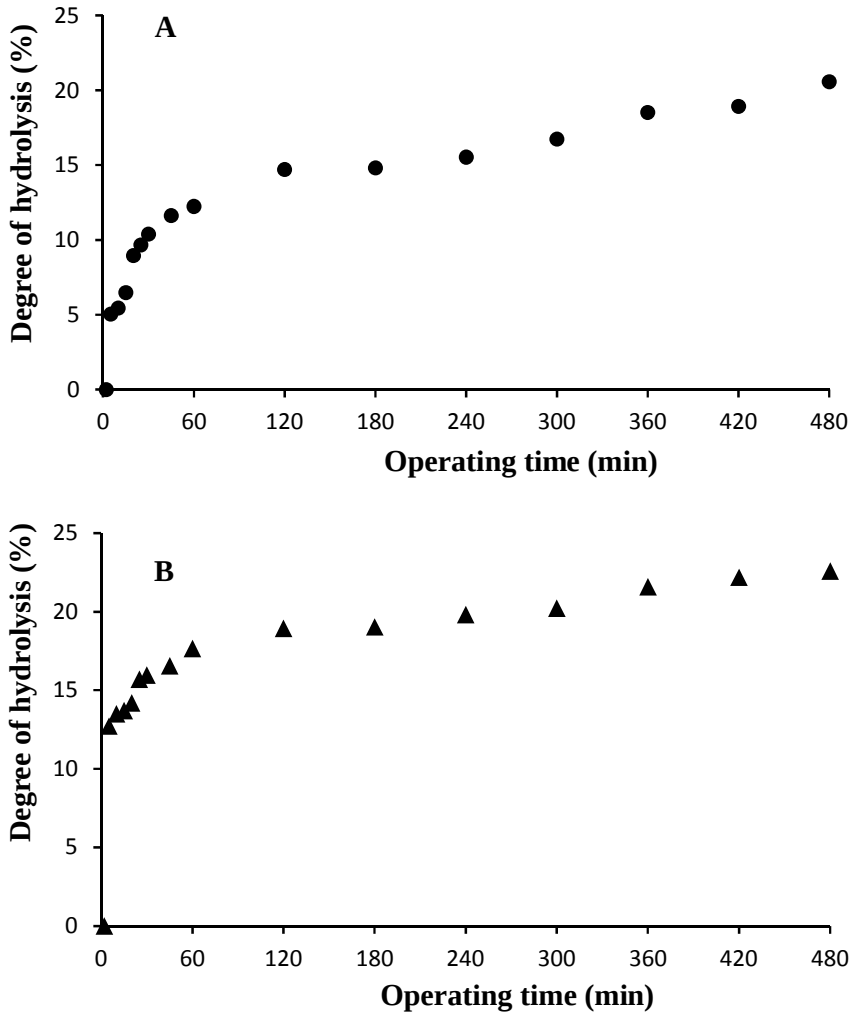

Figure 20. Effect of operating time on degree of hydrolysis during CEMR system by 5 kDa MWCO (A) and 1 kDa MWCO (B) membranes.

The effect of operating time on degree of hydrolysis during CEMR system is shown in Figure 20. The degree of hydrolysis rate increased sharply during the first 60 minutes and then increased gradually as operating time increase. The DH and bioactive activities of permeates increased with the increase operating time. It suggests that hydrolysis was required to release bioactive peptides.

4.3.3 Effect of operating time on protein conversion and productivity; antioxidant, ACE inhibitory activity of peptides during CEMR system

