ฤทธิ์การยับยั้งเอนไซม์แอนจิโอเทนซินและการปรับระบบภูมิคุ้มกันของโปรตีนไฮโดรไลเซทผลิตโดยโปรติเนสจาก *Virgibacillus halodenitrificans* SK1-3-7

นางสาวธิดารัตน์ ธูปแช่ม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
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ANGIOTENSIN I-CONVERTING ENZYME (ACE) INHIBITORY ACTIVITY AND IMMUNOMODULATING ACTIVITY OF PROTEIN HYDROLYSATES PRODUCED BY *VIRGIBACILLUS HALODENITRIFICANS* SK1-3-7 PROTEINASES

Tidarat Toopcham

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

Suranaree University of Technology

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ENHANCEMENT OF ARBUSCULAR MYCORRHIZAL INOCULANT PRODUCTION USING PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ธิดารัตน์ ธูปแช่ม : ฤทธิ์การยับยั้งเอนไซม์แอนจิโอเทนซินและการปรับระบบภูมิคุ้มกันของโปรตีนไฮโดรไลเซทผลิตโดยโปรติเนสจาก *VIRGIBACILLUS HALODENITRIFICANS* SK1-3-7 (ANGIOTENSIN I-CONVERTING ENZYME (ACE) INHIBITORY ACTIVITY AND IMMUNOMODULATING ACTIVITY OF PROTEIN HYDROLYSATES PRODUCED BY *VIRGIBACILLUS HALODENITRIFICANS* SK1-3-7 PROTEINASES) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 198 หน้า.

วัตถุประสงค์ของการศึกษาครั้งนี้คือเพื่อผลิตและศึกษาคุณลักษณะของเพปไทด์ที่มีฤทธิ์ยับยั้งเอนไซม์แอนจิโอเทนซิน (เอซีอี) ที่ได้จากโปรตีนไฮโดรไลเซทจากกล้ามเนื้อปลานิลย่อยโดยโปรติเนสจาก *Virgibacillus halodenitrificans* SK1-3-7 นอกจากนี้ศึกษาฤทธิ์การปรับระบบภูมิคุ้มกันของโปรตีนไฮโดรไลเซทจากแหล่งของโปรตีนต่าง ๆ ซึ่งถูกย่อยโดยโปรติเนสที่ผ่านการทำบริสุทธิ์บางส่วนจาก *V. halodenitrificans* SK1-3-7

เมื่อเตรียมโปรตีนไฮโดรไลเซทจากกล้ามเนื้อปลานิลโดยใช้เนื้อปลาบด เนื้อปลาบดล้างน้ำ และโปรตีนซาร์โคพลาสมิค ที่ย่อยด้วยโปรติเนสจาก *V. halodenitrificans* SK1-3-7 ที่เวลาการย่อย 8 ชั่วโมง พบว่าไฮโดรไลเซทจากเนื้อปลาบดที่ระดับการย่อยสลาย 48% แสดงฤทธิ์การยับยั้งเอซีอีได้สูงสุดโดยมีค่า IC_{50} เท่ากับ 0.54 มิลลิกรัมต่อมิลลิลิตร ในขณะที่ไฮโดรไลเซทจากโปรตีนซาร์-โคพลาสมิคแสดงค่าระดับการย่อยสลายต่ำที่สุด โดยมีค่า IC_{50} เท่ากับ 1.15 มิลลิกรัมต่อมิลลิลิตร ไฮโดรไลเซทจากเนื้อปลาบดแสดงรูปแบบการยับยั้งแบบผสม โดยมีค่าคงที่การยับยั้งเท่ากับ 0.041 มิลลิกรัมต่อมิลลิลิตร ถึงแม้ว่าการย่อยด้วยระบบทางเดินอาหารที่ทดสอบในหลอดทดลองจะลดกิจกรรมการยับยั้งเอซีอีของไฮโดรไลเซทจากเนื้อปลาบด แต่สามารถเพิ่มความสามารถการซึมผ่านชั้นของเซลล์ลำไส้เล็ก Caco-2 เพปไทด์ที่ซึมผ่านได้ถูกบ่งชี้ว่าประกอบด้วยกรดอะมิโน 3-4 โมเลกุล และยังแสดงฤทธิ์การยับยั้งเอซีอีได้เป็นอย่างดี และเปปไทด์ใหม่ที่มีฤทธิ์ยับยั้งเอซีอีได้สูงที่สุดคือ MCS ซึ่งมีค่า IC_{50} เท่ากับ 0.29 ไมโครโมลาร์

การทำบริสุทธิ์เพปไทด์จากไฮโดรไลเซทจากเนื้อปลานิลบดที่ผ่านการย่อย 8 ชั่วโมง ด้วยเทคนิคอัลตราฟิลเตรชั่น การแลกเปลี่ยนประจุลบ การแลกเปลี่ยนประจุบวกและการแยกตามขนาดตามลำดับ พบว่าเพปไทด์ที่มีฤทธิ์การยับยั้งเอซีอีได้สูงที่สุดมีค่า IC_{50} เท่ากับ 0.15 มิลลิกรัมต่อมิลลิลิตร และแสดงรูปแบบการยับยั้งแบบไม่แข่งขัน (uncompetitive inhibition) โดยมีค่าคงที่การยับยั้งเท่ากับ 0.18 มิลลิกรัมต่อมิลลิลิตร เพปไทด์ยังแสดงเสถียรภาพต่อการให้ความร้อนที่อุณหภูมิสูงที่ 100 และ 121 องศาเซลเซียส และพีเอชในช่วง 2-10 และยังคงมีเสถียรภาพหลังจากผ่านการย่อยด้วยระบบทางเดินอาหารที่ทดสอบในหลอดทดลอง เพปไทด์ที่แสดงฤทธิ์การยับยั้งเอซีอีได้สูง

ที่สุดประกอบด้วยกรดอะมิโนไม่มีขั้ว (เมทไทโอนีน ไอโซลิวซีน ลิวซีน และฟีนิลอะลานีน) และมีกรดอะมิโนอาร์จินีนที่ปลายสายซี (C-terminus)

เมื่อย่อยเนื้อปลานิลบด เคซีน และโปรตีนถั่วพี (pea protein) ด้วยโปรติเนสที่ผ่านการทำบริสุทธิ์บางส่วนจาก *V. halodenitrificans* SK1-3-7 เป็นเวลา 8 ชั่วโมง ไฮโดรไลเซทจากเนื้อปลานิลบดส่งเสริมระบบภูมิคุ้มกันโดยกำเนิด (innate immunity) ผ่านการเหนี่ยวนำให้มีการแสดงออกของยีน IL-1β และ COX-2 เมื่อทดสอบด้วยเซลล์แมคโครฟาจทีเอชพี-1 (THP-1 macrophage) ในขณะที่ไฮโดรไลเซทจากเคซีนและโปรตีนถั่วพีแสดงฤทธิ์การต่อต้านการอักเสบโดยไฮโดรไลเซทจากเคซีนกดการตอบสนองของ IL-1β IL-6 IL-8 TNF-α และ COX-2 ในเซลล์ที่ผ่านการกระตุ้นด้วยลิโปโพลีแซคคาไรด์ ส่วนไฮโดรไลเซทจากโปรตีนถั่วพีลดการตอบสนองของ IL-6 และ TNF-α

การศึกษานี้แสดงให้เห็นว่าไฮโดรไลเซทจากเนื้อปลานิลบดที่เตรียมจากโปรติเนสจาก *V. halodenitrificans* SK1-3-7 มีศักยภาพในการเป็นตัวยับยั้งเอซีอีและสามารถนำไปพัฒนาเป็นผลิตภัณฑ์เสริมอาหารที่มีคุณสมบัติควบคุมความดันโลหิตสูง นอกจากนี้โปรตีนไฮโดรไลเซทที่ผลิตโดยโปรติเนสนี้แสดงฤทธิ์การปรับระบบภูมิคุ้มกันอีกด้วย

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2558 ลายมือชื่ออาจารย์ที่ปรึกษา_____

TIDARAT TOOPCHAM : ANGIOTENSIN I-CONVERTING ENZYME (ACE) INHIBITORY ACTIVITY AND IMMUNOMODULATING ACTIVITY OF PROTEIN HYDROLYSATES PRODUCED BY *VIRGIBACILLUS HALODENITRIFICANS* SK1-3-7 PROTEINASES. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 198 PP.

PROTEIN HYDROLYSATE/*V. HALODENITRIFICAN* SK1-3-7/
ACE INHIBITORY ACTIVITY/IMMUNOMODULATORY ACTIVITY

The objectives of this study were to produce and characterize angiotensin I-converting enzyme (ACE) inhibitory peptides derived from tilapia muscle hydrolyzed by *Virgibacillus halodenitrificans* SK1-3-7 proteinases. In addition, immuno-modulatory activity of protein hydrolysates from various protein sources hydrolyzed by partially-purified proteinase from *V. halodenitrificans* SK1-3-7 was investigated.

Tilapia muscle proteins, namely minced (M), washed mince (WM), and sarcoplasmic protein (SP), were hydrolyzed by *V. halodenitrificans* SK1-3-7 proteinases and their ACE inhibitory activity were compared. At 8 h of hydrolysis, M hydrolysate with a degree of hydrolysis (DH) of 48% showed the highest ACE inhibitory activity with an IC_{50} value of 0.54 mg/mL, while SP hydrolysate exhibited the lowest DH with an IC_{50} value of 1.15 mg/mL ($p < 0.05$). The M hydrolysate showed a mixed-type inhibition characteristic with an inhibition constant (Ki) of 0.041 mg/mL. Although *in vitro* gastrointestinal digestion reduced ACE inhibitory activity of the M hydrolysate, it enhanced permeability across Caco-2 cell monolayers. The permeated peptides were identified to contain 3-4 amino acid residues and showed

strong ACE inhibitory activity. The novel ACE inhibitory peptide with the highest inhibition was identified to be MCS with an IC_{50} value of 0.29 μM.

Purification of tilapia mince hydrolysate at 8 h of hydrolysis was performed by ultrafiltration, anion exchange, cation exchange and size exclusion chromatography. The fraction with the most potent ACE inhibitory activity with an IC_{50} value of 0.15 mg/mL was obtained. The peptides showed uncompetitive inhibition characteristics with Ki of 0.18 mg/mL. The peptides also showed high thermal stability at 100 and 121°C and remained inhibitory activity over a wide pH range of 2-10 and *in vitro* gastrointestinal digestion. The presence of hydrophobic amino acids (Met, Ile, Leu, and Phe) in the peptide sequence with Arg residue at the C-terminus enabled high ACE inhibitory potency.

Tilapia mince, casein, and pea protein were hydrolyzed by partially-purified proteinases from *V. halodenitrificans* SK1-3-7 for 8 h. Tilapia mince hydrolysate (TMH) enhanced innate immunity through induction of IL-1β and COX-2 expression based on THP-1 macrophages. Anti-inflammatory activity was found in casein hydrolysate (CH) and pea protein hydrolysate (PPH). CH suppressed IL-1β, IL-6, IL-8, TNF-α, and COX-2 responses on LPS-induced THP-1 macrophages. PPH reduced LPS-induced IL-6 and TNF-α responses.

This study revealed that tilapia mince hydrolysate prepared by *V. halodenitrificans* SK1-3-7 proteinases has the potential to be ACE inhibitor and could be developed as a functional food with antihypertensive property. In addition, protein hydrolysates produced by the proteinase exhibit an immunomodulatory activity.

School of Food Technology Student's Signature_____

Academic Year 2015 Advisor's Signature_____

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LIST OF ABBREVIATIONS

°C	=	Degree celsius
CFU	=	Colony forming unit
d	=	Day
rpm	=	Revolutions per minute
RP-HPLC	=	Reverse phase high performance chromatography
g	=	Relative centrifugal fields
kDa	=	Kilodalton
M	=	Molar
Mg	=	Milligram
µg	=	Microgram
µl	=	Microliter
ml	=	Milliliter
mM	=	Millimolar
mU	=	Milliunit
U	=	Unit activity
Min	=	Minute
mm	=	Millimeter
µm	=	Micrometer
MW	=	Molecular weight
Sec	=	Second

LIST OF ABBREVIATIONS (Continued)

SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
DMSO	=	Dimethyl sulfoxide
M	=	Methionine
I	=	Isoleucine
L	=	Leucine
F	=	Phenylalanine
R	=	Arginine
N	=	Asparagine
Q	=	Glutamine
D	=	Aspartic acid
K	=	Lysine
H	=	Histidine
P	=	Proline
E	=	Glutamic acid
G	=	Glycine
V	=	Valine
A	=	Alanine
C	=	Cysteine
T	=	Threonine
Y	=	Tyrosine

CHAPTER I

INTRODUCTION

1.1 Introduction

The importance of proteins in the diet has been increasingly acknowledged as a result of new scientific findings in the field of nutrition over the last two decades. The value of proteins as an essential source of amino acids is well documented (Korhonen, 2009). In recent years, a considerable amount of research has focused on the release of bioactive peptides which are encrypted within the primary structure of food proteins, in view to investigate such peptides as functional food ingredients aimed to health maintenance. In general, bioactive peptides can be released from the sequence of parent proteins during gastrointestinal digestion, microbial fermentation, food processing, or enzymatic hydrolysis (Hernández-Ledesma, Contreras, and Recio, 2011). Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts and Weiler, 2003). It is well known that the common way to produce bioactive peptides is through enzymatic hydrolysis of whole protein molecules (Korhonen, 2009). Many biological peptides, with health benefits such as opioid, antimicrobial, antioxidative, mineral-binding, enhancement of intestinal, antihypertensive, immunomodulating activities, etc. have been classified and identified from food protein hydrolysates (Korhonen, 2009; Kitts and Weiler, 2003; Ryan, Ross, Bolton, FitzGerald, and Stanton, 2011). Therefore,

enzymatic protein hydrolysates are economically important value-added products as functional foods in the food industry.

High blood pressure or hypertension is a chronic disease affecting 30% of the adult population in the world (Majumder and Wu, 2009), and is one of the major risk factors for stroke coronary heart disease (CHD), kidney dysfunction, and myocardial infarction (Li, Le, Shi, and Shrestha, 2004). It is often called a silent killer because persons with hypertension are often asymptomatic for years (Sheih, Fang, and Wu, 2009). Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is one of the main regulators of blood pressure through its action on two body systems. Firstly, ACE forms part of the renin angiotensin system (RAS), converting angiotensin I to a potent vasoconstrictor, angiotensin II, which also induces the release of aldosterone and therefore, increases the sodium concentration and blood pressure. ACE also takes part of the kallikrein-kinin system as it hydrolyzes bradykinin, which has a vasodilator action (Hernández-Ledesma et al., 2011). Inhibition of ACE can be used for prevention and treatment of hypertension and related diseases (Bougatef et al., 2008). Synthetic ACE inhibitors such as captopril, enalapril, and lisinopril are remarkably effective at regulating blood pressure and are used as clinical antihypertensive drugs (Salvetti, 1990). However, they are not entirely without side effects such as cough, taste disturbances, renal impairment, and angioneurotic edema (Chen et al., 2007). Therefore, production of ACE inhibitory peptides from various food protein sources could be of interest as a functional food product.

More recently, many ACE inhibitory peptides have been isolated from enzymatic hydrolysis of both animal and plant proteins, such as soy (Chiang, Tsou, Tsai, and Tsai, 2006), peanut (Li, Liu, Shi, and Le, 2005; Jamdar, Rajalakshmi,

Pednekar, Juan, Yardi, and Sharma, 2010), rice dregs (Chen et al., 2007), casein (Zhu et al., 2007; Jiang, Tian, Brodkorb, and Huo, 2010), whey protein (Ferreira et al., 2007; Wang, Mao, Cheng, Xiong, and Ren, 2010; Pan, Cao, Guo, and Zhao, 2012), porcine muscles (Arihara, Nakashima, Makai, Ishikawa, and Itoh, 2001), shrimp (He, Chen, Wu, Sun, Zhang, and Zhou, 2006). Apart from them, it has been proven that fish proteins are an excellent source of ACE inhibitory peptides, particularly fish muscle proteins (Charoenphun, Youravong, and Cheirsilp, 2013; Chen, Wang, Zhong, Wu, and Xia, 2012; Raghavan and Kristinsson, 2009; Wijesekara, Qian, Ryu, Ngo, and Kim, 2011). Fish muscle consists of three majors groups of proteins; salt-soluble myofibrillar proteins (60-70 wt%) containing principally myosin, actin, and a smaller amount of tropomyosin and troponin; water-soluble sarcoplasmic proteins (about 30 wt%) consisting of albumins, myoglobin, and enzymes; and insoluble stromal proteins representing 3-10% of total proteins (Suzuki, 1981). A large number of ACE inhibitory peptides derived from fish muscle proteins have been reported. For instance, Wu et al. (2008) found ACE inhibitory activity of the dipeptides CF, EY and FE with IC_{50} values of 1.96, 2.68 and 1.45 μM, respectively, were isolated from shark meat hydrolysate. Ghassem et al. (2011) reported that two peptides VPAAPPK and NGTWFEPP were released from myofilbrillar protein of Huruan (*Channa striatus*) exhibiting ACE inhibitory activity with IC_{50} values of 0.45 and 0.63 μM, respectively.

Tilapia (*Oreochromis niloticus*) is one the most important aquacultured freshwater fish with the global production of about 4 million metric tons in 2011, and is expected to exceed 4.5 million metric tons in 2015 (Fitzsimmons, 2013). It is the third most widely cultured fish, after carp and salmonids. The major farmed-tilapia producing Asian countries are China, Korea, Indonesia, the Phillippines, Taiwan, and Thailand

(Fitzsimmons, 2013). Traditionally, tilapia has been used almost exclusively for fresh consumption and some part is introduced to the fillet industry (Rawdkuen, Sai-Ut, Khamsorn, Chaijan, and Benjakul, 2009). Due to the high current volume of production, protein hydrolysates with ACE inhibitory activity can pave the way for full utilization of tilapia.

Proteinases from different sources are commonly employed to obtain a more selective hydrolysis due to their specificity for peptide bonds adjacent to certain amino acid residues (Khantaphant and Benjakul, 2008). Up to now, several commercial proteinases such as Alcalase (Chen et al., 2012; Charoenphun et al., 2013), pepsin (Wang et al., 2008; Sheih et al., 2009), trypsin (Asoodeh, Yazdi, and Chamani, 2012), thermolysin (Ghassem et al., 2011), and Flavourzyme (Khantaphant, Benjakul, and Kishimura, 2011) have been used to produce protein hydrolysates with ACE inhibitory peptides. However, the use of proteinases from novel sources in protein hydrolysate production has not been widely studied. *Virgibacillus halodenitrificans* SK1-3-7 is a moderately halophilic bacteria, isolated from Thai fish sauce fermentation. Montriwong et al. (2012) found that *V. halodenitrificans* SK1-3-7 proteinase exhibited the highest fibrinolytic and proteolytic activities among 25 bacterial isolates from fish sauce fermentation. ACE inhibitory peptides could be obtained through hydrolytic reaction of *V. halodenitrificans* SK1-3-7 proteinase.

One of the greatest challenges in developing nutraceutical and functional food products is proving the *in vivo* efficacy of their bioactive components. Survival from physiological barriers after oral administration, including gastrointestinal digestion and intestinal epithelial absorption in human tract is the essential prerequisite for the bioactive assessment of peptides (Xie, Wang, Ao, and Li, 2013). After oral ingestion,

gastrointestinal (GI) enzymes may break up peptides, thereby increasing or decreasing their activity. Although animal studies and human clinical trials are the best ways to study the bioactivity and bioavailability of functional ingredients. Nevertheless, they are costly and require strict ethical consideration (Cinq-Mars, Hu, Kitts, and Li-Chan, 2008). *In vitro* simulated GI digestion is being extensively used. It allows for rapid and inexpensive study of bioactive compounds for assessment of their efficacy *in vivo* (Cinq-Mars et al., 2008). Furthermore, to exert their physiological effects, these peptides must be absorbed intact across the brush border membrane. Caco-2 model has been widely used as a predictive tool for the intestine epithelial absorption and permeability of peptides (Cinq-Mars et al., 2008; Guang, Shang, and Jiang, 2012). Caco-2 cell monolayers express characteristics of mature epitheliums, such as microvillus structure, tight junction at apical side, numerous brush border enzymes, and carrier-mediated transport system for di/tri peptides, which are of critical importance for absorption of intact peptides (Sambuy, De Angelis, Ranaldi, Scarino, Stammati, and Zucco, 2005).

The separation and the identification of peptides and amino acids has to be studied in order to obtain better knowledge about the composition of protein hydrolysates (Silvestre, 1997). Amino sequencing and characterization of individual peptides could also be important for studying a mechanism of bioactive peptide (Raghavan, Kristinsson, and Leeuwenburgh, 2008). In addition, amino acid sequences will be useful for the peptide synthesis for either food or pharmaceutical industry. By combining membrane separation, chromatographic techniques and tandem mass spectrometry, bioactive peptides can be purified and identified. In the past, peptide sequencing was achieved using the Edman degradation method, which was time-

consuming, low throughput, and poor sensitivity in comparison with the mass spectrometry. Due to the exceptional molecular specificity and versatility in determining the structure of unknown compounds, mass spectrometric methods play an important role in characterization of peptides. Liquid chromatography (LC) combined with mass spectrometry (MS) using electrospray ionization (ESI) as an interface is a powerful technique for peptide identification (Verardo et al., 2010). Furthermore, the advantages of MS detection include the ability to determine the molecular weight and to obtain structural information (Ghassem et al., 2011). Therefore, liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique is a widely utilized approach in peptide sequencing for isolating and identifying hydrolysate (Liu et al., 2010).

The processing stability of the purified peptides after various temperature and pH treatments is prerequisite in preparing foods with functional peptides. The functional, nutritional and biological properties of peptides in the food matrix are highly influenced by molecular structure, interactions with other components and the conditions of processing (Hwang, 2010). Moreover, pH changes may affect functional properties by modifying specifically one or more amino acids. For example, acidic treatments destroy glutamine and asparagines, whereas alkaline treatments destroy cysteine, serine, and threonine, and produce lysinoalanine and D-amino acids (Anantharaman and Finot, 1993). Furthermore, GI digestion is of particular importance in the bioavailability of ACE inhibitory peptides as mentioned above. Durable peptides obtained after *in vitro* GI digestion would be expected to resist against GI proteinases after oral intake, increasing the likelihood for them to exert ACE inhibitory activity.

Therefore, effects of thermal, pH, and *in vitro* GI tract model on stability of ACE inhibitory peptides should be addressed.