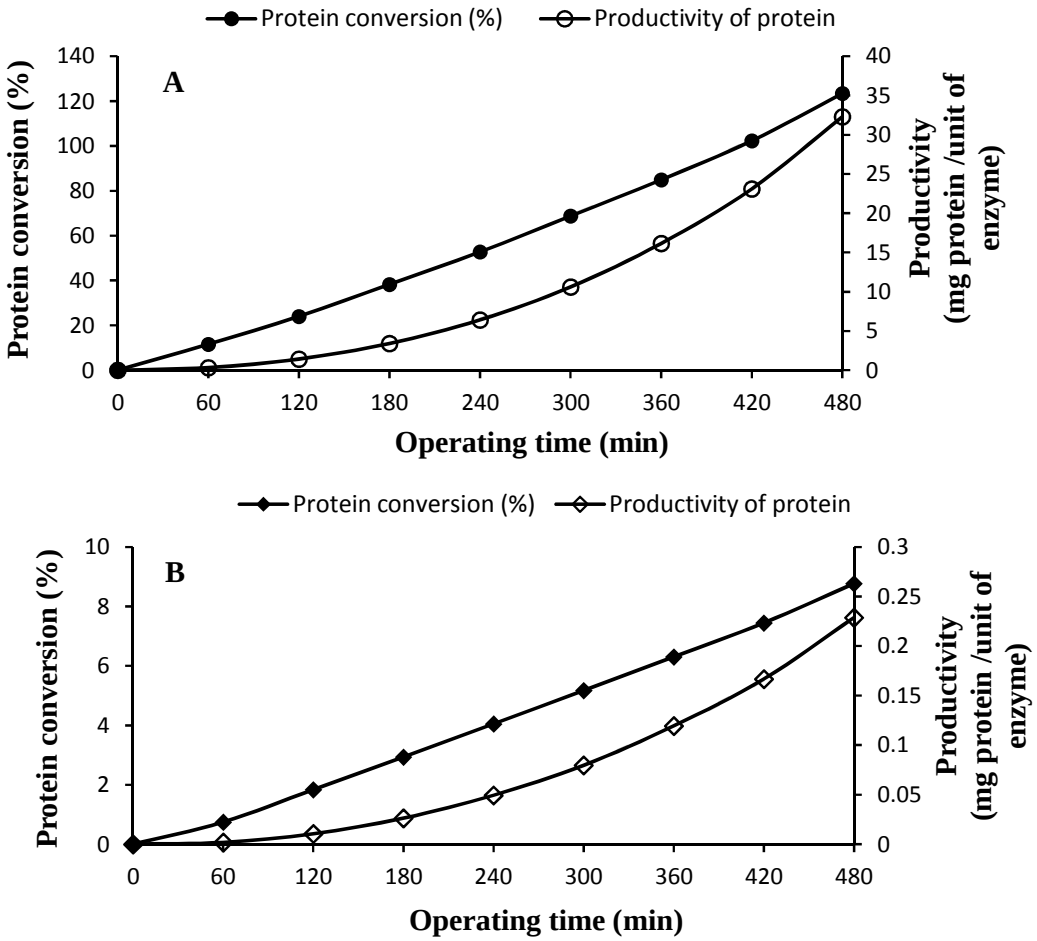

Figure 21. Effect of operating time on protein conversion and productivity of protein during CEMR system by 5 kDa MWCO (A) and 1 kDa MWCO (B) membranes.

The CEMR system provided high protein transmission in both 5 and 1 kDa permeates. However, the protein conversion and productivity of 5 kDa permeate was much higher than that of 1 kDa (Figure 21), associated with permeate flux.

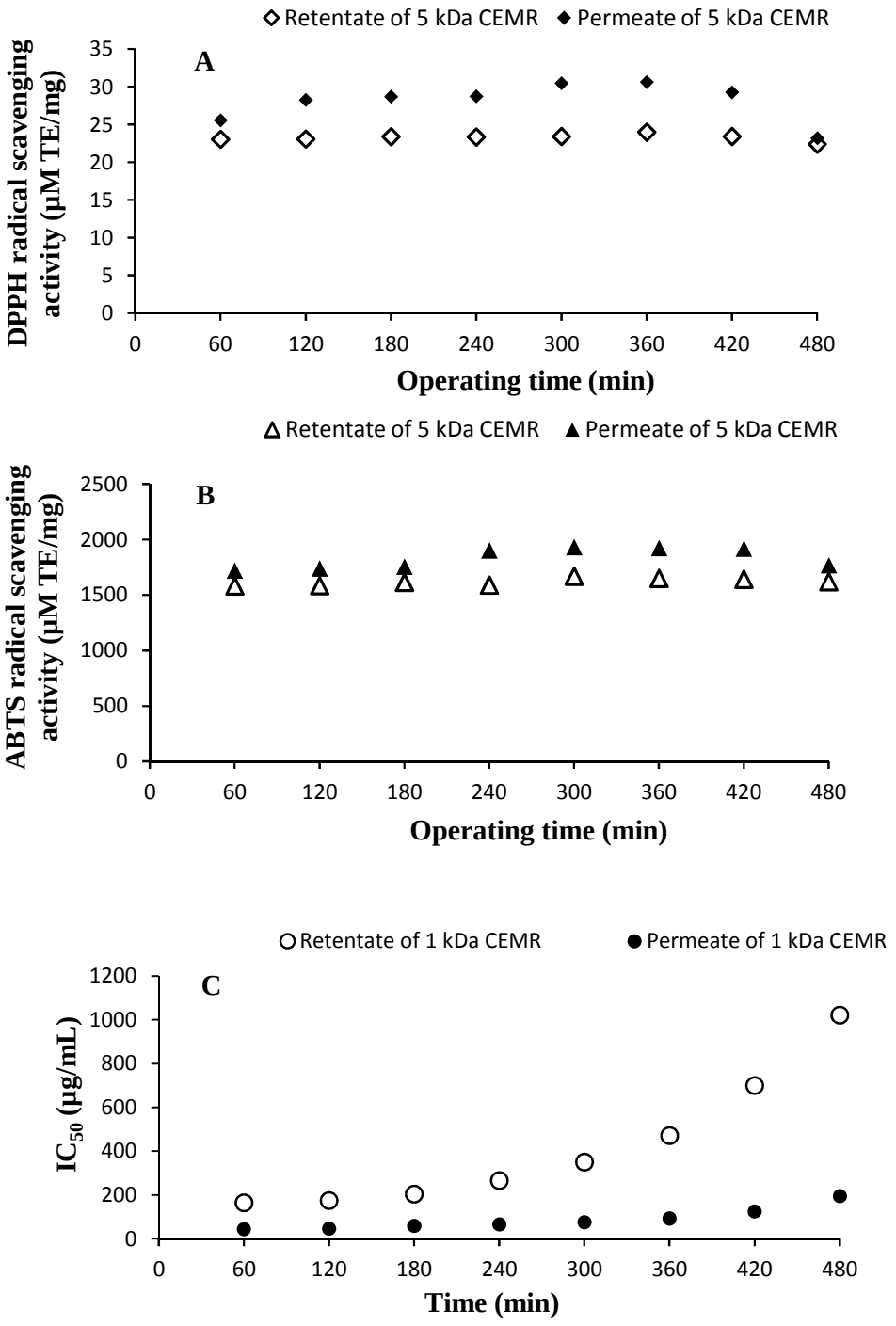

Figure 22. Effect of operating time on antioxidant (A, B) and ACE inhibitory activity (C) of peptides during CEMR system by 5 kDa and 1 kDa MWCO membranes.

Figure 22 show effects of operating time on DPPH, ABTS radical scavenging activity and ACE inhibitory of peptides in permeate and retentate during continuous enzymatic membrane reactor system of protein hydrolysate. The results suggested that bioactive activities of permeate increase when the operating time increases. It results from the increase in conversion of protein into peptides. Table 12 shows protein transmission, bioactive activity of permeates from 5 kDa and 1 kDa CEMR. The results of protein content in retentate and permeate from CEMR operations were shown in appendix 12.

Table 12. Average protein transmission (T_P) and antioxidant, ACE inhibitory activity during CEMR system.

T_P and bioactive activity	5 kDa CEMR system	1 kDa CEMR system
T_P (%)	93.95 ± 1.23 %	82.8 ± 1.76 %
ABTS radical scavenging activity	1762.4 ± 45.32 μM TE mg^{-1}	-
DPPH radical scavenging activity	27.7 ± 1.43 μM TE mg^{-1}	-
ACE inhibitory activity (IC_{50})	-	88.07 ± 3.12 μg mL^{-1}

4.3.4 Effect of operating time on antioxidant, ACE inhibitory activity conversion and productivity of peptides during CEMR system

In the CEMR, the enzyme was introduced only once at the beginning of operation. Afterwards, the volume was replaced continuously. The converting of initial raw material (long-chain peptides or polypeptides) into functionally active protein (short-chain peptides) is called bioactive activity conversion. Productivity was defined as the mass of hydrolysate produced per unit of enzyme. Therefore, high productivity rate have high bioactive production that depends on the activity of enzyme on the substrate. Lee and Kim (1993) reported several factors affected on conversion and productivity including enzyme activity, operating time, enzyme to substrate ratio, substrate feeding pattern.

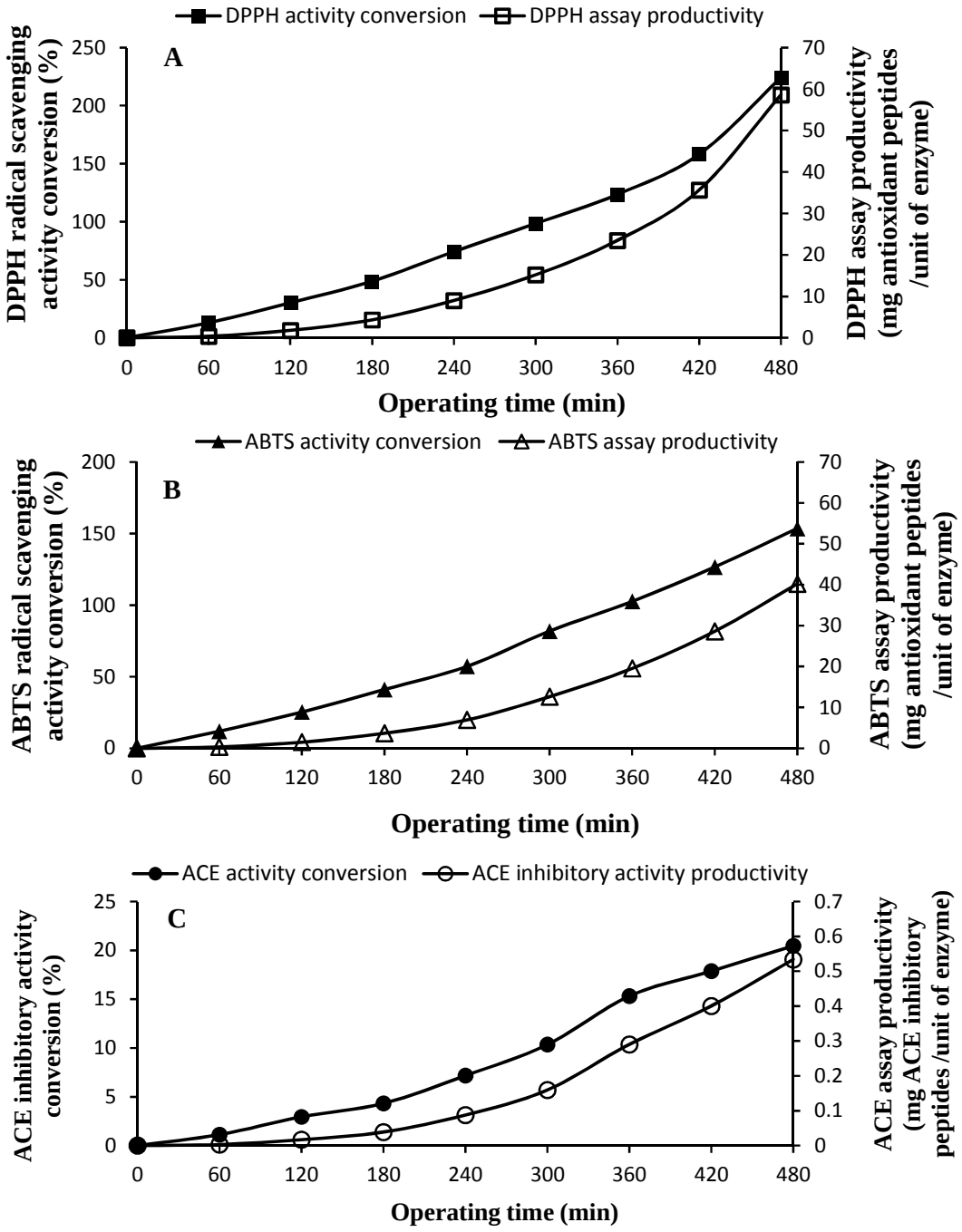

Figure 23. Effect of operating time on antioxidant (A, B) and ACE inhibitory activity I conversion and productivity of peptides during CEMR system by 5 kDa MWCO and 1 kDa MWCO membranes.

The results expressed that antioxidant activity conversion and productivity of peptides during continuous enzymatic membrane reactor system using

5 kDa membrane were much higher than ACE inhibitory activity conversion and productivity of peptides during 1 kDa CEMR system. It was also suggested that continuous enzymatic membrane reactor system was not only able to separate the bioactive products but also to improve the efficiency of the enzymatic reaction (conversion, productivity). In the present study, the substrate was added continuously into the reactor and target peptides removed during 8 h. At the end of operating time, DPPH and ABTS radical scavenging activity conversion were 224 and 154%, respectively; the productivies were 58.6 mg DPPH radical scavenging peptides/unit enzyme and 40.2 mg ABTS radical scavenging peptides/unit enzyme; ACE inhibitory activity conversion and productivity were 20.5% and 0.53 mg ACE inhibitory peptides/unit enzyme.