Apart from ACE inhibitory activity of enzymatic protein hydrolysates, immune-modulating activity of food protein and its protein hydrolysates has been gained interest recently. Many immunostimulating peptides have been isolated from enzymatic digests of various food proteins, including milk proteins (Keyser and Meisel, 1996; Eriksen, Vegarud, Langsrud, Almaas, and Lea, 2008), whey protein (Mercier, Gauthier, and Fliss, 2004; Gauthier, Pouliot, and Saint-Sauveur, 2006), casein (Stuknyte, Noni, Guglielmetti, Minuzzo, and Mora, 2011; Jiehui et al., 2014), fish protein (Duarte, Vinderola, Ritz, Perdigón, and Matar, 2006; Yang et al., 2009), pea protein (Ndiaye, Vuong, Duarte, Aluko, and Matar, 2012) and soy protein (Kong, Guo, Hua, Cao, and Zhang, 2008). These immunomodulatory peptides can enhance immune cell functions, measured as lymphocyte proliferation, natural killer (NK) cell activity, antibody synthesis, cytokine regulation, and nitric oxide (NO) production. The production of immunomodulating peptides can be achieved by several proteinases including Alcalase, thermolysin, pepsin, mixture of trypsin and chymotrypsin (Kong et al., 2008; Ndiaye et al., 2012; Saint-Sauveur, Gauthier, Boutin, and Montoni, 2008; Yang et al., 2009). Utilization of *Virgibacillus halodenitrificans* SK1-3-7 proteinase for producing protein hydrolysates derived from various protein sources with immunomodulating effects could be an alternative means to commercial proteinases.

There were several reports studying inflammation related immune responses of food compounds on monocytes and macrophages (Chanput, Mes, Vreeburg, Savelkoul, and Wichers, 2010; Ndiaye et al., 2012; Montaya-Rodríguez et al., 2014). Since monocytes and macrophages are an important parts of the innate arm of the immune

system. These cells are involved in inflammatory processes, with a profound capacity to synthesize and secrete pro- and anti-inflammatory cytokines (Ross, 1999). Due to either financial or ethical constraints, inevitably connected to animal and human *in vivo* studies, and to the specific mechanistic insights that can be obtained, *ex vivo* or *in vitro* experiments become more relevant for the development of specific applications. Although *ex vivo* systems have the advantage of their natural origin. However, donor viability and high individual variation can make analyses and interpretation of results more complex. Mouse or human cell lines, originating from cancerous cells, are important *in vitro* tools to study cellular functions, mechanisms and responses, as well as signaling pathways and nutrient and drug transport (Chanput, Mes, and Wichers, 2014). THP-1 cells, a human leukemia monolytic cell line, have widely used as *in vitro* model of human monocytes and macrophages in mechanistic studies of inflammatory diseases (Chanput et al., 2010). Thereby, the use of THP-1 cell line to investigate inflammation modulating effect of protein hydrolysates would be employed.

1.2 Research objectives

The objectives of this study were:

1. To investigate ACE inhibitory activity of protein hydrolysates derived from tilapia muscle proteins hydrolyzed by crude *Virgibacillus halodenitrificans* SK1-3-7 proteinases.
2. To assess stability and permeability of ACE inhibitory peptides of tilapia mince hydrolysate, based on *in vitro* intestinal epithelial cell model.
3. To isolate, identify and characterize the most potent ACE inhibitory peptides from tilapia mince hydrolysate.

4. To evaluate pH and thermal stability as well as ACE inhibitory activity after *in vitro* simulated gastrointestinal (GI) digestion, of the purified peptides.
5. To investigate inflammation-related immune responses of protein hydrolysates prepared from tilapia, casein, and pea protein, hydrolyzed by partially-purified proteinases from *V. halodenitrificans* SK1-3-7.

1.3 Research hypotheses

Virgibacillus halodenitrificans SK1-3-7 proteinases can hydrolyze tilapia muscle, yielding protein hydrolysate with ACE inhibitory activity. Different parts of muscle proteins, namely whole mince and washed mince which sarcoplasmic proteins were removed may yield a protein hydrolysate with different ACE inhibitory property. Protein hydrolysate after simulated GI digestion pass across Caco-2 cell monolayers could remain ACE inhibitory activity. ACE inhibitory peptides derived from tilapia muscle exhibit stability towards pH, heat treatments and *in vitro* pepsin-pancreatin simulated GI digestion. Partially-purified proteinases from *V. halodenitrificans* SK1-3-7 can be utilized to produce of protein hydrolysates with different immunomodulatory activity, depending on protein source.

1.4 Scope of the study

Whole mince, washed mince, and sarcoplasmic proteins from tilapia muscle were hydrolyzed by crude *V. halodenitrificans* SK1-3-7 proteinases. Degree of hydrolysis (DH), amino acid composition, and ACE inhibitory activity of protein hydrolysates were determined. Hydrolysate of whole mince showing the highest potent inhibitory activity was digested with pepsin and pancreatin to simulate GI digestion and

remaining ACE inhibitory activity after permeation across Caco-2 cell monolayers of *in vitro* digested hydrolysate was investigated. ACE inhibitory peptides were purified and amino acid sequences were identified by LC-MS/MS. Peptide synthesis of the identified peptides was carried out and their ACE inhibitory activity was tested. Partially-purified proteinases from *V. halodenitrificans* SK1-3-7 were used to hydrolyze tilapia muscle, casein, and pea protein. DH and inflammation-related immune responses on THP-1 macrophages were determined.

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CHAPTER II

LITERATURE REVIEWS

2.1 Fish muscle proteins

The main constituents of fish flesh are water (65-85%), protein (15-24%), fat (0.1-22%), carbohydrate (1-3%), and inorganic substances (0.8-2%). Protein composition of fish muscle varies according to the species, muscle type, feeding period, and spawning, and etc (Suzuki, 1981). Protein composition in the muscle can be classified into 3 main groups based on solubility as follows:

2.1.1 Myofibrillar proteins

Myofibrillar proteins are the major proteins as structural proteins in fish muscle. These proteins account for 65-75% of total protein in muscle, compared with 52-56% in mammals (Venugopal, 2009). These proteins can be extracted from the muscle tissue with neutral salt solutions of ionic strength above 0.15, usually ranging from 0.30-0.70. The myofibrillar proteins are related with the water holding capacity and other functional properties of proteins such as gelation, emulsification, and foaming (Foegeding, Lanier, and Hultin, 1996; Vareltzis, 2000). Contractile proteins which are different in size and location in the muscle, are listed in Table 2.1.

Table 2.1 Contractile proteins in food myosystems.

Protein	Relative abundance (%)	Size (kDa)	Location
Myosin	50-60	470	Thick filaments
Actin	15-30	43-48	Thin filaments
Tropomyosin	8-10	65-70	Thin filaments
Troponins	8-10		Thin filaments
Troponin-C		17-18	
Troponin-I		20-24	
Troponin-T		37-40	
C-protein	-	140	Thick filaments
α-Actin	-	180-206	Z-disc
Z-nin	-	300-400	Z-disc
Connective/Titin	5	700-1,000	Gap filaments
Nebulin	5	~600	N_2-line

Modified from: Ashie and Simpson (1997).

Myofibrillar proteins can be divided into 3 subgroups as follows:

2.1.1.1 Myosin

Myosin is the protein which forms the thick filament. A molecular weight is about 500,000 Daltons. It is the most abundant myofibrillar content. Myosin contains a total of six polypeptide chains, two heavy chains, and four light chains. The six polypeptides chains of myosin are assembled in quaternary structure that resembles a stick (tail) with two pear-shaped heads (Figure 2.1a). The tail regions consists of two alpha-helical heavy chains coiled together into a coiled-coiled alpha-helical supersecondary structure (Foegeding et al., 1996). This structure terminates at the head region. The main secondary structure in the head is alpha-helix, accounting for approximately 48% of amino acids. The myosin head contains the actin binding site,

ATPase site, alkaline light chains site, and DTNB [(5,5-dithiobis)-2-(nitrobenzoic acid)] light chain site. The light chains bind to the alpha-helical regions of the heavy chain. The tail portion of the heavy chain molecule is responsible for its association into thick filament (Foegeding et al., 1996).

2.1.1.2 Actin

The major protein of the thin filaments is actin, which comprises 20% of myofibrillar proteins of muscle. Normally, actin in muscle tissue is associated with troponin and tropomyosin complex. It also contains a myosin binding site, which allows myosin to form temporary complex with it during muscle contractile or the permanent myosin-actin complex rigor mortis in postmorterm (Xiong, 1997). Actin consists of two peanut shaped domains of equal size lying side-by-side. Actin monomers, called globular actin or G-actin, are assembled in a double-helical structure called fibrous actin, or F-actin (Figure 2.1b). G-actin has a molecular mass of 42,000-48,000 Daltons. It binds ATP very firmly and, in the presence of Mg^{2+}, spontaneously polymerized to form F-actin. It also polymerizes in the presence of neutral salts at a concentration of approximately 0.15 M. Filaments of F-actin interact with the head portion of myosin (Foegeding et al., 1996).

2.1.1.3 Troponin

Troponin accounting for 8-10% of myofibrillar proteins consists of three subunits designated troponin C; which is a calcium binding protein and confers calcium regulation to the contractile process via the thin filament, troponin I; which strongly inhibits ATPase activity of actomyosin, and troponin T; which provides a strong association site for binding of tropomyosin (Foegeding et al., 1996).

2.1.1.4 Tropomyosin

Tropomyosin represents approximately 8-10% of myofibrillar protein. It is composed of two alpha-helical polypeptides wound together into a two-stranded coiled-coiled supersecondary structure. It resembles the tail or rod portion of the myosin molecule. In skeletal muscle, two polypeptides, alpha- and beta- tropomyosin polypeptides have molecular masses of 37,000 and 33,000 Daltons, respectively. Tropomyosin aggregates end-to-end and binds to actin filaments along each groove of the actin double helix such that each molecule interacts with seven G-actin monomer (Foegeding et al., 1996).

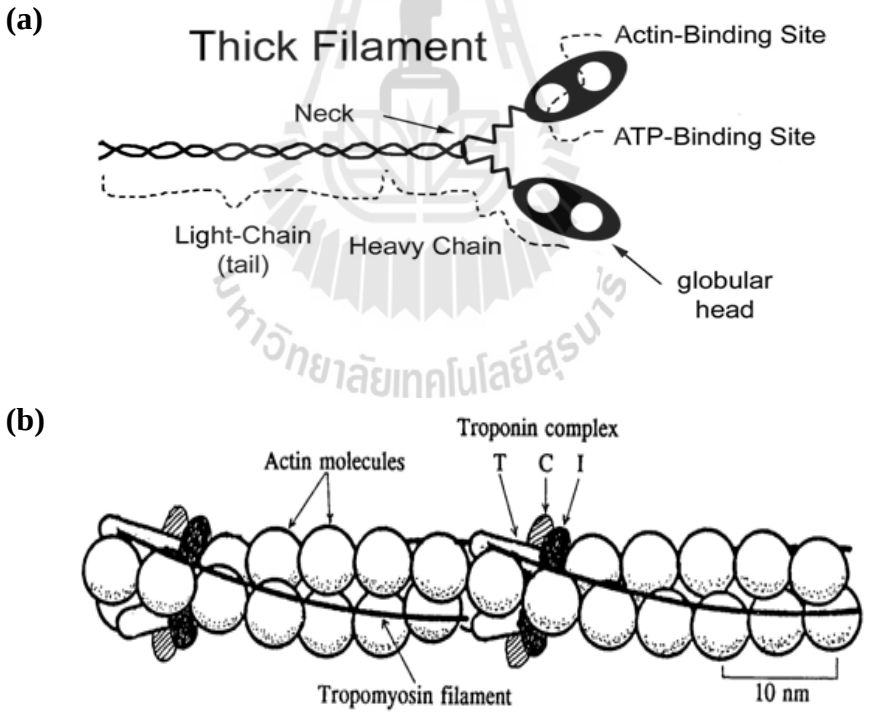

Figure 2.1 (a) The structure of myosin thick filament; (b) Arrangement of actin, troponin, and tropomyosin.

From: www.rohan.sdsu.edu

2.1.2 Sarcoplasmic proteins

Sarcoplasmic proteins are globular in tertiary structure and are commonly called myogens. Fish sarcoplasmic proteins account for 20-40% of the total muscle proteins depending on the species (Okada, 1999). Sarcoplasmic proteins are extracted by homogenizing the muscle tissue with water or neutral salt solutions of ionic strength below 0.15. Table 2.2 showed the sarcoplasmic protein contents of different fish species. Sarcoplasmic proteins comprise several types of proteins including enzymes and heme proteins (Lanier, Carvajal, and Yongsawatdigul, 2005). The heme proteins of red muscle and blood which are responsible for the pigmentation of muscle are myoglobin and hemoglobin, respectively. Much of the heme pigment can be easily removed by separation of dark and light muscles before deboning and washing process or by the conventional washing process. Some of the sarcoplasmic proteins are enzymes. They are mainly enzymes associated with energy-producing metabolism such as creatine kinase, aldolase, and glyceraldehydes-3 phosphate dehydrogenase. However, those proteins have only partially been characterized except for parvalbumin containing large quantities of calcium binding proteins which is common to freshwater fish species (Nakagawa, Watabe, and Hashimoto, 1988).

The sarcoplasmic protein patterns of marine fish differed from those of freshwater fish. Bottom fish (red sea bream), pelagic fish (Pacific mackerel) and freshwater fish (carp) showed similar patterns consisting of 10 components. Molecular weights were estimated to be 94, 60, 49-51, 43, 40, 35, 33, 26, 25, and 23 kDa. The patterns in three major components of sarcoplasmic proteins differed from one fish to another: pelagic fish rich in 43, 40 and 35 kDa, bottom fish rich in 43 and 35 kDa and freshwater fish rich in 43 kDa (Nakagawa et al., 1988).

Sarcoplasmic proteins of sea bass extracts resulted in 13 major protein bands. The SDS-PAGE of those proteins showed molecular weight ranging 97 to 12 kDa. The most abundant protein bands were 97 kDa, a doublet band at 60 kDa, a 51 kDa was assumed to be enolase, a 41-39 kDa huge band that were assumed to be creatine kinase and aldolase, respectively, a 36 kDa was assumed to be glyceraldehydephosphate dehydrogenase (GAPDH), a 34, 27, 25, 21.5, and 17 were supplementary bands. Sarcoplasmic proteins with molecular mass of 13 and 12 kDa could be parvalbumins (Ladrat, Verrez-Bagnis, Noel, and Fleurence, 2003). Sarcoplasmic proteins of mackerel include phosphorelase (94 kDa), enolase (50 kDa), creatine kinase (43 kDa), aldolase (40 kDa) and GAPDH (35 kDa) (Toyohara, Murata, Ando, Kubota, Sakaguchi, and Toyohara, 1999).

Table 2.2 Amount of sarcoplasmic and myofibrillar proteins in different fish (protein nitrogen (mg) in fish meat (g)).

Fish species	**Sarcoplasmic proteins**	**Myofibrillar proteins**
Yellowtail (*Seriola quinqueradiata*)	8.4-8.6	8.9-10.6
Red barracuda (*Sphyraena pinguis*)	7.1	10.7
Mackerel (*Scomber japoincus*)	6.4-8.8	8.6-9.9
Horse mackerel (*Trachurus japonicus*)	5.0-5.6	7.8-8.1
Anchovy (*Engraulis japonica*)	3.8	4.3
Flatfish (*Kareius bicoloratus*)	4.4	7.5
Lizardfish (*Saurida undosquamis*)	5.6	10.8
Grub fish (*Neopercis sexfasciata*)	4.9	11.4
Sea bass (*Steriolepsis ischinagi*)	4.2-5.7	8.3-11.3

From: Haard, Simpson, and Sun Pan (1994).

2.1.3 Stroma proteins

Stroma proteins or connective tissue, which on average account for 3% (w/w) of the total muscle protein, consist predominately of collagen, with the remainder comprising of elastin and keratin (Belitz, Grosch, and Schieberle, 2004; Venugopal, 2009). It cannot be extracted by water, acid, or alkali solution and neutral salt solution of 0.01-0.1 M. Collagen can also be found as a major structural protein in fish skin, bones and scales (Kim and Mendis, 2006). This triple helix protein and its partially hydrolyzed coiled form, gelatin, are rich in non-polar amino acids such as glycine, valine, alanine, proline and hydroxyproline (Kim and Mendis, 2006; Vercruysse, Camp, and Smagghe, 2005). Moreover, the primary sequence of these proteins contain repeated glycine-proline-hydroxyproline-glycine-A-A amino acid sequences (Vercruysse et al., 2005). At least 12 different forms have been identified, each having a unique sequence of amino acids. In its secondary structure, the molecular chain is arranged as a left-handed helix and three of these combine to form a right-handed super helix, called tropocollagen. The tropocollagen molecules assemble to form fibrils and these aggregate to form fibers (Shoulders and Raines, 2009).

2.2 *Virgibacillus* sp.

2.2.1 Morphology and physiology

The genus *Virgibacillus* was firstly proposed by Heyndrickx et al. (1998) and reclassified from *Bacillus pantothenticus* on the basis of amplified DNA restriction analysis, fatty acid profiles, SDS-PAGE pattern of whole-cell proteins and phenotypic characteristics. This genus could be distinguished from members of *Bacillus* rRNA group 1 and from members of *Paenibacillus* and other aerobic endospore-forming

bacteria by routine phenotypic tests. Two species of *Salibacillus* were also reclassified to *Virgibacillus* based on of genotypic and phenotypic characteristics (Heyrman et al., 2003). Additionally, Yoon, Oh, and Park (2004) also proposed to rename *Bacillus halodenitrificans* to *Virgibacillus halodenitrificans* based on 16S rRNA gene comparisons. Bacteria in the genus *Virgibacillus* are Gram-positive rods (0.3-0.7 × 2.0-6.0 µm). Cells arrangement occurred singly, pair, short or long chain. Colony was small, circular, low convex, and slightly transparent-to-opaque. They are aerobic, catalase-positive, motile, and spore forming. *Virgibacillus* bacteria grow at pH 6.0-10.0 (optimum at 7.5-8.0), and 10-55°C (optimum at 30-40°C). Members of this genus are moderately halophilic bacteria able to grow at 0-25% (w/v) NaCl with optimum of 5-10% NaCl (Phrommao, 2010).

2.2.2 Proteinase from *Virgibacillus*

This genus from various sources has been reported to produce a variety of extracellular hydrolytic enzymes. For example, *Virgibacillus marismortui* NB2-1 isolated from Pla-ra, fermented fish in Thailand, secreted five proteinases with molecular weight ranging from 17 to 35 kDa. The enzyme were alkaline serine proteinases and showed optimum catalytic activity at pH 10, 50°C, and 5% NaCl (Chamroensaksri, Akaracharanya, Visessanguan, and Tanasupawat, 2008). Alkaline serine proteinase was produced by *Virgibacillus pantotheticus* MTCC 6729 isolated from fresh chicken meat samples, at 40°C at pH 9.0. The enzyme was thermostable alkaline by retaining its 100% and 85% stability and at 50°C, respectively (Gupta, Joseph, Mani, and Thomas, 2008).

Sinsuwan, Rodtong, and Yongsawatdigul (2007, 2008a, 2008b, 2010a, 2010b, 2012) characterized *Virgibacillus* sp. SK33 and SK37 proteinases isolated from one-

month-old Thai fish sauce. For *Virgibacillus* sp. SK33 proteinases, crude extracellular enzyme showed a subtilisin-like alkaline serine proteinase characteristic with optimal catalytic condition at 50°C and pH 8-11. Enzyme with molecular weight of 19 and 32 kDa were purified and showed NaCl- and $CaCl_2$-activated characteristics. In addition, the 32 kDa enzyme exhibited a high stability in various organic solvents at concentrations up to 25% (v/v) including dimethylsulfoxide, methanol, acetonitrile, and ethanol. *Virgibacillus* sp. SK37 was found to possess both extracellular and cell-bound proteinases which were subtilisin-like alkaline serine proteinase with maximal activity at 65°C and pH 7-9.5. Moreover, the cell-bound proteinases from *Virgibacillus* sp. SK37 with molecular weight of 19, 20, 22, 32, 34, and 44 kDa showed proteolytic activity in either the absence or presence of 10 and 25% NaCl toward fish muscle, soy protein isolate, and casein substrates.

Montriwong, Kaewphuak, Rodtong, Roytrakul, and Yongsawatdigul (2012) found that *Virgibacillus* sp. SK1-3-7 exhibited the highest fibrinolytic activity among 25 bacteria isolates obtained from Thai fish sauce fermentation. Results of 16S rRNA gene sequence analysis showed 99% homology to *Virgibacillus halodenitrificans* ATCC 49067. Therefore, it was identified as *V. halodenitrificans* SK1-3-7. The enzymes with molecular weight of 20 and 36 kDa showed fibrinolytic activity on a fibrin zymogram and showed NaCl and $CaCl_2$-activated characteristics. The enzyme showed high stability at a wide pH range of 4-10 and over a wide range of NaCl concentration of 0-2 M. In addition, the enzyme was completely inhibited by phenylmethanesulfonyl fluoride (PMSF) and preferably hydrolyzed Suc-Ala-Ala-Pro-Phe-pNA, suggesting that it is a subtilisin-like alkaline serine proteinase. Besides commercial proteinases, other microbial proteinases used for the production of

bioactive peptides have rarely been reported. Due to broad substrate specificity of *Virgibacillus* proteinases, angiotensin I-converting enzyme (ACE) inhibitory peptides could be obtained through hydrolytic reaction of *V. halodenitrificans* SK1-3-7 proteinase.

2.3 Enzymatic hydrolysis of food proteins

Enzymatic protein hydrolsates are made by breaking the peptide bonds in proteins to peptides with varying sizes and amino acids. This is achieved by the use of proteolytic enzymes. Up to now, many researchers have started focusing their attention on the nutraceutical properties of protein hydrolysates. It has been recognized that dietary proteins provide a rich source of biologically active peptides. Such peptides are inactive within the sequence of the parent protein and the most common way to release bioactive peptides is through enzymatic hydrolysis (Korhonen and Pihlanto, 2006). He et al. (2007) reported that the hydrolysate obtained by digestion of shark meat with protease SM98011 showed much higher ACE inhibitory activity with IC_{50} value of 0.4 mg/mL, comparing to the untreated shark slurry with IC_{50} value of 10.5 mg/mL.

Although the protein hydrolysates can also be obtained from acid or alkaline hydrolysis, the enzymatic hydrolysis is preferred in food and pharmaceutical products because of a lack of residue organic solvents or toxic chemicals in the products (Kim and Wijesekara, 2010). Enzymatic hydrolysis is carried out under mild conditions of pH and temperature, avoiding the extremes usually required for chemical and physical treatments, and minimizing side reactions. The overall amino acid composition of enzymatic protein hydrolysates is similar to that of the starting material (Clemente, 2000). In parallel, the chemical hydrolysis tends to be a difficult process to control and

yield of products with reduced nutritional qualities. The chemical hydrolysis can destroy L-form amino acids, producing D-form amino acids which are not absorbed by human. In addition, the formation of lysinoalanine, ornithioalanine, lanthionine, and β-amino alanine can be occurred and may lead to the formation of toxic substances (Clemente, 2000). Another drawback of chemical hydrolysis is the destruction of tryptophan, which is an essential amino acid (Kristinsson and Rasco, 2000). Therefore, the enzymatic hydrolysis of food proteins is practiced for the improvement of nutritional characteristics, retarding deterioration, removal of toxic or inhibitory ingredients, and modification of functional properties such as solubility, emulsification, foaming (Damle, Jamdar, and Harikumar, 2006). The biological activities of protein hydrolysates depend on protein substrate, the specific of the enzyme used for proteolysis, the condition used during hydrolysis, and the degree of hydrolysis (Balti, Nedjar-Arroume, Bougatef, Guillochon, and Nasri, 2010).

2.3.1 Protein substrate

Various food proteins have been used as a source of substrate to produce protein hydrolysate with different biological and functional properties, including both animal and plant proteins. For instance, peptides with immunomodulatory and opioid activities have been derived from rice, soybean, wheat, and milk protein sources (Korhonen, 2009; Kitts and Weiler, 2003). In addition, ACE inhibitory peptides have been produced from the hydrolysis of milk, chicken, bovine, wheat, corn, and fish proteins (Arihara, Nakashima, Makai, Ishikawa, and Itoh, 2001; Chen, Wang, Zhong, Wu, and Xia, 2012; Chiang, Tsou, Tsai, and Tsai, 2006; Ferreira et al., 2007; Wijesekara, Qian, Ryu, Ngo, and Kim, 2011; Zhu et al., 2007). The difference among proteins is their primary amino acid sequence. A number of the studies have demonstrated a good correlation between

certain amino acid residues on biofunctional activities of protein hydrolysates. For example, Chen et al. (2012) reported that peptides derived from many protein sources with increased hydrophobicity showed antioxidative and ACE inhibitory properties. Peptides with high contents of His, Ala, Val, Phe, and Leu have been reported to possess strong radical scavenging activity. The hydrophobic and aromatic amino acids in egg white protein hydrolysate showed stronger antioxidant and ACE inhibitory activities (Chen et al., 2012).

2.3.2 Proteinase

Proteinases can be classified, based on their source (animal, plant, and microbial), mode of catalytic action (endopeptidases or exopeptidases), and nature of active site residues involved in mechanism. Endopeptidases cleave the amide bond within the protein or peptide chain, whereas, exopeptidases remove terminal amino acid either at the C-terminus (carboxydases) or at the N-terminus (aminopeptidases). Based on the nature of the active site, proteinases can be classified as aspartic proteinases (e.g. cathepsin D and E, pepsin, chymosin, and renin), cysteine proteinases (e.g. cathepsin B, L, S, K, Q, papain, bromelain, and ficin), metalloproteinases (e.g. gelatinases A and B, collagenase, termoase, neutrase, and thermolysin), serine proteinases (e.g. plasmin, trypsin, chymotrysin, subtilisin, alcalase, and elastase), and threonine proteinases (e.g. proteasome) (Alder-Nissen, 1993; Beynon and Bond, 2001). These enzymes differ in their amino acid specificity. The pH specificity of proteinases depends on the group of the active site; cysteine and metallo proteinases are active at neutral pH, whereas, serine and aspartic proteinases are active at alkaline and acidic pH, respectively (Alder-Nissen, 1993). Table 2.3 shows some commercial proteinases used for protein hydrolysate production.

Table 2.3 Examples of commercial proteinases used for hydrolysis of food proteins.

Enzyme	Temperature (°C)	pH	Source	Specificity
Alcalase 2.4 L FG	55-70	6.5-8.5	*B. licheniformis*	Endopeptidase
Neutrase 0.5 L	45-55	5.5-7.5	*B. subtilis*	Endopeptidase
Protamex	35-60	5.5-7.5	-	Proteinase complex
Alkaline proteinase	60	9-10	*B. licheniformis*	Endopeptidase
Bromelain	50-60	3-9	Pineapple stem	Peptidase
Papain	65-80	5-7	*Carica papaya*	Peptidase
Corolase 7089	< 60	5-7.5	*B. subtilis*	Endopeptidase
Corolase N	< 60	5-7.5	*B. subtilis*	Endopeptidase
Corolase PN-L	< 50	5-8	*A. sojae*	Endo-,exopeptidase
Corolase LAP	< 70	6-9	*A. sojae*	Endopeptidase
Validase TSP	45-55	6.5-8	*B. subtilis*	Endopeptidase
Flavourzyme	50	7.0	*A. oryzae*	Endo-,exopeptidase

Modified from: Gilmartin and Jervis (2002).

2.3.3 Degree of hydrolysis

Degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds broken to the total number of peptide bonds in a given type of protein (Alder-Nissen, 1986), can then be quantified by a variety of different techniques. These include monitoring the reaction of free primary amino groups with trinitrobenzenesulfonic acid (TNBS) or *o*-phthaldialdehyde (OPA), or monitoring the amount of base consumed to keep the hydrolysis reaction at a constant pH (Alder-Nissen, 1986). Alternatively, Hoyle and Merritt (1994) have expressed DH as the percent ratio of 10% trichloroacetic acid soluble nitrogen after hydrolysis to the total nitrogen in the sample as determined by Kjeldahl analysis. DH is one of the basic parameters that describes the properties of protein hydrolysates and needs to be controlled during protein hydrolysis. DH affects size and amino acid composition of the peptides, which are closely related to functional,

biological, and sensory properties (Cheison, Zhang, Wang, and Xu, 2009; Kristinsson and Rasco, 2000).

Jamdar et al. (2010) studied functional properties, antioxidant and angiotensin-converting enzyme (ACE) inhibitory activities of peanut protein hydrolysates (PPHs) prepared using Alcalase, at different DHs of 10%, 20%, 30%, and 40%. As DH increased, ferrous ion chelating activity, DPPH radical-scavenging activity and ACE inhibitory activity of PPH increased. The highest ACE inhibitory activity was found in PPH at 40% DH. Thus, DH affects functional properties, antioxidant and ACE inhibitory activities of peanut protein.

Raghavan and Kristinsson (2009) studied the *in vitro* ACE inhibitory activity of tilapia protein hydrolysates produced by Cryotin-F and Flavourzyme at different of DH (7.5% and 25%). Results showed that both Cryotin and Flavourzyme hydrolysates with 25% DH gave maximum ACE inhibitory activity, indicating that low molecular weight peptides are better ACE inhibitors than high molecular weight counterparts.

Balti et al. (2010) investigated the effect of DH on ACE-inhibitory activity of cuttlefish (*Sepia officinalis*) muscle protein hydrolysate. The undigested cuttlefish protein exhibited low ACE-inhibitory activity (about 1.2%). The DH and ACE-inhibitory activity of protein hydrolysate increased with the increase of hydrolysis time. Higher ACE inhibition activity was obtained at 8% DH.

2.4 Hypertension and ACE

Hypertension or high blood pressure is defined as a systolic blood pressure ≥ 140 mm Hg and a diastolic blood pressure ≥ 90 mm Hg (Ahhmed and Muguruma, 2010). Hypertension is a chronic disease affecting 30% of the adult population in the

world (Majumder and Wu, 2009), and is one of the major risk factors for stroke coronary heart disease (CHD), kidney dysfunction, and myocardial infarction (Li, Le, Shi, and Shrestha, 2004). It is often called a silent killer because persons with hypertension are often asymptomatic for years (Sheih, Fang, and Wu, 2009). Nowadays, hypertension is mainly treated by lifestyle modification and pharmacological treatment with antihypertensive drugs (Hermansen, 2000). While there are many causes of hypertension, it is well recognized that angiotensin I-converting enzyme (ACE) plays important roles in renin-angiotensin and kallikrein-kinin systems for the regulation of blood pressure as well as fluid and salt balance in mammals (Vercruysse et al., 2005).

2.4.1 Renin angiotensin system and kallikrein-kinin system

The rennin-angiotensin system (RAS) is the primary pathway for regulating blood pressure and vascular tone (Daien et al., 2002). The RAS pathway is initiated in the kidney when blood pressure falls with the conversion of prorenin zymogen to the active form, renin. Thereafter, renin is released into the blood stream where it functions in cleaving the N-terminus region of angiotensinogen to release a decapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu), angiotensin-I. This process occurs slowly serving as the first and rate-limiting step that determines the speed of blood pressure regulation by the RAS pathway. Angiotensin-I circulates in the blood until its C-terminus dipeptide His-Leu is cleaved by angiotensin I-converting enzyme (ACE) to form an octapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), angiotensin-II, which acts as a potent vasoconstrictor. Angiotensin-II binds its cell surface receptors to trigger a process that results in the secretion of aldosterone from adrenal glands, leading to an increase in reabsorption of salt and water, and increase in blood pressure by arterial

constriction (Chen et al., 2009; Udenigwe and Mohan, 2014). ACE also inactivates the vasodilator bradykinin, blood pressure lowering nonapeptide in the kallikrein-kinin system, leading to attenuation of the vasodilatory function of bradykinin (Figure 2.2) (Li et al., 2004; Wang et al., 2008). Inhibition of ACE has proven to be an effective strategy in prevention and treatment of hypertension and related diseases.

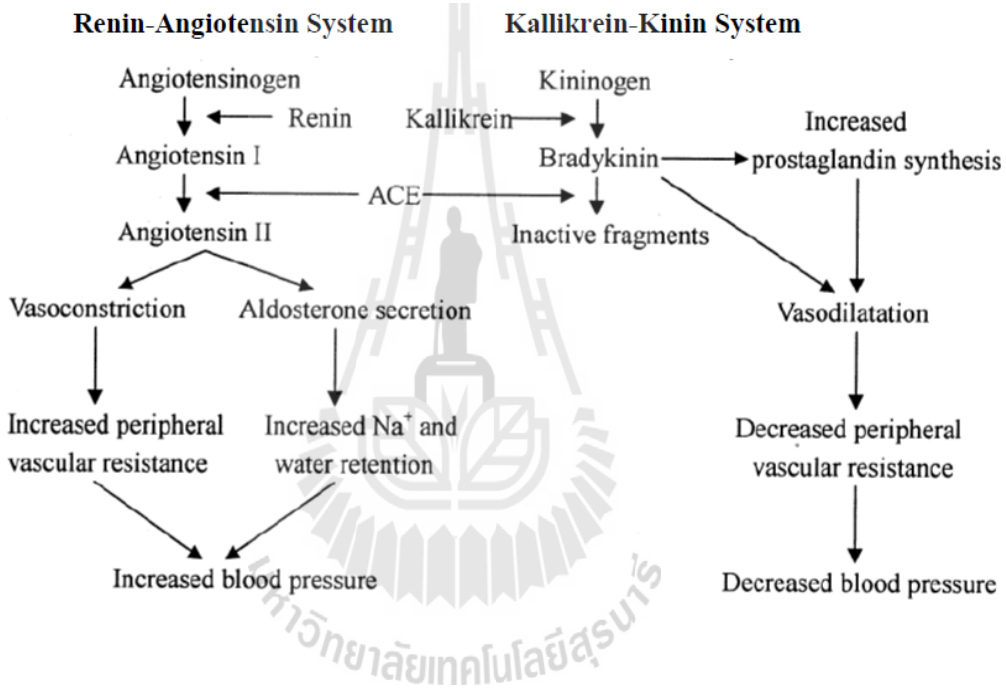

Figure 2.2 Role of angiotensin I-converting enzyme in blood pressure regulation.

From: Li et al. (2004).

2.4.2 Properties and form of ACE

ACE (dipeptidyl-carboxypeptidase, kinase II, EC. 3.4.15.1) is classified as a zinc- and chloride-dependent metallopeptidase (Li et al., 2004). ACE is anchored to the plasma membrane through a single C-terminal transmembrane domain and is oriented in such a way that the catalytic sites are exposed on the extracellular surface of the cell

(Figure 2.3). In mammals, two distinct ACE isoenzymes have been described, an abundant somatic form (170 kDa) found on the endothelial surfaces of the lungs and on brush border membranes of kidneys, intestine, placenta and choroid plexus, and the smaller germinal or testicular form of ACE (100 kDa) found only in the testis (Soubrier et al., 1993). Both ACE isoforms function as ectoenzymes which hydrolyze circulating peptides. While germinal ACE has a crucial role in fertility, tissue bound somatic ACE controls both blood pressure and renal structure and function. A soluble form of ACE, which is derived from the membrane form through the action of a secretase, is also present in serum and other body fluids, but its biological significance is not clarified (Turner and Hooper, 2002). All forms of ACE are heavily glycosylated and it is suggested that various patterns of glycosylation modulate the substrate specificity of ACE (Sturrock, Natesh, Van Rooyen, and Acharya, 2004). Consequently, ACE is one of the glycoproteins that comprise the endothelial cell's unstirred layer.

Somatic ACE is composed of two highly homologous domains, the N-domain and the C domain, suggesting a gene duplication event in the course of evolution. Each domain contains the typical zinc-binding motif, His-Glu-X-X-His, at the active site, which is found in many zinc peptidases. The two histidine residues provide two of the three zinc-coordinating ligands and the carboxyl group of glutamate is the base donor in the catalytic reaction. The third zinc co-ordinating residue is another glutamate. The testicular ACE sequence corresponds to the C-domain of somatic ACE and therefore contains only one active site and one zinc-binding motif (Sturrock et al., 2004).

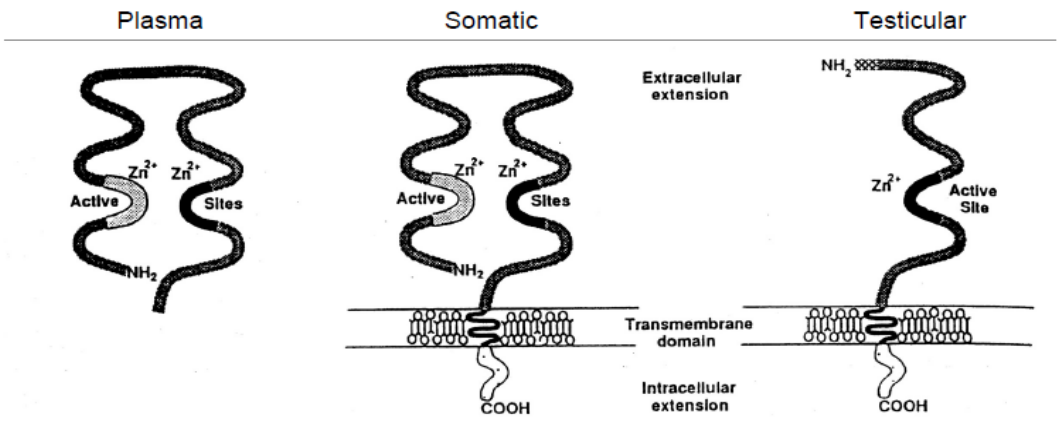

Figure 2.3 Structure and conformation of the angiotensin I-converting enzyme derived from plasma, somatic cell origin and testis, showing the active catalytic sites, zinc dependency and the N- and C-terminal ends.

From: Johnston (1992).

ACE is an unusual zinc-containing exopeptidase that cleaves dipeptide not only from the C-terminal end of angiotensin I but also a variety of peptide substrates including substance P, enkephalins, neurotensin, and luteinzing hormone releasing hormone (LHRH) (Raizada, Ian Phillips, and Colin Sumners, 1993). Table 2.4 indicates the peptide substrates of ACE. It is evident that peptides can serve as substrates for the enzyme. Note that tripeptide and dipeptide products are cleaved by ACE from substance P, LHRH, and enkephalins. Early studies indicated that ACE was a peptidase similar to pancreatic carboxypeptidase A and that both enzymes were zinc-containing metalloproteins since both were inhibited by cyanide and ethylene diamine tetraacetate (EDTA) (Bunning, Holmquist, and Riordan, 1978).

Table 2.4 Peptide substrates of ACE.

Peptides	**Amino acid sequence**
Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Agr
des-Arg^{9}-bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe
Enkephalins:	
Pentapeptide	Tyr-Gly-Gly-Phe-Met
Heptapeptide	Tyr-Gly-Gly-Phe-Met-Arg-Phe
Octapeptide	Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu
β-Neo-endorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro
Dynorphin$_{1-8}$	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile
Dynorphin$_{1-6}$	Tyr-Gly-Gly-Phe-Leu-Arg
Neutrotensin	<Gly-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (4 : 1)
LHRH	<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

Arrows denote primary (↑) and secondary (⇡) sites of cleavage by ACE.

From: Raizada et al. (1993).

ACE has three hydrophobic (S1, S1´, and S2´) and binding Zn^{2+} active sites together with ACE inhibitor in its binding site. The zinc ion of ACE is located between subsites S1 and S1' to participate in hydrolytic cleavage on the peptide bond of the substrate, resulting in a release of the dipeptide product. Hydrogen bond donating group are located between subsite S1' and S2' which can form ionic interaction with the

C-terminal of the substrate (Ondetti and Cushman, 1982). Monovalent anions, particularly chloride ions, can enhance the activity of ACE. The activation of the C-domain is highly dependent on chloride concentration, whereas the activation of the N-domain is not chloride-dependent (Wei, Clauser, Aslhenc, and Corvol, 1992).

2.5 *In vitro* ACE inhibitory assay

The search for *in vitro* ACE inhibitors in the most common strategy followed in the selection of potential antihypertensive peptides derived from food proteins. *In vitro* ACE inhibitory activity is generally obtained by monitoring the conversion of an appropriate substrate by ACE in the presence and absence of inhibitors. In order to facilitate the identification and isolation of ACE inhibitory peptides, establishment of a simple, sensitive and reliable *in vitro* ACE inhibition assay is desirable. Numerous methods for the measurement of ACE inhibitory activity have been reported, including spectrophotometric (Cushman and Cheung, 1971; Li, Liu, Shi, and Le, 2005; Shalaby, Zakora, and Otte, 2006; Vermeirssen, Camp, and Verstraete, 2002), fluorometric (Persson and Wilson, 1977; Sentandreu and Toldrá, 2007), radiochemical (Ryan, Chung, Ammons, and Carlton, 1977), high-performance liquid chromatography (HPLC) (Lahogue, Réhel, Taupin, Haras, and Allaume, 2010; Mehanna and Dowling, 1999; Wu, Aluko, and Muir, 2002), and capillary electrophoresis methods (Wu et al., 2008; He et al., 2007). However, spectrophotometric and HPLC assays are employed most frequently. From the ACE activity in the absence and the presence of an inhibitor, the percent ACE inhibition can be deduced. When this is done for different concentrations of inhibitor, the IC_{50} value, which is the concentration of inhibitor

needed to reduce the ACE activity to half of its initial value, can be calculated. The ACE inhibitory activity is usually expressed as IC_{50} value.

However, there is currently no standard method or substrate for measuring ACE activity *in vitro*. This makes absolute comparison of hydrolysate IC_{50} values between studies difficult due to differences in the K_m of the synthetic substrate used. Even between studies employing the same substrate, the kinetics of the ACE reaction may be altered by different enzyme/substrate ratios, buffer conditions, and inhibitor concentrations. Nonetheless, IC_{50} values, regardless of the assay employed, still give valuable information regarding the relative inhibitory activity of the sample compared to internal study standards such as ACE inhibitory nutraceutical products (PeptACE®) or ACE inhibitory drugs (Captopril®), as well as allow for comparison of the relative effects of fractionation processes.

The precedent-setting publication by Cushman and Cheung (1971), outlining a spectrophotometric assay and the properties of ACE from rabbit lung, still provides the foundation for most of the ACE inhibitory activity assays used today. This method is based on the hydrolysis of the synthetic substrate hippuryl-L-histidyl-L-leucine (HHL) by ACE to hippuric acid (HA) and His-Leu (Figure 2.4), and the extent of HA released is measured after extraction with ethyl acetate by its absorbance at 228 nm. As such, ACE inhibitory activity of an added sample can be determined by the reduction in absorbance compared to a control. However, extraction of reaction product is tedious and may overestimate ACE activity if unhydrolyzed HHL is also extracted.

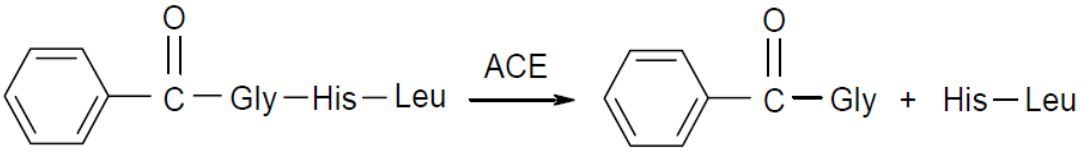

Figure 2.4 ACE catalysed reaction in the method of Cushman and Cheung (1971) with substrate HHL.

From: Vermeirssen (2003).

In order to eliminate the extraction step, Hayakari, Kondo, and Izumi (1978) developed the method of which is based on the specific colorimetric reaction of liberating HA with 2,4,6-trichloro-*s*-triazine (TT) and absorbance read at 382 nm. Serra, Còrtes, Lombardi, Braga de Oliveira, and Braga (2005) described the method based on the cleavage of the substrate hippuryl-glycine-glycine by ACE and subsequent reaction with trinitrobenzenesulfonic acid (TNBS) to form 2,4,6-trinitrophenyl-glycyl-glycine, whose absorbance is determined at 415 nm. However, due to possible elevated absorbance reading resulting from reaction of the colorimetric reagents with amino groups, these methods are not suitable for peptide-based ACE inhibitory samples. A recent method by Li et al. (2005) employed the colorimetric reaction of HA with benzene sulfonyl chloride in the presence of quinoline to quantify ACE inhibition at 492 nm. This method may eliminate the interferences of HHL and peptide-based samples. Another approach is to separate and quantify the HA product from the unhydrolyzed HHL and other interfering compounds by reverse phase (RP)-HPLC (Hernández-Ledesma, Contreras, and Recio, 2011; Mehanna and Dowling, 1999). Moreover, HPLC can be coupled with electrospray-mass spectrometry, that allows the screening of complex food samples (Xiao, Luo, Chen, and Yao, 2006).