4.4 Conclusion and suggestion

The results indicated that the CEMR could provide a steady production of 5 and 1 kDa permeates with antioxidant and ACE inhibitory activity since permeates could maintain their flux and bioactive activity. After 8 h operation, permeates of 5 kDa and 1 kDa CEMR were 17.5 and 1.1 L m^2 h, respectively. At the maximum activity, DPPH and ABTS radical scavenging activity conversion were 224 and 154%, respectively; and ACE inhibitory activity conversion was 20.5%.The present study also suggested that reusing the enzyme has the advantage of high productivity in the continuous membrane reactor with 58.6 mg DPPH radical scavenging peptides/unit enzyme, 40.2 mg ABTS radical scavenging peptides/unit enzyme, and 0.53 mg ACE inhibitory peptides/unit enzyme.

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CHAPTER 5

SUMMARY AND FUTURE WORKS

5.1 Summary

The striped catfish skin from fillet processing industry that still contains amount of fish muscle was extracted protein using an autoclave. The extracted protein showed a high yield of 79.1 %. The 5 h hydrolysate (DH= 40.2 %) exhibited the highest bioactive activities including DPPH radical scavenging activity (41.3 μM TE/mg), ABTS radical scavenging activity (2062 μM TE/mg protein), lipid peroxidation inhibition activity was higher than that of 200 ppm α-tocopherol and 1000 ppm ascorbic acid, and ACE inhibitory activity (IC_{50} = 831 μg/mL).

The ultrafiltration using 5 kDa and 1 kDa was successful for separation antioxidant and ACE inhibitory activity peptides since ultrafiltration of the hydrolysate resulted in a significant increase in its bioactive activity in the permeate fractions with DPPH radical scavenging activity (45.2 μM TE/mg), ABTS radical scavenging activity (2474.7 μM TE/mg protein), and ACE inhibitory activity (IC_{50} = 8.3 μg/mL). Based on gel filtration chromatography results, ACE inhibitory peptides had molecular weight ranging of 307 Da to 429 Da while antioxidant peptides had molecular weight in the range of 829.5 Da to 1355 Da. Furthermore, ACE inhibitory and antioxidant activity of peptides showed little changes by the simulated gastrointestinal digestion.

The continuous enzymatic membrane reactor system using 5 and 1 kDa membranes was successful for production of antioxidant and ACE inhibitor peptides since it gave high conversion, productivity of target peptides and stable operation. At the maximum activity, DPPH and ABTS radical scavenging activity conversion were 224 and 154%, respectively; the productivies were 58.6 mg DPPH radical scavenging peptides/unit enzyme and 40.2 mg ABTS radical scavenging peptides/unit enzyme; ACE inhibitory activity conversion and productivity were 20.5% and 0.53 mg ACE inhibitory peptides/unit enzyme.

5.2 Future works

Enzyme concentration and operating parameters for continuous enzymatic membrane reactor system such as transmembrane pressure, cross flow velocity on the performance of membrane separation and bioactive properties of peptides should be investigated.

It is suggested that antioxidant and ACE inhibitor peptides from striped catfish skin by-product were not affected by the simulated gastrointestinal digestion. However, further study should be done to evaluate *in vivo* antioxidant and antihypertensive effect of peptides.

Peptides derived from the striped catfish skin with antioxidant, ACE inhibitory activity have potential as natural antioxidant, antihypertension that could be utilized as a part of functional food to promote consumer health. Therefore, the development of a new food for health in the form of healthy drink or dietary supplement product using these bioactive peptides as functional ingredients should be studied.

APPENDIX

1. Moisture content (AOAC, 1999)

Method

1. Dry the empty dish and lid in the oven at 105 ^{o}C for 30 min and transfer to desiccator to cool (30 min). Weigh the empty dish and lid.
2. Weigh about 5 g of sample to the dish. Spread the sample with spatula.
3. Place the dish with sample in the oven. Dry for 16 h or overnight at 105 ^{o}C.
4. After drying, transfer the dish with partially covered lid to the desiccator to cool.

   Reweigh the dish and its dried content.

Calculation

$$\% \text{ Moisture} = \frac{W1 - W2}{W1} \times 100$$

Where: W1 = weight (g) of sample before drying

W2 = weight (g) of sample after drying

2. Ash (AOAC, 1999)

Method

1. Place the crucible and lid in the furnace at 550 ^{o}C overnight to ensure that impurities on the surface of crucible are burn off. Cool the crucible in the desicator (30 min).
2. Weigh the crucible and lid to 3 decimal places.
3. Weigh about 5 g of sample into the crucible. Heat over low Bunsen flame with lid half covered. When fumes are no longer produced, place crucible and lid in furnace.
4. Heat at 550 ^{o}C overnight. During heating, do not cover the lid. Place the lid on after complete heating to prevent loss of fluffy ash. Cool down in the desiccator.
5. Weigh the ash with crucible and lid until turning to gray. If not, return the crucible and lid to the furnace for further ashing.

Calculation

$$\% \text{ Ash content} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

3. Fat (AOAC, 1999)

Reagents

Petroleum ether.

Method

1. Place the bottle and lid in the incubator at 105 ^{o}C overnight to ensure that weight of bottle was stable.
2. Weigh about 3-5 g of sample to paper filter and wrap.
3. Take the sample into extraction thimble and transfer into soxhlet.
4. Fill petroleum ether about 250 ml into the bottle and take it on the heating mantle.
5. Connect the soxhlet apparatus and turn on the water to cool them and then switch on the heating mantle.
6. Heat the sample about 14 h (heat rate of 150 drop/min).
7. Evaporate the solvent by using the vacuum condenser.
8. Incubate the bottle at 80-90 ^{o}C until solvent was completely evaporated and bottle was completely dry.
9. After drying, transfer the bottle with partially covered lid to the desiccator to cool. Weigh the bottle and its dried content.

Calculation

$$\% \text{ Fat} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100$$

4. Protein (AOAC, 1999)

Reagents

1. Kjedahl catalyst: Mix 9 part of potassium sulphate (K_2SO_4) anhydrous with 1 part of copper sulphate ($CuSO_4$).
2. Sulfuric acid (H_2SO_4).
3. 40 % NaOH solution (w/v).

4. 0.1 N HCl solution.
5. 4% H_3BO_3 solution (w/v).
6. Indicator solution: Mix 100 ml of 0.1% methyl red (in 95% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol).

Method

1. Place sample (0.5-1.0 g) in digestion flask.
2. Add 5 g Kjedahl catalyst, and 200 ml of H_2SO_4.
3. Prepare a tube containing the above chemical except sample as blank. Place flasks in inclined position and heat gently until frothing ceases. Boil briskly until solution clears.
4. Cool and add 60 ml distilled water cautiously.
5. Immediately connect flask to digestion bulb on condenser, and with tip of condenser immersed in standard acid and 5-7 indicator in receiver. Rotate flask to mix content thoroughly; then heat until all NH_3 has distilled.
6. Remove receiver, wash tip of condenser, and titrate excess standard acid in distilled with standard NaOH solution.

Calculation

$$\% \text{ Protein} = \frac{(\text{A} - \text{B}) \text{ x N x } 14.007 \text{ x } 6.25}{\text{W}}$$

Where: A= volume (ml) of 0.1N HCl used sample titration.

B = volume (ml) of 0.1N HCl used blank titration.

N= Normality of HCl.

W= weight (g) of sample.

14.007=atomic weight of nitrogen

6.25= the protein-nitrogen conversion factor for fish and its by-products.

5. Enzymatic assay of protease – Casein as a substrate

Principle:

Casein + H_2O $\longrightarrow$ Amino acids

Conditions:

T = 37 ^oC, pH = 7.5, $A_{660\ nm}$

Reagents:

A. 50 mM Potassium phosphate buffer, pH 7.5 at 37 ^oC.

B. 0.65% (w/v) Casein solution (Casein).

C. 110 mM Trichloroacetic acid reagent (TCA).

D. Folin & Ciocalteu's phenol reagent (F-C).

E. 500 mM Sodium carbonate solution (Na_2CO_3).

F. 10 mM Sodium acetate buffer with 5mM Calcium acetate, pH 7.5 at 37 ^oC.

G. 1.1 mM L-Tyrosine standard (Std Soln).

H. Protease enzyme solution (0.1 - 0.2 unit/mL).

Procedure:

Pipette the following reagents into suitable vials (in milliliters):

	Test	Blank
Reagent B	5.00	5.00
Equilibrate to 37 ^oC. Then add:		
Reagen H	1.00	-
Mixing and incubate at 37 ^oC for exactly 10 minutes. Then add:		
Reagent C	5.00	5.00
Reagent H	-	1.00

Mix by swirling and incubation at 37 ^oC for about 30 minutes. Filter through Whatman #50 filter paper and use the filtrate in color development.

Color development:

Standard curve:

Prepare a standard curve by pipetting the following reagents into suitable vials (in milliliters).

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std Blank
Reagent G (Std Soln)	0.05	0.10	0.20	0.40	0.50	0.60	0.00
Deionize water	1.95	1.90	1.80	1.60	1.50	1.40	2.00
Reagent E	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Reagent D	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Sample:

Pipette the following reagents into suitable vials (in milliliters).

	Test	Blank
Test filtrate	2.00	-
Blank filtrate	-	2.00
Reagent E (Na_2CO_3)	5.00	5.00
Reagent D (F-C)	1.00	1.00

Read the absorbance at 660 nm for each of the vials in suitable cuvettes.

Calculations:

Standard curve:

$$\Delta A_{660\ nm}\ \text{Standard} = A_{660\ nm}\ \text{Standard} - A_{660\ nm}\ \text{Standard Blank}$$

Plot the $\Delta A_{660\ nm}$ Standard vs μmoles of Tyrosine.

Sample determination:

$$\Delta A_{660\ nm}\ \text{Sample} = A_{660\ nm}\ \text{Test} - A_{660\ nm}\ \text{Sample Blank}$$

Determine the μmoles of Tyrosine equivalents liberated using the standard curve.

$$\text{Units/mL enzyme} = \frac{(\mu\text{mole Tyrosine equivalents released})\ (11)}{(1)(10)(2)}$$

11 = Total volume (in milliliters) of assay.

10 = Time of assay (in milliliters) as per the Unit definination.

1 = Volume of enzyme (in milliliters) of enzyme used.

2 = Volume (in milliliters) used in colorimetric determination.

$$\text{Units/mg solid} = \frac{\text{Units/mL enzyme}}{\text{mg solid/mL enzyme}}$$