Another substrate for ACE is 2-furanacryloyl-L-phenylalanyl-L-glycyl-L-glycine (FAPGG). FAPGG is hydrolyzed to FAP and Gly-Gly, resulting in a decrease in absorbance at 340 nm (Figure 2.5).

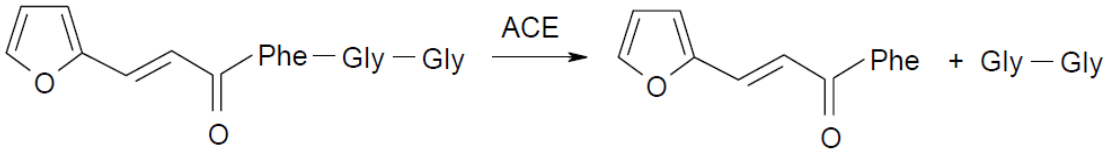

Figure 2.5 ACE catalyzed reaction with substrate FAPGG.

From: Vermeirssen (2003).

In comparing assays of different substrates, Shalaby et al. (2006) found that the IC_{50} of tryptic whey protein isolate digest, when assayed using FAPGG as ACE substrate, was one order of magnitude higher than that found when using HHL. Thus, FAPGG results are more conservative in terms of estimating ACE inhibitory potency. Nevertheless, modifications of Cushman and Cheung (1971) method still remain the most widely used, possibly because cleavage of HA from HHL has been made to mimic the dipeptidase activity of ACE *in vivo* (Meisel, Walsh, Murray, and FitzGerald, 2006), facilitating results comparison.

2.6 *In vivo* antihypertensive assay

The antihypertensive effects can be assessed by *in vivo* experiments using spontaneously hypertensive rats (SHRs) (FitzGerald, Murray, and Walsh, 2004). A great number of studies have addressed the effects of both short-term and long-term administration of potential antihypertensive peptides using this animal model

(Matsufuji et al., 1995; Nakashima et al., 2002; Sipola, Finckenberg, Korpela, Vapaatalo, and Nurminen, 2002; Wang et al., 2008). The change in blood pressure is monitored in conscious and anesthetized rats by the tail-cuff method (Widdop and Li, 1997) or via a catheter in the artery, after administration of the product orally, intravenously or intraperitoneally (Fujita and Yoshikawa, 1999). Normally, ACE inhibitory peptides only produce an antihypertensive effect in SHRs, while no effect is observed in normotensive or Wistar Kyoto (WKY) rats. The results of these tests have highlighted an important lack of correlation between the *in vitro* ACE inhibitory activity and the *in vivo* action. This fact has provided doubts on the use of the *in vitro* ACE-inhibitory activity as the exclusive criteria for potential antihypertensive substances, since physiological transformations may occur *in vivo,* and because other mechanisms of action than ACE inhibition might be responsible for the antihypertensive effect (Hernández-Ledesma et al., 2011).

The antihypertensive effect of some food-derived peptides has been conducted in human studies to determine whether these peptides possess an antihypertensive effect on human subjects with high-normal blood pressure and mild hypertension (Fujita, Yamagami, and Ohshima, 2001; Kawasaki et al., 2000). The most substantiated antihypertensive activity in humans has been obtained for the commercial fermented milk products and hydrolysates containing the ACE-inhibitory peptides IPP and VPP after long term administration. The antihypertensive effect of the sour milk product Calpis®, commercialized in Japan, was tested in a clinical study with mildly hypertensive patients (Hata et al., 1996). Recently, a study has been conducted among patients with high-normal blood pressure and mild hypertension, evaluating the effect

of different doses of a casein hydrolyzate produced by *Aspergillus oryzae* containing IPP and VPP, and commercialized as AmealPeptide® by Calpis (Mizuno et al., 2005).

2.7 Commercially synthetic ACE inhibitors

ACE inhibitors were first discovered in snake venom of *Bothrops jararaca* in 1970. These ACE inhibitors contained 5-13 amino acid residues per molecule, and most of them had a C-terminal sequence of Ala-Pro or Pro-Pro, in which the nonapeptide SQ 20881 (Tiprotide) with the amino acid sequence Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro was the most effective for lowering blood pressure in animal models of hypertension, and was eventually shown to be effective for treatment of human hypertension when administered parenterally (Li et al., 2004). The key idea in the development of oral ACE inhibitors was that ACE is similar to carboxypeptidase A and the construction of a hypothetical model of ACE with the zinc atom appropriately positioned to induce vulnerability of the penultimate peptide bond (Figure 2.6). These efforts culminated in the development of captopril (SQ14225, *D*-3-mercapto-2-methylpropanyl-proline), the first orally active inhibitor by Ondetti, Rubin, and Cushman (1977).

Currently, specific inhibitors of ACE are one of several classes of pharmacological agents that have been widely used to treat hypertension, congestive heart failure and myocardial infarction, endothelial dysfunction and renal disease (Turner and Hooper, 1999). ACE inhibitory drugs are considered as competitive and reversible, slow and tight-binding inhibitors of ACE (Cushman and Ondetti, 1999). Three kinds of synthetic ACE inhibitors were designed. They are grouped by their ligand for the active site on ACE. Captopril, the major representative of this group, has a sulfhydryl moiety, lisinopril and enalapril have a carboxyl moiety, and fosinopril has

a phosphorus group (Vercruysse et al., 2005), and the structure of these commercial ACE inhibitors are shown in Figure 2.7. Although, these synthetic ACE inhibitors are remarkably effective at regulating blood pressure and are used as clinical antihypertensive drugs (Chen et al., 2007). However, they have demonstrated diverse side effects, such as allergic reactions, skin rashes, cough, taste disturbances, and angioneurotic edema (Chen et al., 2007; Bougatef et al., 2008). Thus, food-derived ACE inhibitory peptides are particularly attractive due to the limited side effects (Chen et al., 2012).

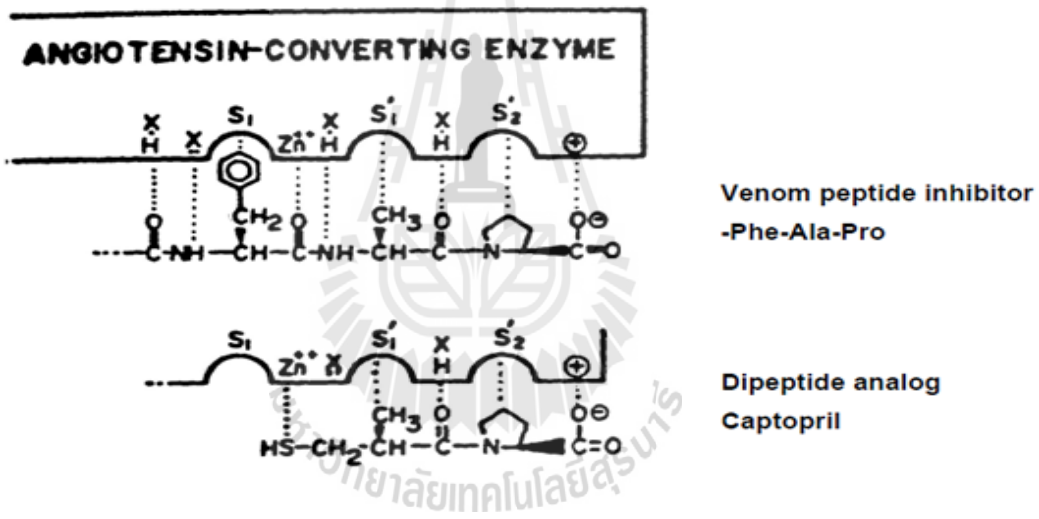

Figure 2.6 Hypothetical binding of competitive inhibitors to the active site of ACE. The subsites S_1, S'_1, S'_2 interact with side chains of terminal, penultimate and antepenultimate amino acids or residues of inhibitors. The residue X-H donates a hydrogen bond and X accepts a hydrogen bond.

From: Cushman et al. (1987).

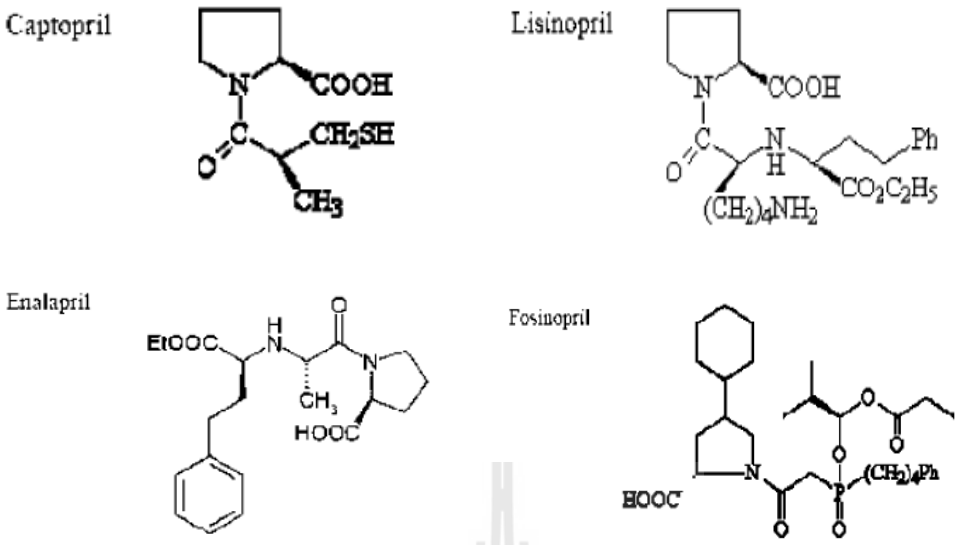

Figure 2.7 Chemical structure of captopril, lisinopril enalapril, and fosinopril.

From: Vercruysse et al. (2005).

2.8 ACE inhibitory peptides derived from food proteins

The discovery of ACE inhibitory peptides in snake venom indicated the presence of inhibiting sequences in natural proteins. This was soon confirmed when ACE inhibitory peptides were isolated from a collagenase hydrolysate of gelatin and a trypsin hydrolysate of casein (Maruyama and Suzuki, 1982). Since then, small peptides with ACE inhibitory efficacy have been widely explored. Generally, three sources are available to obtain novel ACE inhibitory peptides: (1) direct isolation from animal and plant materials; (2) isolation from *in vitro* enzymatic digests of precursor proteins; (3) chemical synthesis based on combinatorial library designs of peptides which have ACE inhibitory effect (Minervini et al., 2003). Besides, there were reports that some fermented foods contained small ACE inhibitory peptides (Gómez-Ruiz, Ramos, and Recio, 2002; Je, Park, Jung, Park, and Kim, 2005; Quirós Hernández-Ledesma, Ramos, Amigo, and Racio, 2005; Shin, Yu, Park, Chung, Nam, and Kim, 2001).

Numerous ACE inhibitory peptides have been isolated from food proteins in both animal and plant sources including soy protein (Chiang et al., 2006), peanut protein (Li et al., 2005; Jamdar et al., 2010), rice dregs protein (Chen et al., 2007), casein (Zhu et al., 2007; Jiang, Tian, Brodkorb, and Huo, 2010), whey protein (Ferreira et al., 2007; Wang, Mao, Cheng, Xiong, and Ren, 2010; Pan, Cao, Guo, and Zhao, 2012), porcine muscles (Arihara et al., 2001), shrimp (He et al., 2006), and fish proteins (Nakajima, Yoshie-Stark, and Ogushi, 2009; Raghavan and Kristinsson, 2009; Samaranayaka, Kitts, and Li-Chan, 2010). These peptides with *in vitro* ACE inhibitory activities have been well demonstrated having *in vivo* inhibitory properties on ACE and antihypertensive effects without side effects in SHRs. Although the ACE inhibition potencies of these peptides are not as great as drugs commonly used in the treatment of hypertension, they are naturally derived from food protein sources, and considered to be milder and safer without the side effects as compared with drugs. At the same time, these peptides usually have multifunctional properties and are easily absorbed (Chen et al., 2007). Therefore, food protein-derived ACE inhibitory peptides show great promise in the development of novel physiologically functional food for preventing hypertension as well as for therapeutic purposes (Li et al., 2005; Chen et al., 2007; Kim and Wijesekara, 2010).

2.8.1 ACE inhibitory peptides derived from plants

ACE inhibitory peptides have been identified from various plant food sources including soybean (Chen, Okada, Muramoto, Suetsuna, and Yang, 2003; Gouda, Gowda, and Rao, 2006; Kuba, Tanaka, Tawata, Takeda, and Yasuda, 2003; Wu and Ding, 2002; Shin et al., 2001; Kodera and Nio, 2006), mung bean (Li, Wan, Le, and Shi, 2006), sunflower (Megías et al., 2004), rice (Li, Qu, Wan, and You, 2007), corn

(Yang et al., 2007), wheat (Matsui, Li, and Osajima, 1999), buckwheat (Li, Matsui, Matsumoto, Yamasaki, and Kawasaki, 2002), and spinach (Yang, Marczak, Yokoo, Usui, and Yoshikawa, 2003) (Table 2.5). Although the active peptides have not been sequenced, peanut (Quist, Phillips, and Saalia, 2009), chickpea (Péfroche, Yust, Girón-Calle, Alaiz, Millán, and Vioque, 2002; Yust, Pedroche, Girón-Calle, Alaiz, Millán, and Vioque, 2003), and potato (Pihlanto, Akkanen, and Korhonen, 2008) protein hydrolysates also display strong ACE inhibitory activity. *In silico* gastrointestinal digestion of vicilin and albumin PA2 in pea directly releases a number of potent peptides, indicating that pea protein is a rich source of ACE inhibitory peptides (Vermeirssen, Camp, and Verstraete, 2004). Sesame peptide powder (SPP) could competitively inhibit ACE activity. A reconstituted sesame peptide mixture according to their content ratio in SPP showed a strong antihypertensive effect on SHRs (Nakano et al., 2006). The peptide, which was isolated from wheat gliadin hydrolysate, inhibited the hypertensive activity of angiotensin I with intravenous injection, and decreased the blood pressure significantly with intraperitoneal administration (Motoi and Kodama, 2003).

Table 2.5 Potent ACE inhibitory peptides derived from plant foods.

Source	Parent protein	Enzyme	Amino acid sequence	IC_{50} (μM)
soybean	whole protein	alcalase	DG	12.3
			DLP	4.8
		pepsin	IA	153
			FFL	37
			IYLL	42
			YLAGNQ	14
			VMDKPQG	39
		fermentation	WL	29.9
			IFL	44.8

Table 2.5 Potent ACE inhibitory peptides derived from plant foods (Continued).

Source	Parent protein	Enzyme	Amino acid sequence	IC_{50} (μM)
soybean	whole protein	alcalase	DG	12.3
			DLP	4.8
		pepsin	IA	153
			FFL	37
			IYLL	42
			YLAGNQ	14
			VMDKPQG	39
		fermentation	WL	29.9
			IFL	44.8
	protein isolate	protease D3	YVVFK	44
			NWGPLV	21
			PNNKPFQ	33
			IPPGVPYT	64
	glycinin	protease P	VLIVP	1.69
	glycinin	acid proteinase	WL	65
mung bean	protein isolate	alcalase	KDYRL	26.5
			VTPALR	82.4
			KLPAGTLF	13.4
sunflower	protein isolate	pepsin-pancreatin	FVNPQAGS	6.9
rice	protein isolate	alcalase	TQVY	18.2
corn	gluten	alcalase	AY	14.2
wheat	germ protein	alcalase	IVY	0.48
			VFPS	0.46
			DYVGN	0.72
			TYLGS	0.86
			GGVIPN	0.74
spinach	rubisco	pepsin-pancreatin	MRW	0.6
			MRWRD	2.1
			LRIPVA	0.38
			IAYKPAG	4.2
buckwheat	whole protein	pepsin-chymotrypsin	VK	13
		- trypsin	YQY	4
			FY	25
			LGI	29

Modified from: Guang and Phillips (2009).

2.8.2 ACE inhibitory peptides derived from milk proteins

It is well established that *in vitro* incubation of milk proteins with gastrointestinal proteinase preparations enriched in pepsin, trypsin, and chymotrypsin activities results in the release of ACE inhibitory peptides (Abubakar, Tadao, Kitazawa, Kawai, and Itoh, 1998; Maruyama, Mitachi, Tanaka, Tomizuka, and Suzuki, 1987). Therefore, hydrolysate of whole milk protein, caseinates, whey proteins, and fractions enriched in individual milk proteins are potentially good sources of ACE inhibitory peptides. Individual caseins from αs1, αs2 or β-casein are a potent *in vitro* inhibitor of ACE (Meisel and FitzGerald, 2000). The casein hydrolysate produced a significant reduction in blood pressure in a population of subjects with high-normal blood pressure or mild hypertension without any adverse event (Korhonen and Pihlanto, 2006).

The proteinases in various bacterial strains, many of which are used in the manufacture of fermented dairy products are capable of releasing ACE inhibitory peptides from milk proteins (Gobbetti, Ferranti, Smacchi, Goffredi, and Addeo, 2000; Meisel, 1997). Pihlanto, Rokka, and Korhonen (1998) reported that the *in vitro* release of ACE inhibitory peptides from casein or whey by commercial yoghurt starters required further incubation with pepsin and trypsin activity. *Lactobacillus helveticus* strains were capable of releasing ACE inhibitory peptides into fermented milk drinks or yoghurt-type products (Fuglsang, Nilsson, and Nyborg, 2002; Leclerc, Gauthier, Bachelard, Santure, and Roy, 2002; Sipola et al., 2002). A number of studies have shown that ACE inhibitory peptides can be produced during cheese making (Ryhänen, Pihlanto, and Pahkala, 2001; Saito et al., 2000). It was also found that the release of ACE inhibitory peptide increases during cheese ripening.

A limited number of human studies have been performed on the hypotensive effect of different milk protein hydrolysates and fermented dairy products which contain ACE inhibitory peptides *in vitro*. The majority of *in vivo* studies attributed the

antihypertensive effects to casokinins (casein-derived ACE inhibitors). Sekiya, Kobayashi, Kita, Imamura, and Toyama (1992) were the first to demonstrate that the consumption of 20 g/day of a tryptic hydrolysate of casein could bring about some reduction in both diastolic blood pressure (DBP) and systolic blood pressure (SBP) in hypertensive human volunteers. More recently it was reported that a tryptic hydrolysate of casein containing a potent ACE inhibitory 12 residue (C12) αs1-casein peptide f(23-34) could also reduce blood pressure in hypertensive patients (Hong et al., 2008). An increasing number of ingredients containing specific bioactive peptides based on casein or whey protein hydrolysates have been launched on the market within the past few years are currently under development by international food companies (Table 2.6).

Table 2.6 Commercial dairy products and ingredients with function claimed on blood pressure reduction.

Brand name	Type of product	Claimed functional bioactive peptide	Producer
Calpis	Sour milk	VPP, IPP from β-casein and κ-casein	Calpis Co., Japan
Evolus	Calcium enriched fermented milk drink	VPP, IPP from β-casein and κ-casein	Valio Oy, Finland
BioZate	Hydrolyzed whey protein isolate	β-lactoglobulin fragments	Davisco, USA
C12 peptide	Ingredient -hydrolysate	Casein derived peptide	DMV International, The Netherlands

Modified from: Korhonen and Pihlanto (2006).

2.8.3 ACE inhibitory peptides derived from egg

Antihypertensive peptides isolated from eggs may be obtained by enzymatic hydrolysis of ovalbumin, including ovokinin and ovokinin. The hydrolysis of crude egg

white with pepsin, trypsin, and chymotrypsin produced peptides with ACE inhibitory properties. Ovokinin had higher antihypertensive potency than ovokinin in SHRs. Some other ACE inhibitory peptides obtained from this hydrolysate also showed antihypertensive activity in these rats (Miguel and Aleixandre, 2006). The most active hydrolysates were obtained after treatment with pepsin, with the fraction having a molecular weight lower than 3000 Da, giving the highest ACE inhibitory activity (Miguel, Recio, Gómez-Ruiz, Ramos, and Lopez-Fandino, 2004). While most orally active anti-hypertensive peptides derived from food proteins inhibit the ACE, ovokinin has been shown to induce nitric oxide-dependent vasorelaxation in an isolated mesenteric artery as well as an antihypertensive effect after oral administration in SHRs. Furthermore, oral administration of the peptide lowered the blood pressure of SHRs but not of normotensive Wistar-Kyoto rats. Table 2.7 shows antihypertensive peptides obtained from egg proteins.

Table 2.7 Peptides with possible antihypertensive activity obtained from egg proteins.

Amino acid sequence	**Origin**	**Activity demonstrated**
FRADHPFL	Ovalbumin	Vasorelaxing/Antihypertensive
RADHPF	Ovalbumin	Vasorelaxing/Antihypertensive
RADHPFL	Egg white	ACEI/Antihypertensive
YAEERYPIL	Egg white	ACEI/Antihypertensive
IVF	Egg white	ACEI/Antihypertensive
FGRCVSP	Ovalbumin	ACEI
ERKIKVYL	Ovalbumin	ACEI
FFGRCVSP	Ovalbumin	ACEI
LW	Ovalbumin	ACEI/Antihypertensive
FCF	Ovalbumin	ACEI

Table 2.7 Peptides with possible antihypertensive activity obtained from egg proteins (Continued).

Amino acid sequence	Origin	Activity demonstrated
NIFYCP	Ovalbumin	ACEI
RADHP	Egg white	ACEI/Antihypertensive

ACEI, angiotensin I-converting enzyme inhibitory activity.

From: Hong et al. (2008).

2.8.4 ACE inhibitory peptides from animal muscle proteins

Up to now, an increasing number of ACE inhibitory peptides have been detected in protein hydrolysate prepared from animal muscle proteins including fish muscle, porcine skeletal muscle, chicken breast, and etc (Arihara et al., 2001; Fujita, Yokoyama, and Yoshikawa, 2000; Katayama et al., 2003; Khantaphant, Benjakul, and Kishimura, 2011; Raghavan and Kristinsson, 2009; Matsui et al., 1993; Wang et al., 2008; Vercruysse et al., 2005). Table 2.8 shows the amino acid sequence of ACE inhibitory peptides derived from animal muscle proteins, their origin, the enzyme used for hydrolysis, and the IC_{50} value. These studies indicate that the ACE inhibitory peptides have been generated not only from myofibrillar structural proteins (myosin and actin) and regulatory proteins (tropomyosin and troponin) but also from stroma protein, such as collagen and gelatin.