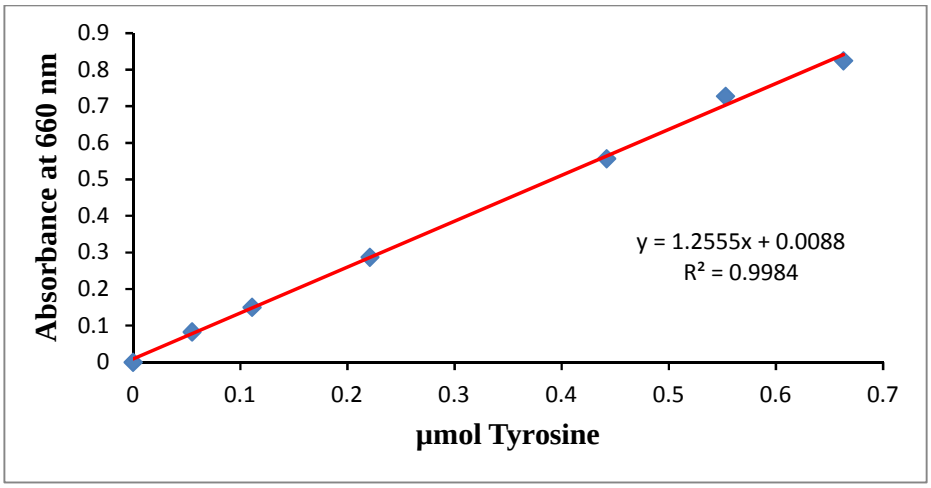

Figure-Appendix 5. The Tyrosine standard curve at wavelength 660 nm.

6. Determination of soluble protein content by Lowry method (1951)

Reagents

1. **A**: 2% sodium carbonate in 0.1 N NaOH.
2. **B**: 0.5% $CuSO_4$. $5H_2O$ in 1% sodium citrate.
3. **C**: Folin-Ciocalteu`s phenol reagent + distilled water with the ratio 1: 1
4. **D**: 50 mL reagent A + 1 mL reagent B
5. Standard reagent: Bovine serum albumin (BSA) at a concentration 250 µg/mL.

Method

1. To each of seven disposable cuvettes, add the following reagents according to the table.
2. To tubes 500 µL of standards and unknown protein sample were added and mix well by using the vortex mixer.
3. Add 2 mL reagent D to each of the standards and unknown protein sample tube and then vortex immediately.
4. Incubate precisely 10 min at room temperature.
5. Add 0.2 mL reagent C (previously dilute 1:1 with distilled water) and vortex immediately.

6. Incubate 30 min at room temperature (sample incubated longer than 60 min should be discarded).
7. Read absorbance at 750 nm. Blank was DI water.
8. Plot standard curve and calculate the unknown.

Table experimental set up for the Lowry's assay:

Tube	DI Water (µL)	250 µg/mL BSA (µL)	BSA concentration (µg/mL)
1	500	0	0
2	400	100	50
3	300	200	100
4	200	300	150
5	100	400	200
6	0	500	250
7		500 µL sample	unknown

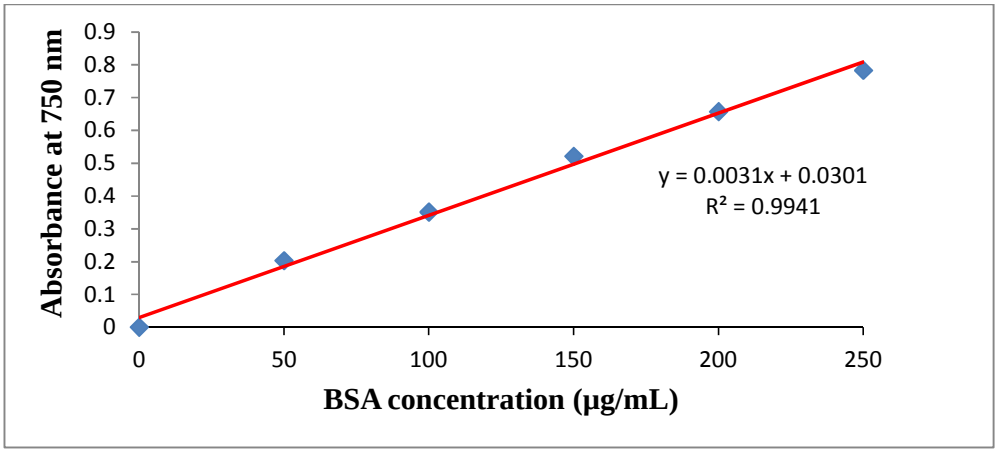

Figure-Appendix 6. The bovine serum albumin standard curve at wavelength 750 nm.

7. Determination of degree of hydrolysis (Nielsen *et al*., 2001)

The OPA reagent, was prepared daily, consists of 6 mM OPA (first dissolve in 97% v/v ethanol) and 5.7 mM DL-dithiothreitol in 0.1 M sodium tetraborate decahydrate containing 1 % (w/v) sodium dodecyl sulphate (SDS). Samples (0.4 mL) from the hydrolysis reactions was mixed with 3 mL of OPA

reagent and incubated for 2 min at room temperature before measuring the absorbance at 340 nm using a spectrophotometer.

The amount of dissociated α-NH_2 groups in the hydrolysate was calculated as Serine-NH_2 moieties using L-Serine as the standard. The mass of peptides was defined as the amount of dissociated α-NH_2 groups. Total number of primary amino groups in the protein was determined by acid hydrolysis of the protein (6 N HCl at 110 °C for 24 h). The degree of hydrolysis (DH %) was calculated according to the below equation:

$$\text{DH } \% = \frac{[NH_2]_{t_x} - [NH_2]_{t_0}}{[NH_2]_{total} - [NH_2]_{t_0}} \times 100$$

Where $[NH_2]_{t_0}$= Number of α-NH_2 groups at 0 min of hydrolysis

$[NH_2]_{t_x}$= Number of α-NH_2 groups in the supernatant after x min of protease 103atalyzed hydrolysis for each experimental point.

$[NH_2]_{total}$ = Number of α-NH_2 groups resulting from acid hydrolysis (complete hydrolysis of protein is assumed).