A large number of bioactive peptides have been derived from fish proteins. The first report of ACE inhibitory peptides derived from fish muscle proteins was made by Suetsuna and Osajima (1986). They reported that denazyme AP (a protease from *Aspergillus oryzae*) hydrolysates of sardine and hair tail meat contained ACE inhibitory peptides with IC_{50} values *in vitro* of 3.79 and 9.01 mg/l. Additionally, *in vitro* ACE inhibitory peptides derived from fish proteins exerted antihypertensive activity in SHRs

and human trials. Yokoyama, Chiba, and Yoshikawa (1992) detected ACE inhibitory activity of protein hydrolysate derived from dried bonito (Katsuobusi), hydrolyzed by various proteinases. A thermolysin digest showed the most potent inhibitory activity with an IC_{50} value of 29 μg/mL. Eight peptides were isolated and four of them were found in the primary structure of actin, by searching for sequence homology. One of these 8 peptides, LKPNM (IC_{50} = 2.4 μM), was found to be hydrolyzed by ACE, producing another, more potent ACE inhibitory peptide, LKP (IC_{50} = 0.32 μM) (Fujita et al., 2000). Both peptides, intravenously administrated to SHRs, exerted antihypertensive activity. Oral administration in SHRs showed that LKPNM had maximum reduction of blood pressure 6 h after administration, while LKP exerted a maximum decrease after 4 h. LKPNM is a prodrug-type ACE inhibitory peptide which is converted into LKP by ACE *in vivo*. Therefore, the 2 h delay in maximal activity is explained by the time lag required for enzymatic conversion of LKPNM. In a small scale clinical study, the thermolysin digest evidenced long-lasting antihypertensive activity after oral administration in hypertensive and borderline hypertensive subjects. This digest has been officially approved as a "Food for Specified Health Use" in Japan.

Matsui et al. (1993) investigated ACE inhibitory activity of sardine muscle hydrolysate prepared by alcalase. The ACE inhibitory activity is detected with an IC_{50} value of 260 μg/mL. After fractionation of the alcalase hydrolysate, a more potent inhibitor fraction is observed, with an IC_{50} value of 83 μg/mL. The ACE inhibitory activity of this fraction did not change after being treated with gastrointestinal proteases. Thirteen ACE inhibitory peptides were isolated with IC_{50} values ranging from 1.63 to 330 μM (Matsufuji et al., 1994). In SHRs, VY decreased the blood pressure after oral administration (Matsufuji et al., 1995). A randomized double-blind placebo-controlled study, carried out on 29 volunteers, presented a significant antihypertensive effect on mild hypertensive subjects via ACE inhibition (Kawasaki et al., 2000).

So far, the fish peptides showing ACE inhibitory and antihypertensive activities have been obtained. Nakajima et al. (2009) evaluated ACE inhibitory activity of fish protein hydrolysates derived from fish including Atlantic salmon, coho salmon, Alaska pollack, and southern blue whiting using pepsin, pancreatin, and thermolysin. ACE was inhibited by thermolysin-hydrolyzed Atlantic salmon and coho salmon at IC_{50} values of 0.078 and 0.138 mg/mL, respectively. Wu et al. (2008) reported that shark meat hydrolysate obtained with protease SM98011 digestion showed high ACE inhibitory activity (IC_{50} value of 0.4 mg/mL), comparing to the untreated shark slurry (IC_{50} value of 10.5 mg/mL). The sequences of CF, EY, and FE were confirmed to be novel ACE inhibitory peptides, with IC_{50} values of 1.96, 2.68 and 1.45 μM, respectively. All of which were dipeptides and have a hydrophobic amino acid residue, Phe or Tyr, at the C-terminal position. Balti et al. (2010) showed ACE inhibitory activity of the sequences of VYAP, VIIF and MAW (IC_{50} values of 6.1, 8.7 and 16.32 μM, respectively), isolated from cuttlefish (*Sepia officinalis*) muscle hydrolysate. These purified peptides also showed the stability under gastrointestinal enzymes (pepsin, trypsin, and chymotrypsin) digestion.

In the enzymatic hydrolysates of the water-insoluble protein fraction of porcine skeletal muscle (containing mainly myosin, actin, and collagen) and of myosin, ACE inhibitory peptides were released by thermolysin digestion with the highest inhibitory activity. Two peptides of MNPPK and ITTNP were isolated (Arihara et al., 2001). Moreover, the thermolysin hydrolysates of the water-insoluble proteins and myosin from porcine skeletal muscle exhibited antihypertensive activity after single oral administration to SHRs (Nakashima et al., 2002). Katayama et al. (2003) revealed that crude troponin did not exhibit ACE inhibitory activity, while the peptic hydrolysate of troponin showed strong ACE inhibitory activity.

Table 2.8 ACE inhibitory peptides derived from animal muscle proteins.

Source	Parent protein	Enzyme	Amino acid sequence	IC_{50} (μM)	References
Bonito	muscle	thermolysin	IKPLNY	43	Yokoyama et al.
			DYGLYP	62	(1992)
	actin	thermolysin	IVGRPRHQG	2.4	
			ALPHA	10	
			FQP	12	
	actin	thermolysin	IWHHT	5.8	Fujita and Yoshikawa,
			IY	2.31	(1999)
	muscle	thermolysin	LKPNM	2.4	
	muscle	thermolysin	LKP	0.32	
			IKP	6.9	
	actin	thermolysin	IWH	3.5	
	actin	thermolysin	IVGRPR	300	Fujita et al. (2000)
Salmon	muscle	thermolysin	WA	277.3	Ono et al. (2003)
			VW	2.5	
			WM	96.6	
			MW	9.9	
			IW	4.7	
			LW	17.4	

Table 2.8 ACE inhibitory peptides derived from animal muscle proteins (Continued).

Source	Parent protein	Enzyme	Amino acid sequence	IC_{50} (μM)	References
Sardine	muscle	alcalase	MF	44.7	Matsufuji et al. (1994)
			RY	51	
			MY	193	
			LY	38.5	
			YL	82	
			IY	10.5	
			VF	43.7	
			GRP	20	
			RFH	330	
			AKK	3.13	
			RVY	205.6	
			GWAP	3.86	
			KY	1.63	
	muscle	alcalase	VY	10	Kawasaki et al. (2000)
Huruan	myofilbrillar	thermolysin	VPAAPPK	0.45	Ghassem et al. (2011)
			NGTWFEPP	0.63	
Lizard fish	muscle	neutral proteinase	SPRCR	41	Wu et al. (2012)
Rainbow trout	muscle	pepsin	KVNGPAMSPNAN	63.9	Kim and Byun, (2012)

Table 2.8 ACE inhibitory peptides derived from animal muscle proteins (Continued).

Source	Parent protein	Enzyme	Amino acid sequence	IC_{50} (μM)	References
Skipjack roe	muscle	alcalase	MLVFAV	3.07	Intarasirisawat et al. (2013)
Snake head fish	sarcoplasmic protein	alcalase	LYPPP	1.3	Ghassem et al. (2014)
			YSMYPP	2.8	
porcine	myosin	thermolysin	ITTNP	549	Arihara et al. (2001)
			MNPPK	945.5	
	myosin	synthesized	MNP	66.6	
			NPP	290.5	
			PPK	>1000	
			ITT	678.2	
			TTN	672.7	
			TNP	207.4	
	troponin C	pepsin	RMLGQTPTK	34	Katayama et al. (2003)
			RMLGQTP	503	
	myosin B	pepsin	KRVIQV	20.3	Mugurama et al. (2009)
	actin	pepsin	VKAGF	6.1	
	troponin T	pepsin	KRQKYDI	26.2	Katayama et al. (2008)

Table 2.8 ACE inhibitory peptides derived from animal muscle proteins (Continued).

Source	Parent protein	Enzyme	Amino acid sequence	IC_{50} (μM)	References
porcine	titin	pepsin + pancreatin	KAPVA	46.56	Escudero et al. (2010)
			PTPVP	256.41	
chicken	creatine kinase	thermolysin	LKA	8.5	Fujita et al. (2000)
			FKGRYYP	0.55	
	muscle	thermolysin	LAP	3.2	
			IKW	0.21	
	aldolase	thermolysin	LKP	0.32	
	myosin	thermolysin	FQKPKR	14	
	actin	thermolysin	IVGRPRHQG	2.4	
	collagen	Aspergillus proteinase + proteinase FP, A, G, N	GAXGLXGP	29.4	Saiga et al. (2008)
			GAXGPAGPGGIXGERGL XG	45.6	
			GLXGSRGERGERGLXG	60.8	
			GIXGSRGERGPVGPSG	43.4	
cow	muscle	thermolysin + proteinase A	VLAQYK	32.06	Jang et al. (2004)
			FHG	52.9	Jang et al. (2008)
			GLSDGEWQ	117	

Table 2.8 ACE inhibitory peptides derived from animal muscle proteins (Continued).

Source	Parent protein	Enzyme	Amino acid sequence	IC_{50} (μM)	References
cow		proteinase K	GFHI	64.3	Jang et al. (2008)
		alcalase	DFHING	50.5	
	skin gelatin	alcalase + pronase E	GPV	4.67	Kim et al. (2001)
		+ collagenase	GPL	2.55	
Antartic krill	muscle	pepsin + trypsin	KLKFV	30	Kawamura et al. (1992)
oyster	muscle	denazyme AP	LF	126	Matsumoto et al. (1994)
	muscle	pepsin	VVYPWTQRF	66	Wang et al. (2008)
shrimp	muscle	*Bacillus* sp. SM98011 proteinase	FCVLRP	12.3	Hai-Lun et al. (2006)
			IFVPAF	3.4	
			KPPETV	24.1	

Modified from: Vercruysse et al. (2005).

2.9 Structure requirement of ACE inhibitory peptides

Several structural features that influence the potency of ACE inhibitory peptides derived from food have been identified. Recently, it has been reported that artificial neural networks and quantitative structure–activity relationship (QSAR) modelling may be used to develop statistical computer models potentially capable of identifying ACE-inhibitory peptides based on structure-activity data (Gu, Majumder, and Wu, 2011; Majumder and Wu, 2010; Pripp et al., 2004). Several descriptor variables such as molecular mass and shape, hydrophobicity, charge and electronic properties have been recognized as critical in this QSAR modelling.

The majority of ACE inhibitory peptides are relatively short sequences containing from 2 to 12 amino acids. This is in agreement with the results of Natesh, Schwager, Sturrock, and Acharya (2003), who demonstrated from crystallography studies, that the active site of ACE cannot accommodate large peptide molecules. 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 contain Tyr, Phe, Trp, and/or Pro at the C-terminal (Cheung, Wang, Ondetti, Sabo, and Cushman, 1980; Byun and Kim, 2002). Gómez-Ruiz et al. (2004) suggested that Leu may contribute significantly to increase ACE-inhibitory potential. Furthermore, other branched chain aliphatic amino acids such as Ile and Val are predominant in highly peptide inhibitors. In addition, structure-activity data suggest that the positive charge of Lys (ε-amino group) and Arg (guanidine group)

as the C-terminal residue may contribute to the inhibitory potency (Ondetti et al., 1977; Chueng et al., 1980; Ariyoshi, 1993). Other characteristics have also been found to play important roles for ACE inhibition. It has been recognized that ACE-inhibitory peptides possess a characteristic pattern different from that of inactive peptide molecules (FitzGerald and Meisel, 2000). For long chain peptides, it is expected that peptide conformation, will influence binding to ACE. It has also been demonstrated that ACE has a requirement for the L-configuration of the amino acid at position three from the C-terminal. Moreover, changes in cis-trans conformations of Pro at the C-terminal position of an ACE-inhibitory peptide may cause significant changes in its interaction with the enzyme (Gómez-Ruiz et al., 2004).

However, there are many ACE substrates in the enzymatic hydrolysates of food proteins, because the primary activity of ACE is to cleave the C-terminal dipeptide of oligopeptide substrates with a wide specificity. Thus, the substrate of ACE also show seemingly ACE inhibitory activities tentatively in the assay used for screening of ACE inhibitory peptides. To discriminate the substrates from true inhibitors, peptides are generally preincubated with ACE before measurement of ACE inhibitory activity (Li et al., 2004). In this way, ACE inhibitory peptides from various sources can generally be classified into three groups (Li et al., 2004; Hwang, 2010). The first is the *inhibitor type*. The IC_{50} values of this type of peptides are not affected after preincubation with ACE, such as peptides Ile-Tyr and Ile-Lys-Trp. The second is the *prodrug-type inhibitor*. These peptides are converted to true inhibitors by ACE or gastrointestinal proteinases. Fujita and Yoshikawa (1999) reported that Leu-Lys-Pro-Asn-Met (IC_{50} = 2.4 μM) is the prodrug-type ACE inhibitory peptide derived from the thermolysin-digest of dried bonito, and may be hydrolyzed further to produce Leu-Lys-Pro (IC_{50} =

0.32 μM), which was an 8-fold higher ACE inhibitory activity than the parent peptide. The third is the *substrate type*. These peptides are hydrolyzed by ACE to give peptides with weaker or no activity at all; for example, Phe-Lys-Gly-Arg-Tyr-Tyr-Pro (IC_{50} = 0.55 μmol/L) was hydrolyzed by ACE into Phe-Lys-Gly, Arg-Tyr, and Tyr-Pro with the IC_{50} value increasing to 34 μmol/L (Li et al., 2004).

2.10 *In vitro* digestion and intestinal permeability

One of the greatest challenges in developing nutraceutical and functional food products is proving the *in vivo* efficacy of their bioactive components. Survival from physiological barriers after oral administration, including gastrointestinal digestion and intestinal epithelial absorption in human tract is the essential prerequisite for the bioactive assessment of peptides (Xie, Wang, Ao, and Li, 2013). Lot of bioactive peptides exerting their potencies have been evaluated for their bioavailability by *in vitro* models, because of strict ethical considerations of animal studies and high costs of clinical trials (Cinq-Mars, Hu, Kitts, and Li-Chan, 2008). Furthermore, estimation of the *in vitro* bioavailability of bioactive peptides could significantly support their *in vivo* functional properties (Xie et al., 2013). Up to now, many researchers have started focusing on stability of bioactive peptides against gastrointestinal (GI) digestion, intestinal absorption and permeation using *in vitro* models. The purpose of the *in vitro* digestion model is to simulate in a simplistic manner the digestion processes that take place in the mouth, stomach, and small intestine. To investigate the effect of GI enzymes on the ACE inhibitory activity of peptides, the peptides are usually incubated at 37°C by hydrolysis of pepsin, trypsin, α-chymotrypsin or pancreatin to mimic the digestion on gastric phase and intestinal phase (Gómez Ruiz et al., 2004; Guang, Shang,

and Jiang, 2012; Sheih et al., 2009; Xie et al., 2013). However, not all peptides exhibiting *in vitro* ACE inhibitory activity may be effective *in vivo*. For example, the digest of dried bonito obtained after pepsin-trypsin-chymotrypsin treatment (Fujita et al., 2000) and the tripeptide TNP (Nakashima et al., 2002) did not significantly lower blood pressure in SHRs despite potent *in vitro* ACE inhibitory activity with IC_{50} values of 41 µg/mL and 207.4 µM, respectively. On the other hand, the isolated peptide TQPKTNAIPY from manchego cheese exhibited an activity 6 times greater after pepsin-pancreain digestion (Gómez-Ruiz et al., 2004).

Another important factor that will determine the actual efficacy of orally consumed ACE inhibitory peptides to lower blood pressure *in vivo* concerns the bioavailability or absorption efficiency across the intestinal epithelium (Cinq-Mars et al., 2008). *In vitro* cell culture models can offer advantages in terms of practicality and reproducible of experiments. It can also be applied to medium to high throughput screening of large number of compounds (Mortensen et al., 2008). Furthermore, cell culture models can also be used to evaluate cytotoxicity of bioactive compounds at concentrations used to exert the desired bioactivity in the body (Samaranayaka et al., 2010). An accepted model system for the enterocyte of the human small intestine is the Caco-2 cell line. This cell line has been widely used as a predictive tool for the intestinal epithelial absorption and permeability of bioactive compounds (Cinq-Mars et al., 2008; Samaranayaka et al., 2010). Several studies in drug transport have applied the Caco-2 cell model for permeability measurement. The Caco-2 cell line, originally derived from human colon carcinoma, can spontaneously differentiate into intestinal epithelium under standard culture conditions (Xie et al., 2013).

When cultured as a monolayer, the differentiated cells express characteristics of mature epithelium, such as microvillus structure, tight junction at apical side between cells to serve as a model of paracellular movement of compounds across the monolayer, numerous brush border enzymes (including aminopeptidase N, aminopeptidase P, aminopeptidase W, dipeptidyl peptidase IV, endopeptidase-24.11, γ-glutamyl transpeptidase, microsomal dipeptidase, and peptidyl dipeptidase A), and carrier-mediated transport system for di/tri peptides, which are of critical importance for absorption of intact peptides (Guang et al., 2012; Xie et al., 2013). Caco-2 cell permeability study, are performed using Caco-2 cell monolayers grown on semi-permeable plastic supports in multi-well insert systems (Figure 2.8). Test compounds can be added to either the upper (apical) or lower (basolateral) chamber to measure permeability in the absorptive (apical to basolateral) or secretive (basolateral to apical) directions, respectively. Samples are then taken from the opposite chamber at various time intervals to measure the amount of test compound passing through the cell monolayer. The amount of peptide passing through the monolayers can be monitored using TNBS method (Samaranayaka et al., 2010; Xie et al., 2013), HPLC analysis (Cinq-Mars et al., 2008), or UV absorbance at 215 and 225 nm (Guang et al., 2012).

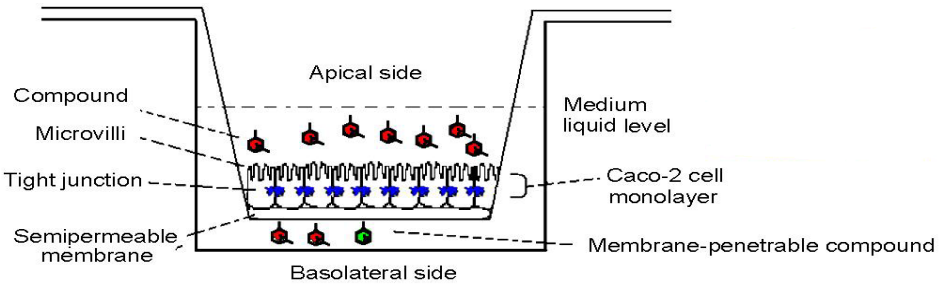

Figure 2.8 Schematic representation of a Caco-2 permeability assay.

From: Yang et al. (2007).

In order to ensure integrity of Caco-2 cell monolayers, function of tight junctions (TJ) can be determined and monitored by the measurement of transepithelial electrical resistance (TEER) (Cinq-Mars et al., 2008; Samaranayaka et al., 2010). In addition, the integrity of cultured monolayers is also detectable by carrying out transport studies using water-soluble reference compounds that can be absorbed by TJ channels between the cells such as radio-labeled mannitol, phenol red, Lucifer Yellow, and fluorescein. Quantification is then made by detecting the reference substance in the basolateral compartment (Konishi, 2006).

2.11 Overview of immune system

The immune system is a network of cells, tissue, and organs that work together defend the body against million of bacteria, microorganisms, viruses, toxin, and parasite. In addition, the immune system is claimed to play a role in overall homeostasis in the body, and as such be able to respond not only to exogenous, but also endogenous danger signal (MacPherson and Austyn, 2012). Although it is a very effective defending machine against infection, however, disorder of immune system can result in immunodeficiencies, autoimmunity (e.g. diabetes type I, rheumatoid arthritis, and systemic lupus erythematosus (SLE)), and hypertensitivity (Ghaffar, 2006; MacPherson and Austyn, 2012).

The immune system consists of several lymphatic organs (bone marrow, thymus gland, lymph nodes, and spleen) and lymphatic tissues (adenoids, tonsils, and appendix) throughout the body (Delves and Roitt, 2000). All immune cells, tissue cells and white blood cells or leucocytes, develop from haematopoietic stem cells in the bone marrow. The stem cells also give rise to erythocytes (or red blood cells) and thrombocytes (or

platelets). The production of leucocytes is through two main pathways of differentiation (Figure 2.9). The lymphoid progenitor produces T lymphocytes (T cells), B lymphocytes (B cells), and Natural killer (NK) cells. The myeloid pathway gives rise to monocytes, granulocytes (neutrophils, eosinophils, and basophils), and mast cells (MacPherson and Austyn, 2012).

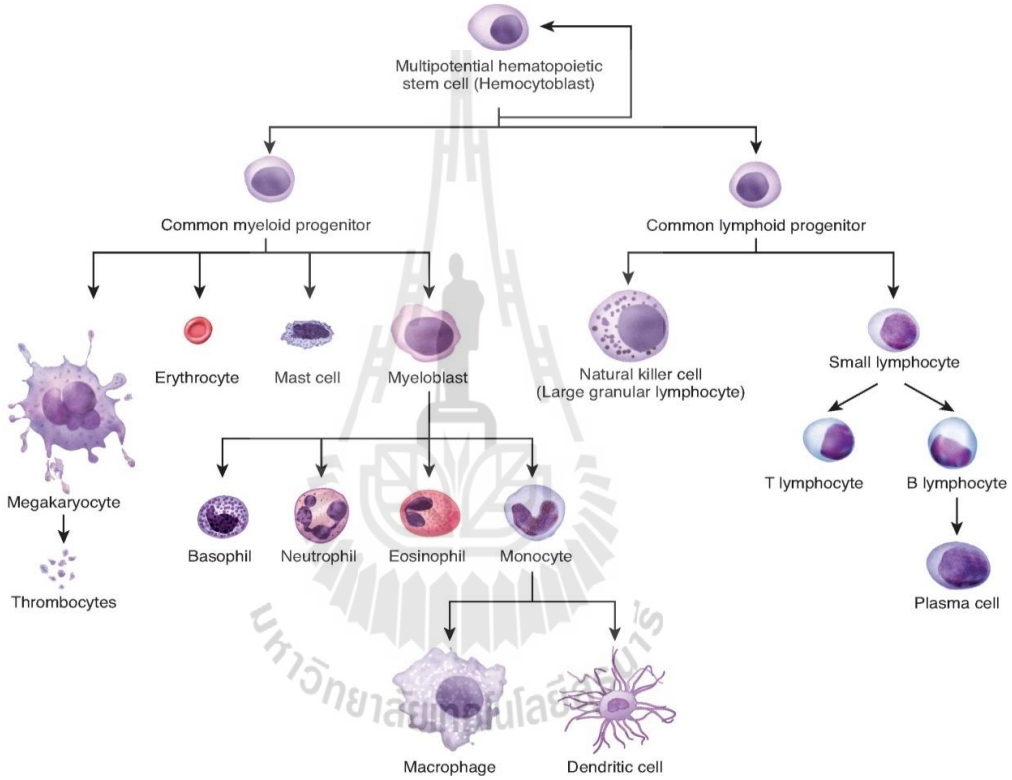

Figure 2.9 Development of cells in the immune system.