Prepare OPA reagent

1. The amount of 7.62 g sodium tetraborate decahydrate and 200 mg sodium dodecyl sulphate (SDS) were dissolved in 150 mL deionized water.
2. The amount of 160 mg O-phthaldialdehyde 97% (OPA) was dissolved in 4 mL ethanol. The OPA solution was then transferred quantitatively to the above-mentioned solution by rinsing with deionized water. 176 mg dithiothreitol 99% (DTT) was added to the solution by rinsing with deionized water. The solution was made up to 200 mL with deionized water.
3. The serine standard was prepared as follows: 10 mg serine was diluted in 100 ml deionized water to get a concentration of 100 μg/mL Serine.
4. The sample solution was prepared in the concentration of 0.1 to 1.0 mg/mL.

Procedure

All spectrophotometer readings were performed at 340 nm using deionized water as the control. 3 ml OPA reagents were added to all test tubes. As absorbance changes somewhat with time, it is important the samples stand for exactly

the same time (2 min) before measuring. The assay was carried out at room temperature. Blank was prepared from 400 µL deionized water. Samples were prepared 400 µL.

Calculation

The amount of free amino groups was calculated as serine-NH_2 moieties. Acid hydrolysis of the hydrolysate in 6 M HCl for 24 h at 110 ^{o}C was carried out for determination of total free amino acid groups of proteins. The degree of hydrolysis (DH %) was calculated as the ratio of released free amino groups due to enzymatic hydrolysis to total amino groups released due to complete acid hydrolysis per unit of protein and expressed as a percentage value:

$$\text{DH (\%)} = \frac{\text{Free amino groups released due to enzymatic hydrolysis}}{\text{Total free amino groups from acid hydrolysis}} \times 100$$

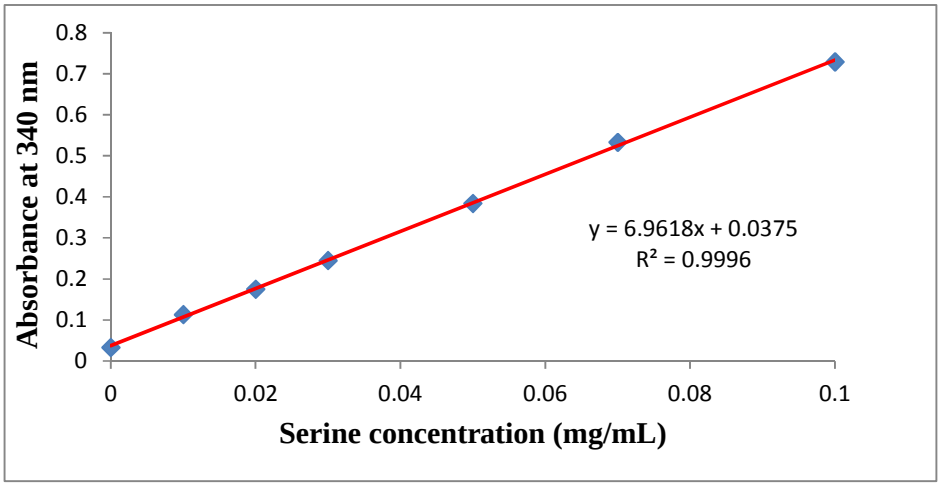

Figure-Appendix7. The Serine standard curve at wavelength 340 nm.

8. Trolox standard curve for determination of DPPH radical scavenging activity

1. The amount of 5.9145 mg 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was dissolved in 100 mL ethanol 50 % (v/v) to prepare 0.15 mM DPPH solution.
2. Trolox standards in the concentration of 0 to 60 µM were prepared in ethanol 95%.
3. Add 1.5 mL of 0.15 mM DPPH solution to 1.5 mL of Trolox standards and then vortex immediately.
4. Incubate 30 min at room temperature in the darkness.

5. Read absorbance at 517 nm. Blank was DI water.
6. Plot standard curve.

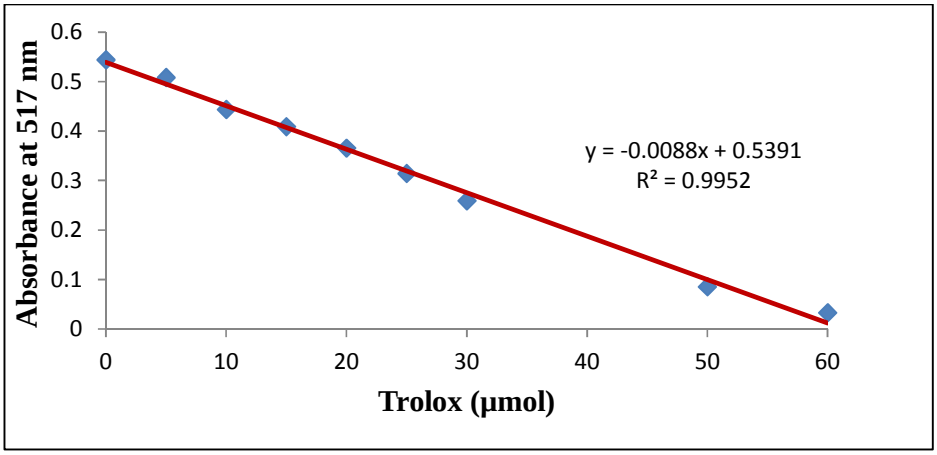

Figure-Appendix 8. The Trolox standard curve 0-60 µM at wavelength 517 nm.

9. Trolox standard curve for determination of ABTS radical scavenging activity

1. The amount of 40.602 mg 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was dissolved in 10 mL DI water to prepare 7.4 mM ABTS reagent.
2. The amount of 7.03 mg potassium persulphate was dissolved in 10 mL DI water to prepare 2.6 mM potassium persulphate solution.
3. Mix ABTS reagent and potassium persulphate solution with the ratio 1: 1. Incubate 12-16 h at room temperature in the darkness.
4. Fresh ABTS solution was prepare for assay by mixing ABTS solution with methanol in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm.
7. Trolox standards in the concentration of 0 to 600 µM were prepared in ethanol 95%.
8. Add 2850 µL of ABTS solution to 150 µL of Trolox standards and then vortex immediately.
9. Incubate 2 h at room temperature in the darkness.
10. Read absorbance at 734 nm. Blank was DI water.
11. Plot standard curve.

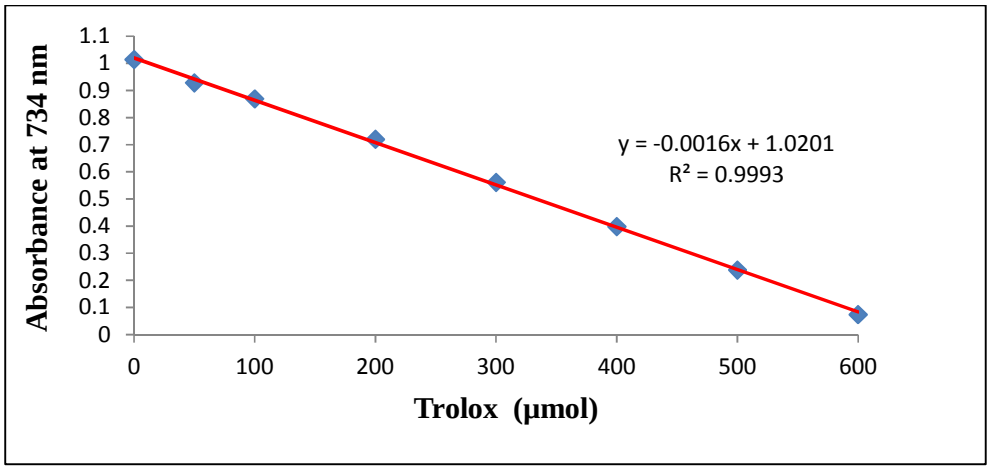

Figure-Appendix 9. The Trolox standard curve 0-600 µM at wavelength 734 nm.

10. Protein content in retentate and permeate from UF processes with 10, 5, and 1 kDa.

Operating time (h)	Protein content (mg/mL)					
	10 kDa		5 kDa		1 kDa	
	Feed	Permeate	Feed	Permeate	Feed	Permeate
1	3.681 ± 0.09	3.667 ± 0.07	4.255 ± 0.16	3.689 ± 0.11	4.530 ± 0.34	3.193 ± 0.08
2	3.598 ± 0.07	3.565 ± 0.08	3.905 ± 0.18	3.307 ± 0.14	4.144 ± 0.37	2.879 ± 0.12
3	3.519 ± 0.09	3.458 ± 0.12	3.590 ± 0.12	2.965 ± 0.15	3.749 ± 0.38	2.491 ± 0.11
4	3.469 ± 0.11	3.368 ± 0.09	3.524 ± 0.23	2.865 ± 0.10	3.724 ± 0.33	2.455 ± 0.09
5	3.418 ± 0.12	3.278 ± 0.11	3.339 ± 0.25	2.695 ± 0.12	3.514 ± 0.33	2.278 ± 0.08
6	3.367 ± 0.14	3.118 ± 0.10	3.144 ± 0.22	2.459 ± 0.19	3.335 ± 0.36	2.151 ± 0.07

11. Protein content of peaked fractions from Sephadex^{M} G-25 and G-15 columns

Fraction No.	Protein content (mg/mL)	
	Sephadex^{M} G-25	Sephadex^{M} G-15
47	3.579 ± 0.11	-
55	2.297 ± 0.15	-
59	1.793 ± 0.29	-
96	0.073 ± 0.23	-
15	-	3.412 ± 0.26
16	-	0.883 ± 0.30
22	-	2.307 ± 0.10

12. Protein content in the feed and permeate from CEMR operation with 5 and 1 kDa

Operating time (h)	Protein content (mg/mL)			
	5 kDa CEMR		1 kDa CEMR	
	Feed	Permeate	Feed	Permeate
1	4.515 ± 0.17	4.334 ± 0. 27	5.234 ± 0.75	4.232 ± 0.74
2	4.512 ± 0.71	4.346 ± 0.29	5.043 ± 0.11	4.268 ± 0.36
3	4.351 ± 0.55	4.358 ± 0.24	5.000 ± 0.22	4.335 ± 0.47
4	4.326 ± 0.38	4.419 ± 0.23	4.973 ± 0.43	4.377 ± 0.52
5	4.254 ± 0.15	4.448 ± 0.16	4.926 ± 0.64	4.394 ± 0.34
6	4.225 ± 0.48	4.468 ± 0.47	4.922 ± 0.89	4.398 ± 0.28
7	4.178 ± 0.16	4.510 ± 0.30	4.913 ± 0.56	4.427 ± 0.39
8	4.110 ± 0.17	4.634 ± 0.13	4.905 ± 0.14	4.518 ± 0.37

VITAE

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Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Food Technology)	Can Tho University	2001

Scholarship Awards during Enrolment

1. The Mekong 1000 scholarship for training staff abroad for graduate levels to promote the process of industrialization and modernization in the Mekong Delta, Vietnam.
2. Thesis financial support from the Graduate School, Prince of Songkla University, Thailand.

List of Publication and Proceedings

Publication

1. Hue, Q. H., Youravong, W., Sirinupong, N. 2013. Antioxidant activities of protein hydrolysate from the skin of striped catfish (*Pangasius hypophthalmus*) fillet processing waste. J. Fish. Sci. Technol. Special Issue: P. 70-77.

Proceedings

1. Hue, Q. H., Youravong, W., Sirinupong, N. 2013. Antioxidant peptides derived from the skin of striped catfish (*Pangasius hypophthalmus*) fillet processing waste. Utilization of waste/rest raw materials and by-products in the fish processing industry: Opportunities and Challengs. 9-10 December 2013. Nha Trang, Vietnam. Oral presentation.