From: www.textbookofbacteriology.net

2.11.1 Innate and adaptive immune responses

The immune system is basically composed of two compartments, innate and adaptive immunity. The innate immunity provides immediate but less-specific responses and can be found in all multi-cellular organisms. The skin, mucosal

secretions. While the adaptive immunity is found only in vertebrates and is activated by the innate response, gives stronger and very specific responses and develops a memory, by which it remembers specific invaders and mounts a faster and a stronger response in later challenges. This process of the adaptive immunity the basis of vaccination. Next to cellular immunity, humoral immune responses are critical to make the immune system function effectively. Humoral immunity is mediated by the secretion of antibodies produced by B cells with the aid of T cells. Table 2.9 shows the innate and adaptive immune cells and theirs functions.

Table 2.9 Functions of immune cells.

Immune cells	Function
Innate immune cells	
- Neutrophils	Engulfing and inflammation
- Basophils	Release histamine and inflammation
- Eosinophils	Destroy worms; hypersensitivity reactions
- Monocytes	Engulfing invading pathogens and differentiate into macrophages
- Macrophages	Engulfing invading pathogens and activating T cells
- Mast cells	Trigger inflammatory response
- Natural killer cells	Kill cells with guidance from antibodies
- Dendritic cells	Potent antigen presenting cells to T cells
Adaptive immune cells	
- B cells	Recognize foreign antigens, differentiate to plasma cells and memory cells, secrete antibodies to guide attack
- Cytotoxic T cells	Recognize and attack cancerous and infected cells
- Helper T cells	Help activate B cells and cytotoxic T cells

From: Chanput (2012).

2.11.2 Inflammatory response

Macrophages are part of the innate immunity which detect and eliminate invading microorganisms and toxic molecules. Their response towards these targets are rapid and triggered by specific molecular structures, commonly referred to as Pathogen-Associated Molecular Patterns (PAMPs). Macrophages play a critical role in the initiation, maintenance, and resolution of inflammation (Fujiwara and Kobayashi, 2005). Major roles of macrophages are (i) recognition of foreign pathogens such as bacteria, fungi, and viruses via interaction of their surface structures with different types of Pattern Recognition Receptors (PRRs), (ii) proliferation to increase the amount of cells that are able to eliminate pathogens, (iii) production of pro-inflammatory chemokines and cytokines and anti-inflammatory cytokines when the infection is under control, and (iv) phagocytosis to engulf and digest pathogens (Chanput, Mes, and Wichers, 2014). Circulating monocytes differentiate into macrophages once they arrive in tissues, such as intestine, adipose tissue, alveolar space, which then constrains the functional properties of macrophage. The main functions of macrophages are involved in wound healing, resolution of inflammation, coordinating cell migration, and tissue remodeling (Chanput, 2012). Macrophages that encourage inflammation are called M1 macrophages, whereas those that decrease inflammation and encourage tissue repair are called M2 macrophages (Mills, 2012). They are activated and deactivated in the inflammatory process. They are activated by stimuli such as interferon (INF)-γ, bacterial lipopolysaccharides (LPS) in Gram negative bacteria, extracellular matrix proteins, and other chemical mediators (Ahn, Cho, and Je, 2014). Activated macrophages are deactivated by anti-inflammatory cytokines (IL-10 and TGF-β) and cytokine antagonists that are mainly produced by macrophages (Fujiwara and

Kobayashi, 2005). Inhibition of inflammation by removal or deactivation of mediators and inflammatory effector cells permits the host to repair damages tissues.

Research on the mechanisms of the inflammatory response has identified various mediators, cytokines, and protein kinases that act as vital signaling components, which represent potential therapeutic targets (Matthias, Alexey, and Michael, 2009). Inflammation can be measured by the use of different markers such as interleukin (IL), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), tumour necrosis factor-α (TNF-α), cyclooxygenase (COX), and nitric oxide (NO), and etc (Oseguera-Toledo, de Mejia, Dia, and Amaya-Llano, 2011). NF-κB has been interested for inflammatory-mediated responses due to several mediators and cytokines cause the activation of this transcription factor, leading to the activation of inflammation, cell proliferation, differentiation, migration, and cell survival (Sharif, Bolshakov, Raines, Newham, and Perkins, 2007). NF-κB induces the transcription of pro-inflammatory mediators, such as inducible nitric oxide synthase (iNOS), COX-2, TNF-α, IL-1β, IL-6, and IL-8 (Alderton, Cooper, and Knowles, 2001). TNF-α is an important mediator in the pathogenesis of lethal systemic inflammation in septic shock and also induces apoptosis in other cells and tissue damage (Cohen, 2002). High serum IL-6 levels are observed in inflammation, trauma, and autoimmune diseases (Yoon et al., 2007). IL-8 plays critical roles in the development of pain and its expression increases in the brain after peripheral inflammation (Cui et al., 2012). Moreover, large amounts of pro-inflammatory mediators, NO and prostaglandin E_2 (PGE_2) are generated by iNOS and COX-2, respectively (Oseguera-Toledo et al., 2011). Both mediators are strongly involved in inflammation and carcinogenesis (Ahn et al., 2014). Although, inflammation is an important normal immune response during lesions and infections,

an excessive inflammation can contribute to several acute and chronic diseases (e.g. rheumatoid arthritis, inflammatory bowel disease, artherosclerosis, and cancer) (Millán-Linares, Bermúdez, Yust, Millán, and Pedroche, 2014). Therefore, inhibitors of the pro-inflammatory mediators have been considered as a candidate of anti-inflammatory drugs and controlling of excessive inflammation is important to maintain health and wellness.

2.11.3 THP-1 cell line model for immune modulation study

There are limitations to study monocyte and macrophage functions and responses *ex vivo* due to the small number and donor-related differences of cells obtained from whole blood isolation and *in vivo* due to ethical restriction and high cost (Chanput et al., 2014). Thus, monocyte cell lines can be an alternative to *ex vivo* and *in vivo* studies. THP-1 is a human leukemia monocytic cell line, which were derived from the peripheral blood of a 1-year-old male with acute monolytic leukemia (Tsuchiya, Yamabe, and Yamaguchi, 1980). Early studies indicated that THP-1 cells resemble primary monocytes and macrophages in morphological and differentiation properties. THP-1 cells are round suspension cells expressing distinct monocyte markers (Tsuchiya et al., 1980). THP-1 monolytic cell line can be differentiated into the macrophages state using either phorbol-12-myristate-13-acetate (PMA) and 1α,25-dihydroxyvitamin D3 (vD3) (Park et al., 2007; Schwende, Fitzke, Ambs, and Dieter, 1996). However, PMA and vD3 regulate different signaling pathways, in which PMA recruits protein kinase C to the intracellular side of the plasma membrane, whereas vD3 up-regulates expression of mitogen-activated protein kinase phosphatase-1 (MKP-1). In addition, PMA treatment resulted in a more differentiated phenotype than vD3, according to adherence, loss of proliferation, phagocytosis of latex beads, and

expression of CD11b and CD14 (Schwende et al., 1996). Therefore, PMA-differentiated THP-1 macrophages are widely used to study macrophage functions and responses (Chanput, 2012). THP-1 cells have also been widely used to study monocytes and macrophages responses to various stimuli including food derived and non-food derived compounds, in which tested either at the resting or inflammation-activated state (Chanput, Reitsma, Kleinjans, Mes, Savelkoul, and Wichers, 2012; Iio et al., 2012; Xu, et al., 2012).

2.11.4 Immunomodulation by food-protein-derived peptides

Development of immunomodulators from natural sources for diet supplementation both animal and human, is an active area of research (Duarte, Vinderola, Ritz, Perdigón, and Matar, 2006). The influence of peptides on the immune system has been well documented. Examples include early work on muramyl dipeptides and lauryl tetrapeptide of microbial origin, and peptides derived from immunoglobulin G (tuftsin) and thymopentin (TP-5). Many immunostimulating peptides have been isolated from enzymatic digests of various food proteins, including milk proteins (Keyser and Meisel, 1996; Eriksen, Vegarud, Langsrud, Almaas, and Lea, 2008), whey protein (Mercier, Gauthier, and Fliss, 2004; Gauthier, Pouliot, and Saint-Sauveur, 2006), casein (Stuknyte, Noni, Guglielmetti, Minuzzo, and Mora, 2011; Jiehui et al., 2014), fish protein (Duarte et al., 2006; Yang et al., 2009), pea protein (Ndiaye, Vuong, Duarte, Aluko, and Matar, 2012) and soy protein (Kong, Guo, Hua, Cao, and Zhang, 2008).

The immunomodulatory peptides can enhance immune cell functions, measured as lymphocyte proliferation, natural killer (NK) cell activity, antibody synthesis, cytokine regulation, and NO production. For instance, milk proteins and their

enzymatically derived peptides have been demonstrated to have modulating capacities on *in vitro* lymphocyte activation and proliferation. In addition, the modulating ability of whey proteins and peptides also includes cytokine secretion, antibody production, granulocyte and natural killer (NK) cell activity and phagocytic activity (Eriksen et al., 2008). Mercier et al. (2004) determined immunomodulating effects of whey proteins and their enzymatic digests (trypsin/chymotrypsin) and reported that some short-chain (MW < 5 kDa) and neutral/basic peptides from whey proteins are effective in stimulating immune cell proliferation. Kong et al. (2008) reported the immunomodulating effect of soy protein hydrolysates (SPHs) which was evaluated *in vitro* by measuring their impact on lymphocyte proliferation and the phagocytic activity of peritoneal macrophages. The SPHs prepared with Alcalase, had the highest immunomodulating activities by enhancing the proliferation of murine spleen lymphocytes and phagocytic activity of peritoneal macrophages. In addition, lower molecular weight and higher content of positively charged peptides from soy proteins were effective in stimulating immunomodulating activity.

Recently, anti-inflammatory activity of enzymatic protein hydrolysates derived from various protein sources have also been reported. Casein hydrolysate from yak milk prepared by Alcalase showed significant down-regulation of nitric oxide (NO) production, as well as pro-inflammatory cytokines, IL-6, TNF-α, and IL-1β secretion in a dose-dependent manner in LPS-stimulated murine peritoneal macrophages. Moreover, yak milk casein hydrolysate exhibited significantly higher pro-inflammatory cytokine-attenuating activity compared with non-hydrolyzed yak casein (Mao, Cheng, Wang, and Wu, 2011). Ndiaye et al. (2012) found that pea protein hydrolysate (PPH) hydrolyzed by thermolysin showed significant inhibition of NO production in activated

macrophages. PPH also inhibited secretion of pro-inflammatory cytokines (TNF-α and IL-6). Oral administration of PPH in mice enhanced the phagocytic activity of their peritoneal macrophages. Millán-Linares et al. (2014) found that lupine protein hydrolysates (LPHs) prepared by Izyme AL and Alcalase attenuated expression of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) and increased expression of anti-inflammatory marker genes (chemokine (C-C motif) ligand 18) compared to control without LPHs. Furthermore, LPHs significantly decreased NO production. Peptide fraction with molecular weight (MW) above 5 kDa derived from almond protein hydrolysate showed the inhibition of relative gene expressions of pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α in the activated macrophages (Udenigwe, Je, Cho, and Yada, 2013). Moreover, this fraction decreased expression of iNOS and COX-2 in the activated cells.

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CHAPTER III

ANGIOTENSIN I-CONVERTING ENZYME (ACE) INHIBITORY ACTIVITY OF PROTEIN HYDROLYSATES OF TILAPIA MUSCLE PROTEINS AND PERMEABILITY ACROSS INTESTINAL EPITHELIAL CELLS

3.1 Abstract

Angiotensin I-converting enzyme (ACE) inhibitory activity of protein hydrolysates from tilapia muscle proteins, namely mince (M), washed mince (WM), and sarcoplasmic protein (SP), were investigated. Each fraction was hydrolyzed by *Virgibacillus halodenitrificans* SK1-3-7 proteinases for up to 24 h. After 8 h of hydrolysis, M hydrolysate with 48% degree of hydrolysis (DH) showed the highest ACE inhibitory activity with an IC_{50} value of 0.54 mg/mL, while SP hydrolysate exhibited the lowest DH with an IC_{50} value of 1.15 mg/mL ($p < 0.05$). The M hydrolysate showed a mixed-type inhibition characteristic with an inhibition constant of 0.041 mg/mL. Although *in vitro* gastrointestinal digestion reduced ACE inhibitory activity of the M hydrolysate, it enhanced permeability across Caco-2 cell monolayers. The permeated peptides were identified to contain 3-4 amino acid residues showing strong ACE inhibition. The novel ACE inhibitory peptide with the highest inhibition was identified to be MCS with an IC_{50} value of 0.29 µM. Therefore, tilapia mince

hydrolyzed by *V. halodenitrificans* proteinases contained ACE inhibitory peptides that are potentially bioavailable.

Keywords: tilapia, muscle proteins, protein hydrolysates, ACE inhibitory activity, permeability

3.2 Introduction

Hypertension is identified as a cardiovascular risk factor and currently affects 30% of the adult population in the world (Majumder and Wu, 2009). Angiotensin I-converting enzyme (ACE) is a key enzyme in the regulation of peripheral blood pressure and electrolyte homeostasis (Vercruysse, Camp, and Smagghe, 2005). It elevates blood pressure by cleaving a dipeptide His-Leu from inactive decapeptide angiotensin I into the potent vasoconstrictor angiotensin II via the renin-angiotensin system. Additionally, it also converts the vasodilator bradykinin into an inactive peptides via the kallikrein-kinin systems (Li, Le, Shi, and Shrestha, 2004; Wang et al., 2008). Thus, inhibition of ACE activity is a major target to reduce mortality in patients with hypertension.

Numerous ACE inhibitory peptides have been produced from enzymatic hydrolysis of various food proteins (Iwaniak and Dziuba, 2009; Murray and FitzGerald, 2007). Among them, it is well established that fish muscle proteins are an excellent source of ACE inhibitory peptides (Charoenphun, Youravong, and Cheirsilp, 2013; Chen, Wang, Zhong, Wu, and Xia, 2012; Wijesekara, Qian, Ryu, Ngo, and Kim, 2011). Fish muscle proteins compose of myofibrillar proteins, sarcoplasmic proteins, and stromal proteins (Suzuki, 1981). It has not been clearly determined which type of fish muscle is served as a good source of ACE inhibitory peptide.

Tilapia (*Oreochromis niloticus*) is one of the important aquacultured freshwater fish worldwide and its farming has increased in the last three decades due to ease of aquaculture, marketability, and stable market prices (Wang and Lu, 2015). Global tilapia production was 4.8 million metric tons in 2014, and is expected to reach 5 million metric tons in 2015 (Fitzsimmons, 2015). Thus, production of the new value-added protein hydrolysate from tilapia muscle proteins may be an alternative use for preventing hypertension.

Until now, several commercial proteinases, such as Alcalase (Chen et al., 2012; Charoenphun et al., 2013), pepsin (Wang et al., 2008; Sheih, Fang, and Wu, 2009), trypsin (Asoodeh, Yazdi, and Chamani, 2012), thermolysin (Ghassem, Arihara, Babji, Said, and Ibrahim, 2011), and Flavourzyme (Khantaphant, Benjakul, and Kishimura, 2011) have been used to produce protein hydrolysates with ACE inhibitory peptides. However, the use of proteinases from novel sources in protein hydrolysate production has not been widely studied. *Virgibacillus halodenitrificans* SK1-3-7 is a moderately halophilic bacteria isolated from Thai fish sauce fermentation. Montriwong, Kaewphuak, Rodtong, and Yongsawatdigul (2012) found that *V. halodenitrificans* SK1-3-7 proteinases exhibited the highest fibrinolytic and proteolytic activities among 25 bacterial isolates obtained from fish sauce fermentation. Due to the broad substrate specificity of *Virgibacillus* proteinases (Sinsuwan, Rodtong, and Yongsawatdigul, 2010), ACE inhibitory peptides could be obtained through hydrolytic reactions using *V. halodenitrificans* SK1-3-7 proteinases.

One of the great challenges of bioactive peptides for physiological functions is their bioavailability. Survival from gastrointestinal digestion and intestinal epithelial absorption in human tract is the essential prerequisite for bioactive assessment of

peptides. A bioactive peptide needs to be absorbed from the intestine before entering to blood circulation and exerting ACE inhibition (Foltz et al., 2008). Caco-2 cells derived from a human colon carcinoma have been widely accepted as an intestinal epithelial cell model for predicting intestinal permeability of bioactive molecules due to their similarity to the intestinal epithelium cells (Cinq-Mars, Hu, Kitts, and Li-Chan, 2008). Transport of bioactive peptides based on this cell model has been investigated in hydrolysates from milk (Fernández-Musoles et al., 2013; Regazzo et al., 2010), fish (Samaranayaka, Kitts, and Li-Chan, 2010), and collagen (Shimizu et al., 2010).

This study aimed to evaluate ACE inhibitory activity of tilapia mince (M), washed mince (WM), and sarcoplasmic protein (SP), hydrolyzed by *V. halodenitrificans* SK1-3-7 proteinases. In addition, bioavailability of tilapia hydrolysate with high ACE inhibitory activity was evaluated based on the Caco-2 cell monolayers. Moreover, identification of the permeable peptides exhibiting ACE inhibitory activity was carried out.

3.3 Materials and methods

3.3.1 Materials

Fresh tilapia (*Oreochromis niloticus*) were purchased about 12 h after the catch from a local market in Nakhon Ratchasima, Thailand. Fish were packed in a polystyrene foam box filled with ice and transported to the laboratory at Suranaree University of Technology within 30 min. Dorsal muscle was manually separated upon arrival. Angiotensin I-converting enzyme (ACE) from rabbit lungs, hippuryl-histidyl-leucine (HHL), hippuric acid (HA), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, α-chymotrypsin from bovine mucosa, and trinitrobenzenesulfonic

acid (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile and trifluoroacetic acid (TFA) were of HPLC grade. Other reagents and chemicals used were of analytical grade.

3.3.2 Production of *Virgibacillus halodenitrificans* SK-1-3-7 proteinases

Inoculum was prepared by transferring a loopful of pure *V. halodenitrificans* SK1-3-7 culture into yeast extract broth (2.5% NaCl, 1.1% yeast extract, 0.3% trisodium citrate, 0.2% KCl and 2.5% $MgSO_4 \cdot 7H_2O$, pH 7.0) as described by Montriwong et al. (2012) and incubated at 35°C for 2 d. The optical density (OD) of the culture at 600 nm reached approximately 0.3, which was equivalent to a cell count of 10^7 colony forming units (CFU)/mL. Fifty mL of inoculum was transferred to 450 mL of the culture medium and incubated at 35°C for 5 d in a shaking incubator at 100 rpm. Crude extracellular proteinases were collected by centrifugation at 10,000×g and 4°C for 20 min. Supernatants were filtered through 0.45 µm membrane to eliminate cells debris, and filtrates were lyophilized. Proteinase activity was measured at 450 nm using azocasein as a substrate. One unit (U) of proteinase activity was defined as the amount of enzyme that produced an absorbance change of 0.01 per min at 450 nm, 60°C and pH 9.0, for 60 min. Lyophilized crude proteinases were kept at -20°C throughout the study.

3.3.3 Preparation of protein substrates

Three types of substrate were prepared, namely mince (M), washed mince (WM), and sarcoplasmic protein (SP). The dorsal muscle was ground using a meat grinder (Model 8-22, Marblehead, Ohio, USA) with a perforated-plate screen size of 1.5 mm. This ground tissue was referred to as the M sample. The WM was prepared using a typical three wash cycle process. Mince was added to cold distilled water (4°C)

at a ratio 1:3 (w/v) and stirred for 5 min. The second and third washing cycles were carried out using the same volume of distilled water and a 0.3% NaCl solution, respectively. The mixture was filtered through three layers of cheesecloth and manually dewatered. This was referred to as the WM sample. The water from the first wash was centrifuged at 10,000×g for 20 min at 4°C. Some floating matter, including muscle tissues and fat, were manually removed. The supernatant containing sarcoplasmic proteins was lyophilized and used as the SP sample. All samples were defatted using isopropanol at a ratio of sample to solvent of 1:4 at 40°C for 20 min. Fat extraction was performed three times. Defatted samples were dried in a vacuum oven set at 60°C for 3 h, ground into a powder and passed through an 18-mesh sieve. The defatted powder samples were vacuum-packed and kept at -20°C until use.

Moisture, ash, crude fat and protein content of the defatted samples were determined according to the method of AOAC (2000). Protein expression patterns were determined using SDS-PAGE (Laemmli, 1970). One gram of sample was solubilized in 9 mL of 5% sodium dodecyl sulfate (SDS) solution heated to 95°C. Stacking and separating gels were made of 4 and 10% polyacrylamide, respectively. Protein samples (25 µg) were loaded and gels were run at a constant voltage of 120 V. Gels were stained with 0.125% Coomassie Brilliant Blue R-250 and then destained in a solution containing 25% ethanol and 10% acetic acid.

3.3.4 Production of protein hydrolysates

Dried M, WM, and SP samples were suspended in deionized water (1% w/v) and adjusted to pH 9.0 using 1 M NaOH. The mixtures were incubated at 60°C for 5 min. Lyophilized crude proteinases were added to the mixtures at concentration of 5 U/g of sample. Hydrolysis was carried out at 60°C in a shaking water bath. At each

time interval (0, 2, 4, 8, 12, and 24 h), samples from the mixtures were taken and heated at 90°C for 10 min to terminate proteinase activity. The heated samples were then centrifuged at 10,000×g for 20 min at 4°C. The supernatants were then collected and used for analyses.

3.3.5 Characteristics of protein hydrolysates

3.3.5.1 Degree of hydrolysis (DH)

DH was determined using the TNBS method according to Adler-Nissen (1979). Fifty µL of hydrolysate were mixed with 0.5 mL of 0.2125 M phosphate buffer, pH 8.2 and 0.5 mL of 0.05% TNBS reagent. The reaction mixture was incubated at 50°C for 1 h in a water bath. Subsequently, one mL of 0.1 M HCl was added to stop the reaction, and the mixture was incubated at room temperature for 30 min. The absorbance of the samples was monitored at 420 nm. Leucine was used as a standard. To determine the total amino acid content, the defatted TM powder was hydrolyzed with 6 M HCl at a sample to acid ratio of 1:100 at 120°C for 24 h using an autoclave. The DH was calculated using the following the equation (Benjakul and Morrissey, 1997):

$$\%DH = ([(L_t - L_0)/ (L_{max} - L_0)] \times 100$$

where L_t is the amount of α-amino group released at time t, L_0 is the amount of α-amino group in the supernatant at 0 h, and L_{max} is the total amount of α-amino group obtained after acid hydrolysis.

3.3.5.2 Amino acid analysis by GC-MS

Amino acid profiles of hydrolysates from the M, WM, and SP samples, after 8 h of hydrolysis, were analyzed according to the method of AOAC (2000). In

brief, 0.05-0.10 g of each sample was mixed with performic acid for 16 h in an ice bath to oxidize cysteine and methionine to cysteic acid and methionine sulfone, respectively. Subsequently, sodium metabisulfite was added to decompose the performic acid. Samples were then digested with 6 M HCl at 110°C for 24 h under nitrogen. The digested samples were derivertized with propyl chloroformate. Quantitative analysis of the total amino acid composition was performed using a gas chromatography–mass spectrometry system (GC-MS, Agilent 6890N GC/5973 Inert MSD, Agilent Technologies, Darmstadt, Germany) equipped with a ZB-AAA column (10 m × 0.25 mm I.D., 0.25 µm film thicknesses, Phenomenex, Torrance, USA). The amino acid composition was expressed as g/100 g protein.

3.3.5.3 ACE inhibitory activity

The ACE inhibitory activity analysis was performed using an HPLC according to Cushman and Cheung (1971), with some modifications. Hippuryl-Histidyl-Leucine (HHL) was dissolved in 50 mM sodium borate buffer (pH 8.3) containing 0.5 M NaCl to achieve a final concentration of 8.3 mM. A reaction mixture containing 50 µL of ACE solution (25 mU/mL) and 50 µL of sample were pre-incubated at 37°C for 5 min. Subsequently, 150 µL HHL was added and the mixture was incubated at 37°C for 60 min. The reaction was terminated by the addition of 250 µL of 1 M HCl. The released hippuric acid (HA) was extracted by the addition of 1.5 mL ethyl acetate followed by centrifugation at 800×g and 4°C for 15 min. The upper layer (1 mL) was transferred into a new tube and evaporated at 80°C until dry; then, 1 mL of deionized water was added to the HA. The amount of HA was determined via RP-HPLC on a Zorbax Eclipse XDB C18 column (4.6 mm i.d. × 150 mm, Agilent, Palo Alto, CA) at 25°C. The column was connected to an Agilent 1260 HPLC system. The dissolved

samples were filtered through 0.45-µm syringe filters, and 20 µL was injected. The column was eluted with mobile phase (A) of 0.05% TFA in water and (B) of 0.05% TFA in acetonitrile at flow rates of 0.5 mL/min. The separation was carried out using 20-60% of mobile phase B for the first 13 min, maintained at 60% of mobile phase B for 2 min, and then returned to 20% of mobile phase B for 2 min. The elution was observed at 228 nm. A series of standard HA solutions was prepared to construct a calibration curve. The degree of ACE inhibition was calculated as follows;

$$\text{Degree of inhibition (\%)} = \frac{[(A-B)-(C-D)]}{(A-B)} \times 100$$

where A is the HA concentration of a reaction containing ACE without peptide, B is the HA concentration of a reaction containing ACE previously inactivated by adding HCl in the absence of peptide, C is the HA concentration of a reaction in the presence of ACE and peptide, and D is the HA concentration of a reaction containing ACE previously inactivated by adding HCl in the presence of peptide. The IC_{50} was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity. Specific inhibitory activity was calculated as ACE inhibition (%) divided by total peptide content (mg).

3.3.5.4 ACE inhibition pattern

Since the M hydrolysate prepared by 8 h hydrolysis exhibited the strongest ACE inhibitory activity, the kinetics of ACE inhibition of this sample were investigated. Various substrate concentrations (0.5, 1.0, 3.0, 5.0, and 7.0 mM) were incubated with ACE in the absence or presence of 0.002 and 0.020 mg/mL of the M hydrolysate. Each reaction mixture was assayed as described above. The K_m and V_{max}

values were determined using a Lineweaver-Burk plot. The inhibitor constant (K_i) was determined from the Dixon plot of the slope of Lineweaver-Burk plot against inhibitor concentration.

3.3.6 *In vitro* gastrointestinal digestion

In vitro gastrointestinal (GI) digestion of M hydrolysate samples was performed by the method of Vreeburg et al. (2011) with some modifications. One gram of lyophilized sample was dissolved in 10 mL of 140 mM NaCl and 5 mM KCl, and the pH was adjusted to 2.0 with 6 M HCl. Pepsin (at an enzyme to substrate ratio of 1:35 w/w) was added, and the mixture was incubated in a shaking incubator for 1 h at 37°C. The pH was then adjusted to 5.8 with 1 M $NaHCO_3$. Subsequently, pancreatin (at an enzyme to substrate ratio of 1:25 w/w) and α-chymotrypsin (0.52 U/mg substrate) were added to the mixture. This was followed by the addition of 0.5 mL of a mixture of sodium taurocholate and sodium glycodeoxycholate (176 mM each). The pH of the mixture was then adjusted to 6.5 with 1 M $NaHCO_3$. The headspace was flushed with nitrogen and the sample was subsequently incubated for 2 h at 37°C with shaking. Subsequently, the pH of sample was adjusted to 7.5 with 1 M $NaHCO_3$. The digest, referred to as MH-GI, was filtered through a 0.45 μm cellulose acetate membrane and then flushed with nitrogen and stored at -20°C until use. A solution of 140 mM NaCl and 5 mM KCl was used instead of the M hydrolysate for preparing the *in vitro* digestion control.

3.3.7 Evaluation of the cytotoxicity and permeability to Caco-2 cells

3.3.7.1 Cell culture

Cells from the Caco-2 human colon cancer cell line (American Type Culture Collection, Rockville, MD, USA) were cultured in Dulbecco's Modified

Eagle's Medium (DMEM) containing 4.5 g/L glucose, 4 mM L-glutamine, 25 mM HEPES, and no pyruvate (Gibco BRL, Grand Island, NY, USA) and were supplemented with 10% fetal bovine serum (FBS; Hyclone, Thermo Fisher Scientific, Northumberland, UK). Cells were maintained in a humidified incubator with 5% CO_2 at 37 °C and were sub-cultured once a week when the cells reached 80-90% confluence. Cells were detached using 0.25% trypsin-EDTA (Gibco BRL). Caco-2 cells were used at a passage number between 30 and 40.

The obtained Caco-2 cells were grown in 24-well suspension culture plates on transwell inserts of 33.6 mm^2, 0.4 µm pore size, 1×10^8 pore/cm^2 (Greiner Bio-one, The Netherlands). Cells were seeded with 150 µL of 0.225×10^6 cells/mL per insert (apical side) and 700 µL of DMEM was added to the basolateral side. Cell culture medium was carefully replaced every other day for 21 days, until the Caco-2 cells were fully differentiated as monolayer. Monolayer integrity was measured by transepithelial electrical resistance (TEER) using a MilliCell-ERS volt-ohm meter (Millipore Co., Molsheim, France). Monolayers with TEER values exceeding 200 Ω.cm^2 were used for the cytotoxicity and permeability assays of the M hydrolysate and the MH-GI digest.

3.3.7.2 Cytotoxicity assay

Cytotoxicity was determined using MTT assay. From the selected transwells, medium was removed from the apical and basolateral compartments prior to adding 150 µL of culture medium containing test samples at concentrations of 1, 10, 100, and 1000 µg/mL to the apical compartment and 700 µL of culture medium to the basolateral compartment. The cell culture plates were incubated for 6 h at 37°C in 5% CO_2 in a humidified incubator. TEER values were also monitored after incubation. Both the apical and basolateral media were removed and cells were gently washed with

phosphate-buffered saline (PBS; Gibco BRL, Grand Island, NY, USA). The cells were trypsinized with 50 µL of 0.25% trypsin-EDTA for 10 min at 37°C and 5% CO_2 in air, and then 150 µL of fresh medium was added into the apical compartment. The cell suspension was moved to a 96-well culture plate and centrifuged at 500×g for 5 min and the supernatants were discarded. One hundred microliters of fresh medium containing 0.5 mg/mL MTT was then added into each well and incubated for 2 h at 37°C and 5% CO_2 in air. Subsequently, the plate was centrifuged at 500×g for 5 min and the MTT solution was discarded. Fifty microliters of DMSO:ethanol (1:1) were added into each well, after which, the plate was mildly shaken on a shaker for 5 min. The absorbance was measured at 570 nm using a microplate reader (Tecan Austria GmbH, Groedig, Austria). The relative cell viability was expressed as a percentage relative to the control (non-treated cells).

3.3.7.3 Caco-2 cell-permeability assay

DMEM (without FBS and phenol red) was used as a transport medium in the permeability study. The M hydrolysate or the MH-GI digest (1000 µg/mL for each) was diluted with the transport medium at a ratio of 1:4. Fully differentiated cell monolayers were gently washed twice with the transport medium in both the apical and basolateral compartments. After transferring the transwell inserts to new wells, the cells were pre-incubated with the transport medium for 1 h at 37°C in 5% CO_2 in a humidified incubator. After measuring TEER values and removing the transport medium, 150 µL of each sample (1000 µg/mL in the transport medium) was added to the apical compartment, and 700 µL of the transport medium was added to the basolateral compartment. The cell culture plates were incubated for 6 h at 37°C and 5% CO_2 in air. After measuring the TEER values at the end of the incubation, the peptide

permeates in each basolateral compartment were collected. Milli-Q water and *in vitro* digestion control were added to the transport medium instead of the M hydrolysate and the MH-GI digest, respectively, for preparing the cell permeability controls. Peptide content was estimated according to the TNBS method (Samaranayaka et al., 2010; Xie, Wang, Ao, and Li, 2013). The efficiency of peptide transport was expressed as the percent permeability and was calculated according to the following equation;

$$\text{Permeability (\%)} = \frac{Ps - Pc}{Pi} \times 100$$

where Ps is the peptide content detected in the basolateral compartment of the samples, Pc is the peptide content detected in the basolateral compartment of the control, and Pi is the initial peptide content in the apical compartment. The collected basolateral solutions of the MH-GI digest and its control were lyophilized and used for peptide identification and the determination of ACE inhibitory activity.

3.3.8 Separation and identification of Caco-2 permeated peptides

The lyophilized samples obtained from the basolateral compartment were dissolved in deionized water before being applied to a Superdex peptide 10/300 GL column (10 × 300 mm, GE Healthcare Biosciences Co., Uppsala, Sweden). The column was equilibrated and eluted with deionized water in an isocratic mode at a flow rate of 0.4 mL/min. The eluate was monitored at 215 nm and collected in 0.5 mL fractions. Fractions were pooled and analyzed for ACE inhibitory activity. The pooled fraction showing the highest ACE inhibitory activity was further identified by LC-MS/MS.

Identification of amino acid sequences was determined using an Ultimate 3000 nano LC System (Dionex Ltd., Surrey, UK) coupled with an ESI-Ion Trap MS (HCT

Ultra PTM Discovery System, Bruker, Germany) with electrospray. Peptides were separated on Acclaim PepMap 100 C18 column (3 µm particle size, 100A, 75 µm id × 150 mm) and elution was performed using a linear gradient of 80% acetonitrile containing 0.1% formic acid at a flow rate of 300 nL/min (0-70% for 13 min). The *de novo* amino acid sequence identification was carried out by Pepnovo and can be accessed by the URL http://proteomics.ucsd.edu/LiveSearch/. The identified peptides were subjected to an *in silico* ACE inhibitory activity analysis using the BIOPEP database (http://www.uwm.edu.pl/biochemia). The BLAST program was used for homology searches between the obtained sequences against the non-redundant protein sequence (nr) NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Six identified peptides, including LLP, AHL, PQP, MCS, GTY, and ALSC were chemically synthesized using a solid phase peptide synthesis method (GL Biochem Ltd., Shanghai, China). The purity of the synthesized peptides was higher than 98% as measured by HPLC analysis. ACE inhibitory activity of each synthesized peptide was evaluated as previously described.

3.3.9 Statistical analysis

All the experiments were carried out in triplicate. Results were analyzed using analysis of variance (ANOVA) and means comparison was performed using Duncan's multiple range test (DMRT), at 95% confidence level using a SPSS package (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA).

3.4 Results and discussion

3.4.1 Proximate composition

All samples showed relatively high crude protein contents with WM being the highest ($p < 0.05$, Table 3.1). The samples also showed relatively low crude fat contents, as the majority of the fat was removed by solvent extraction. A decrease in lipid content in the protein hydrolysates may help an increase stability of the material towards lipid oxidation (Ovissipour, Abedian, and Motamedzadegan, 2009). The highest ash content was found in the SP fraction, as soluble inorganic matter was leached out by water washing. Proximate compositions indicated that these muscle protein fractions are a rich source of protein that can be utilized to produce value-added products.

Table 3.1 Proximate compositions (% dry basis) of defatted M, WM, and SP powder.

Compositions	M	WM	SP
Moisture	4.04 ± 0.09^{a}	3.21 ± 0.05^{b}	1.51 ± 0.10^{c}
Crude protein	86.23 ± 0.05^{b}	92.09 ± 0.11^{a}	78.76 ± 0.15^{c}
Ash	5.30 ± 0.01^{b}	1.35 ± 0.06^{c}	17.57 ± 0.12^{a}
Crude fat	1.22 ± 0.01^{a}	0.77 ± 0.07^{b}	0.15 ± 0.05^{c}

Mean ± SD values with different letters in the same rows are significantly different ($p < 0.05$). M is mince; WM is washed mince; SP is sarcoplasmic protein.

3.4.2 SDS-PAGE pattern

Differences in protein expression patterns were noticeable among the samples (Figure 3.1). The M and WM fractions contained myosin heavy chain (MHC) (200 kDa) and actin (45 kDa) as the major proteins. In addition, troponin and tropomysin were detected in both of these protein substrates. Higher band intensities for MHC, actin, troponin, and tropomysin were found in the WM fraction because water washing

concentrates myofilbrillar proteins by removing the water soluble materials. Apart from both the protein substrates, soluble sarcoplasmic proteins of tilapia contained various proteins with molecular weights ranging from 16 to 97 kDa. The most abundant protein bands were 97 kDa, a doublet band at 61 kDa, a 51 kDa (enolase), a 51-41 kDa huge band (creatine kinase and aldolase), a 36 kDa (glyceraldehydephosphate dehydrogenase, GAPGH), a 28, 27, 25, and 16 kDa were supplementary bands.

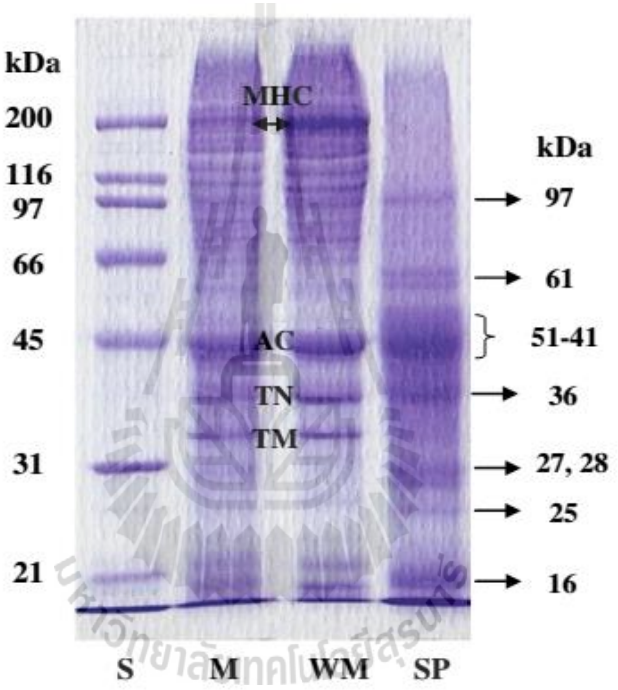

Figure 3.1 SDS-PAGE pattern of defatted mince (M), washed mince (WM), and sarcoplasmic protein (SP). S, standard markers; MHC, myosin heavy chain; AC, actin; TN, troponin; TM, tropomyosin.

3.4.3 Degree of hydrolysis (DH)

The DH of the 3 different protein substrates rapidly increased in the first 4 h and reached a plateau after 8 h of hydrolysis (Figure 3.2). This decrease in the reaction rate may be due to reduced enzyme activity and/or a decrease in peptide bonds available for

hydrolysis (Guerard, Guimas, and Binet, 2002). The M hydrolysate showed the highest DH throughout the course of hydrolysis ($p < 0.05$). This might be due to a broad specificity of the *V. halodenitrificans* SK1-3-7 proteinases. The M fraction was composed of myofibrillar, sarcoplasmic, and stroma proteins, while the W and SP fractions mainly consisted of myofibrillar and sarcoplasmic proteins, respectively. Moreover, the lowest DH was observed in the SP hydrolysate, which may be attributed to proteinase inhibitors present in the SP fraction. Piyadhammaviboon and Yongsawatdigul (2010) found that proteinase inhibitors contained in the sarcoplasmic protein fraction of threadfin bream were able to inhibit serine proteinases, especially trypsin. Siriangkanakun and Yongsawatdigul (2012) also found proteinase inhibitors in the sarcoplasmic protein fraction of common carp.

From the DH profile, each hydrolysate at 2, 4, and 8 h of hydrolysis were selected to evaluate the effect of DH and protein substrates on ACE inhibitory activity.

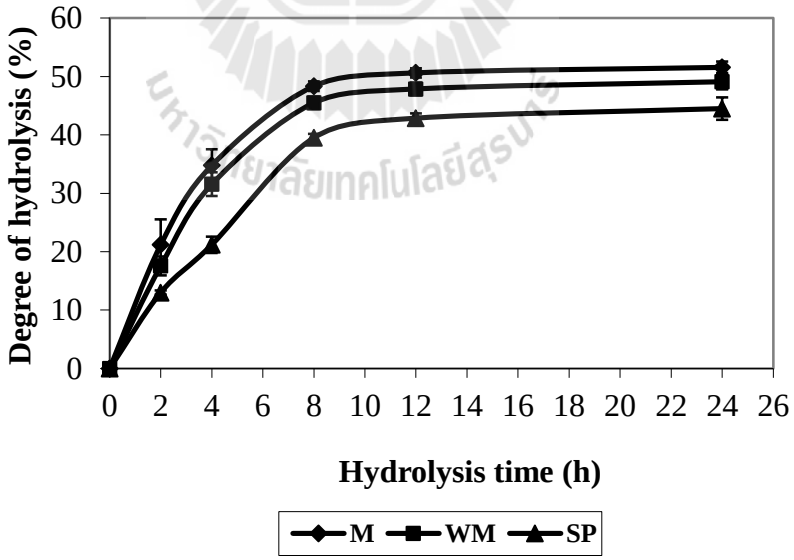

Figure 3.2 Degree of hydrolysis of mince (M), washed mince (WM), and sarcoplasmic protein (SP) hydrolysates at different hydrolysis times. Values are expressed as the means ± S.D. (n = 3).

3.4.4 ACE inhibitory activity of hydrolysates

The ACE inhibitory activity of all hydrolysates increased with hydrolysis time (Figure 3.3). The hydrolysates with higher DH contained more low-molecular weight peptides and exhibited higher ACE inhibitory activities. This is consistent with the results of Jamdar et al. (2010) who reported that changes in amino acid composition, as affected by DH, could also modulate the biological activity of the peptides formed during hydrolysis. Balti et al. (2010) found that the undigested cuttlefish protein exhibited lower ACE inhibitor activity (approximately 1.2%) and that a higher ACE inhibition activity was obtained with an increase in DH of 8%. After 8 h of hydrolysis, the M hydrolysate (48% DH) exhibited the highest ACE inhibitory activity, with an IC_{50} value of 0.54 mg/mL, followed by the W (45% DH) and SP (39% DH) hydrolysates with IC_{50} value of 0.76 mg/mL and 1.15 mg/mL, respectively ($p < 0.05$).

The M hydrolysate was richer in Lys, Tyr, and Glu and in hydrophobic amino acids, including Phe, Leu, and Ile, than the other hydrolysates (Table 3.2). Moreover, all protein hydrolysates contained low levels of Hly, Hyp, and Arg. Both Hly and Hyp are typically found in collagen, which is less significant in fish muscle than in that of land animals. Based on total amino acid content, all hydrolysates showed a high amount of essential amino acids. Therefore, they could serve as an excellent source of nutrients. It has been reported that the most effective ACE-inhibiting peptides identified contain Tyr, Phe, Trp, and/or Pro at the C-terminus (Byun and Kim, 2002). Gómez-Ruiz, Recio, and Belloque (2004) have suggested that Leu may contribute significantly to an increase in ACE-inhibitory potential. Furthermore, other branched chain aliphatic amino acids, such as Ile and Val, are predominant in peptide inhibitors. The presence of hydrophobic amino acids and positively charged amino acids (Lys and Arg) also contribute to a

peptide's inhibitory potency (Guang and Phillips, 2009). Because the M hydrolysate, after 8 h of hydrolysis and with 48% DH, showed the most potent ACE inhibitory activity, it was chosen for further evaluation.

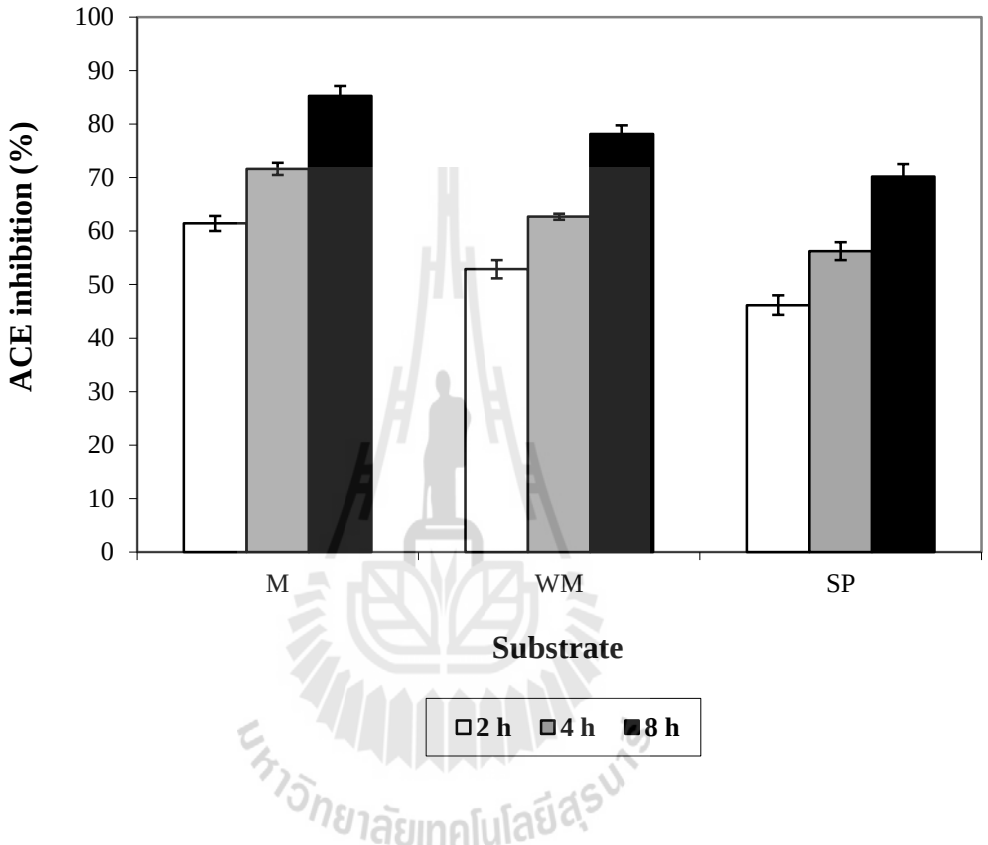

Figure 3.3 ACE inhibitory activity of protein hydrolysates derived from tilapia muscle protein fractions (M: mince, WM: washed mince, SP: sarcoplasmic protein) hydrolyzed by *V. halodenitrificans* SK1-3-7 proteinases for 2, 4, and 8 h. The hydrolysates had a peptide concentration equivalent to 9 mM leucine, as determined by the TNBS method. Values are expressed as the means ± S.D. (n = 3).

Table 3.2 Amino acid composition (g/100 g protein) of hydrolysates from various muscle fractions of tilapia after 8 h of hydrolysis.

Amino acid	MH	WMH	SPH
Ala	2.75	5.56	6.06
Arg	0.22	0.47	0.49
Asp	3.25	3.56	4.94
Cys	2.42	4.75	4.23
Glu	7.13	6.63	5.77
Gly	3.53	4.93	4.06
His[a]	5.39	6.01	5.69
Hly	0.01	0.01	0.01
Hyp	0.01	0.01	0.01
Ile[a]	6.33	5.67	4.10
Leu[a]	11.14	8.11	6.86
Lys[a]	23.12	18.05	14.35
Met[a]	2.04	2.87	2.96
Phe[a]	16.08	10.67	15.37
Pro	2.12	6.79	8.42
Ser	0.55	2.23	2.76
Thr[a]	0.94	2.02	2.22
Trp[a]	1.00	1.75	1.68
Tyr[a]	7.54	4.45	3.79
Val[a]	4.46	5.44	6.02
TEAA[b]	78.04	65.04	63.04
Total	100	100	100

Note: MH is mince hydrolysate; WMH is washed mince hydrolysate; SPH is sarcoplasmic protein hydrolysate; [a] Essential amino acid according to FAO/WHO (1975); [b] Total essential amino acids.

3.4.5 ACE inhibition mode

The M hydrolysate, after 8 h of hydrolysis, showed a mixed-type inhibition characteristic to ACE (Figure 3.4). This indicated that peptides presenting in the M hydrolysate can bind at the active site (competitive) and allosteric site (non-competitive) of ACE. The M hydrolysate composed of mixture of peptides with a wide range of amino acid sequences. However, the structure-activity relationship of food-derived ACE inhibitory peptides has not been fully established. The different structure of the inhibitory peptides can lead to various ACE inhibition patterns. Most of isolated peptides from food protein hydrolysates showed a competitive inhibitor characteristic (Chen et al., 2012; Rawendra et al., 2013; Wu and Ding, 2002). Noncompetitive, uncompetitive, or mixed ACE inhibitors have also been reported (Asoodeh et al., 2012; Li et al., 2004; Sheih et al., 2009; Tsai, Chen, and Pan, 2008; Wang et al., 2008). In this study, K_m of ACE was determined to be 1.24 µM and V_{max} was 0.30 µM/min. In the presence of 0.002 and 0.020 mg/mL hydrolysate, K_m^{app} values were 1.31 and 1.60 µM, with V_{max}^{app} values were 0.27 and 0.24 µM/min, respectively. The K_i value of the M hydrolysate was 0.041 mg/mL, which was less effective compared to the mixed inhibitor derived from hard clam hydrolysate with the K_i value of 0.027 mg/mL (Tsai et al., 2008). Higher value of K_i indicates a lower affinity between enzyme and inhibitor.

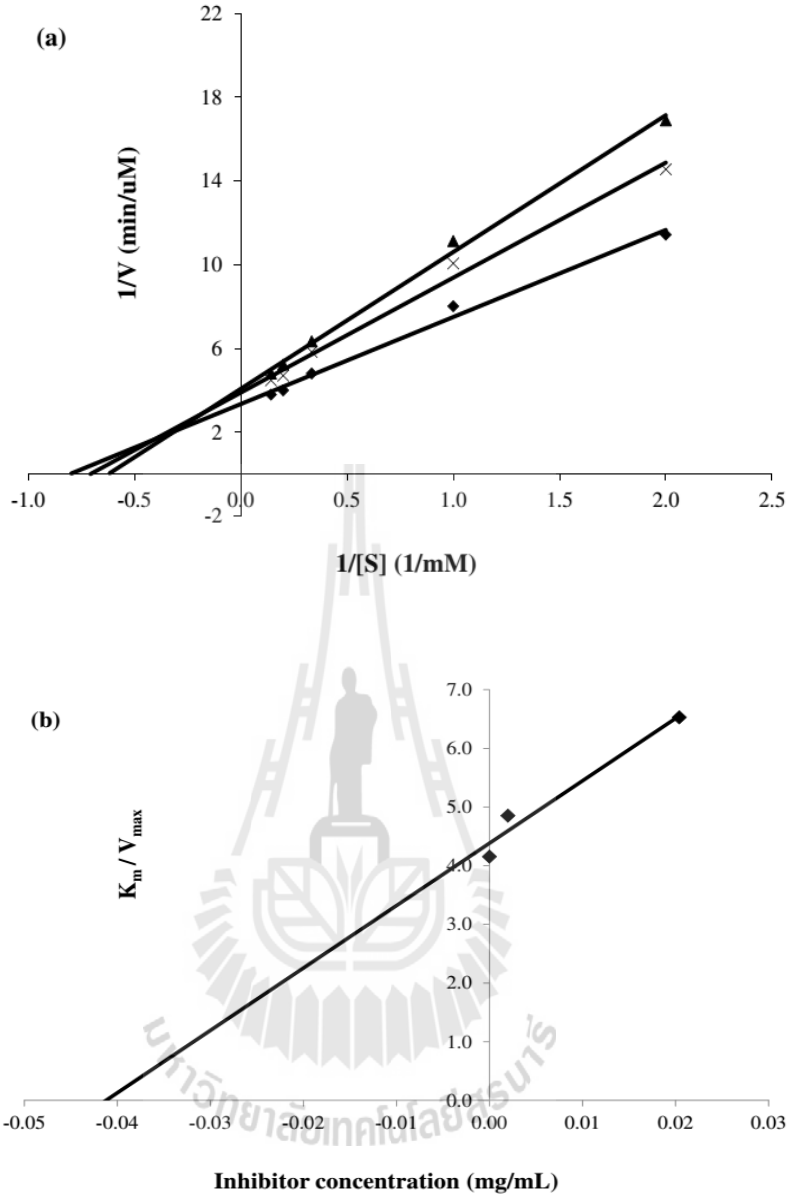

Figure 3.4 (a) Lineweaver-Burk plot of ACE inhibition by mince hydrolysate after 8 h of hydrolysis. The ACE activities were measured in the absence and presence of the hydrolysate (♦, control; ×, 0.002; ▲, 0.020 mg/mL). 1/V and 1/S represents the reciprocal of velocity and substrate, respectively. (b) The Dixon plot for measuring the inhibitor constant (K_i).

3.4.6 Permeability of tilapia hydrolysate across Caco-2 cells

The M hydrolysate and its digest (MH-GI) showed comparable effects on Caco-2 cell viability at concentrations ranging 1-1000 µg/mL ($p > 0.05$, Figure 3.5a). In addition, the integrity of the monolayers was maintained at concentrations up to 1000 µg/mL, as observed by the relative TEER values (Figure 3.5b). Thus, a nontoxic concentration (1000 µg/mL) of the M hydrolysate and the MH-GI digest was used in Caco-2 cell-permeability assay.

In this study, DMEM (without FBS and phenol red) was used as a transport medium instead of Hank's balanced salt solution (HBSS). Although many studies have used HBSS as a transport medium (Cinq-Mars et al., 2008; Fernández-Musoles et al., 2013), some negative effects of HBSS on the integrity of cell monolayers have been reported. The cell monolayer integrity was negatively affected by HBSS medium, either non-buffered or buffered with HEPES, resulting in decreased TEER values (< 200 $\Omega.cm^2$), which indicates a lack of tight biological barriers (Toydermir et al., 2013). In contrast, DMEM medium, either with or without a supplement of 9.1% FBS, provided good tight junctions in Caco-2 cell monolayers (Toydermir et al., 2013). As proteins in FBS and phenol red could interfere with determination of peptide concentration in the basolateral compartment, they were not included in the DMEM used in this study.

The permeability of peptides in the MH-GI digest was found to be 14.8 ± 0.7%, which was higher than that of the original hydrolysate (10.6 ± 0.9%), indicating that simulated GI digestion increased the permeability of the peptides. The enhancement of permeability might be due to the smaller peptides generated after the sequential digestions of pepsin and pancreatin. This is consistent with the results of Cinq-Mars et al. (2008) who reported that the percent of apical-to-basolateral transport of peptides in

Pacific hake fillet hydrolysate was less than 20% and that simulated GI digestion increased the permeability of peptides. Guang, Shang, and Jiang (2012) found that the peptide permeability of *in vitro* digested traditional Chinese pimple milk was 7.8%, which was higher than that for its non-digested counterpart (5.5%). Caco-2 cell monolayers are known to be tighter than *in vivo* human intestinal cells (Vermeirssen, Camp, and Verstraete, 2004). Therefore, *in vivo* peptide absorption is expected to be higher than that obtained from Caco-2 cell monolayers.

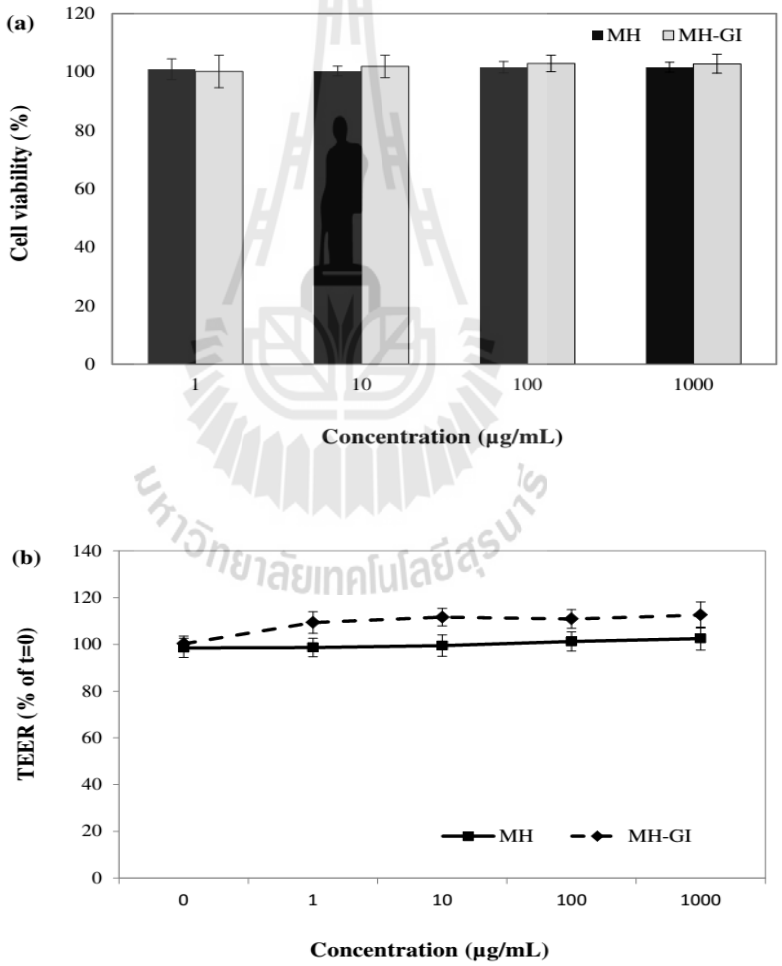

Figure 3.5 (a) Viability of Caco-2 cell monolayers using MTT assay and (b) TEER values after exposure with mince hydrolysate (MH) and MH-GI for 6 h. Values represent the mean ± SD (n = 3).

3.4.7 Identification of permeable ACE inhibitory peptides

Size exclusion chromatograms of peptide and DMEM permeates collected from the basolateral compartment showed similar profiles, with only a small peak of P3 being observed in the peptide permeate (Figure 3.6a and b). In addition, only P3 exhibited ACE inhibitory activity. Retention times of P1, P2, and P4 peaks corresponded with those of M1, M2, and M3 of the DMEM permeates. These three peaks were likely peptides in the DMEM that passed through the Caco-2 cell monolayers, while P3 represents peptides derived from the MH-GI digest.

The ACE inhibitory potency of the M hydrolysate, the MH-GI digest, and the permeable peptides are compared in Table 3.3. Pepsin-pancreatin digestion appeared to decrease ACE inhibitory activity. Hydrolysis of ACE inhibitory peptides into smaller peptides or free amino acids during *in vitro* GI digestion can result in a decrease in ACE inhibitory potency (Samaranayaka et al., 2010). Wu and Ding (2002) found that IC_{50} value of soy protein hydrolysate increased from 65 µg/mL to 73 µg/mL after *in vitro* pepsin-pancreatin digestion. Moreover, Hernandez-Ledesma, Amigo, Ramos, and Recio (2004) reported that the ACE inhibitory activity of whey infant formula hydrolysate significantly decreased at the intestinal stage of simulated GI digestion. However, the permeable peptides in P3 exhibited a higher specific inhibitory activity than that of the MH-GI digest. Further digestion of the MH-GI digest by brush-border membrane peptidases is expected to take place, forming peptides with structural features common of ACE inhibitors.

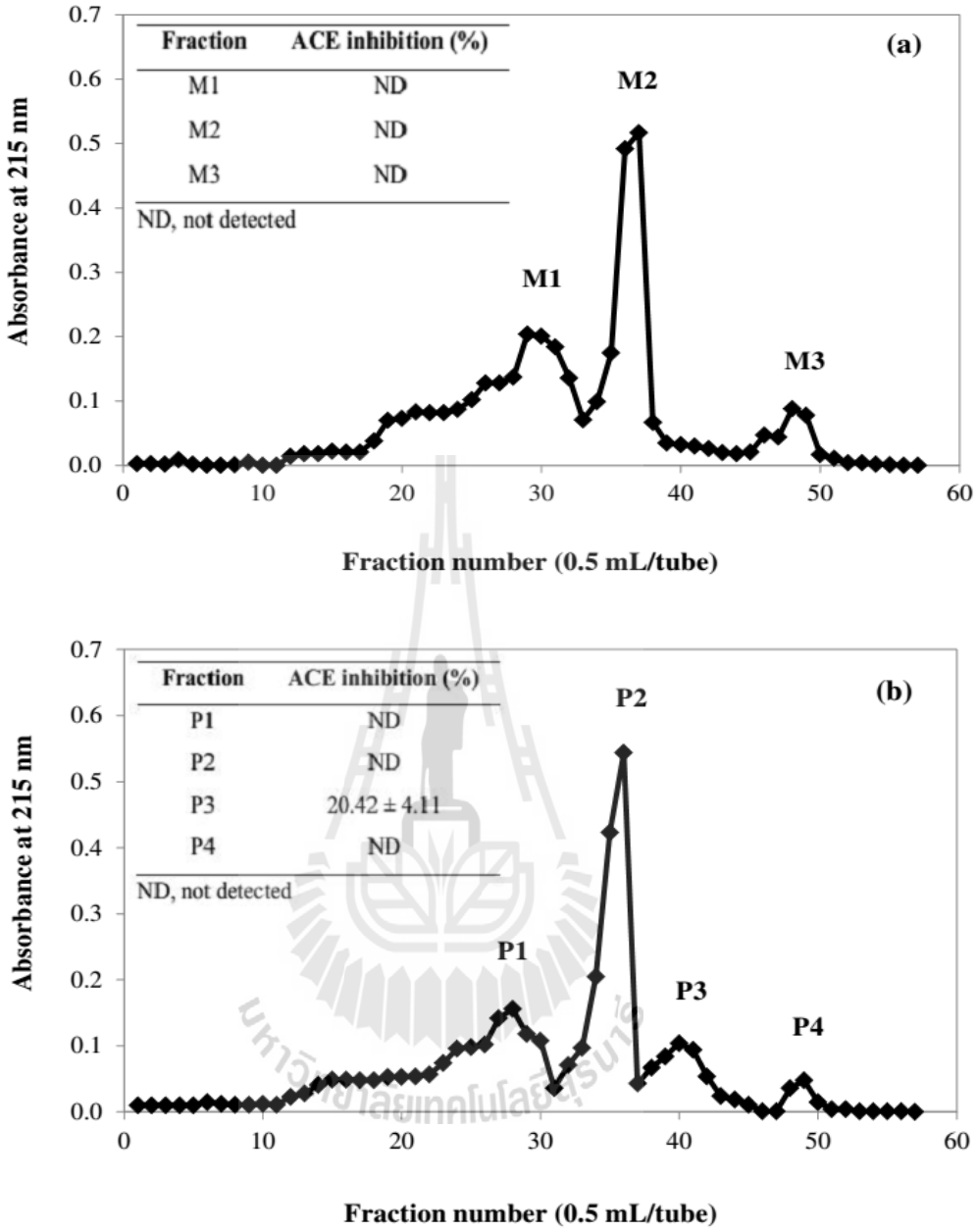

Figure 3.6 Size exclusion chromatograms of (a) the collected basolateral solution containing DMEM as a control and (b) the collected basolateral solution containing peptides of MH-GI. ACE inhibitory activity of each peak is illustrated in the left panels.

Table 3.3 Changes in ACE inhibitory activity after *in vitro* gastrointestinal digestion and permeation across Caco-2 cell monolayers.

Sample	ACE inhibition (%)	Peptide content (mg)	Specific inhibitory activity (%/mg peptide)
Mince hydrolysate	89.34	0.45	198.53 ± 1.35^{a}
MH-GI	56.05	0.45	124.56 ± 4.02^{c}
Permeated peptides in P3 fraction	20.42	0.12	170.17 ± 6.11^{b}

Mean ± SD values with different letters are significantly different ($p < 0.05$).

Based on *de novo* sequencing, 6 peptides were identified in the P3 fraction. The identified peptides contained 3-4 amino acids. Amino acid sequence homology analyses of the identified peptides with protein BLAST analysis revealed a high homology with proteins from skeletal muscle. It has been demonstrated that di- and tri peptides, especially those with C-terminal proline or hydroxylproline residues, are generally resistant to degradation by digestive enzymes (Vermeirssen et al., 2004). The well documented ACE inhibitory milk peptides IPP and VPP were readily transported across the intestinal epithelium when subjected to *in vitro* permeability studies (Foltz et al., 2008). It was reported that IPP is not digested by gastrointestinal enzymes and that it can enter the circulatory system of humans (Hata, Yamamoto, Ohni, Nakajima, Nakamura, and Takano, 1996). There are three mechanisms for transepithelial oligopeptide transport in the intestinal tract, namely, H^{+}-dependent peptide transporter 1 (PepT1) carriers, the paracellular pathway through intercellular junctions, and absorptive transcytosis (Miguel et al., 2008). Some oligopeptides, such as FVNPQAGS and GGYR, have been found to resist intestinal cellular peptidases, and GGYR is transported mainly via the paracellular pathway without being hydrolyzed by peptidases (Megías et al., 2009; Shimizu, Tsunogai, and Arai, 1997). In addition, YPI

was absorbed as an intact molecule through the PepT1 system (Miguel et al., 2008). Permeation of tilapia peptides across intestinal epithelial cells as it related to their structure, deserves further investigation.

This study revealed that cell monolayer permeates from the MH-GI digest were mainly tripeptides and tetrapeptide (Table 3.4). When these peptides were synthesized, they all showed ACE inhibitory activity, with MCS being the most potent inhibitory peptide (Table 3.4). The tripeptide MCS appears to be a novel ACE inhibitory peptide. Other tripeptides also showed relatively higher inhibitory activities compared to that of the tetrapeptide. The presence of hydrophobic amino acids with aromatic or branched side chains, including Pro (P), Ala (A), Leu (L), Tyr (Y), and Met (M) seemed to positively influence the ACE inhibitory activity of these synthetic peptides. The TPTQQS peptide was found to be a non-competitive inhibitor. Its C-terminal Ser (S) pushes the zinc ion away from the active site through the coordination bonds between Ser and the zinc ion, resulting in the conformational change of the ACE active site (Ni, Li, Liu, and Hu 2012). The serine residue of the tripeptide MCS could have a similar effect on the ACE active site. It should be noted that the novel tripeptide MCS showed a stronger ACE inhibition than that of the other tripeptides isolated from fish muscle proteins reported thus far, including IWH derived from dried bonito (IC_{50} value of 3.5 μM) (Fujita, Yokoyama, and Yoshikawa, 2000), and MAW from cuttlefish muscle (IC_{50} value of 16.32 μM) (Balti et al., 2010).

The relationships between the structure and activity of ACE inhibitory peptides has not yet been completely established thus far (Ryan, Ross, Bolton, FitzGerald, and Stanton, 2011). The C-termini of peptides have been suggested to be a controlling factor of the ACE inhibitory activity via interactions with the S_1, S_1', and S_2' subsites at the active site of ACE (Ondetti and Cushman, 1982), which typically contains hydrophobic

amino acid residues. Moreover, many studies have shown that di- or tripeptides with high potent inhibitory activities have Trp (W), Phe (F), Tyr (Y), or Pro (P) at the C-terminus and a branched aliphatic amino acid at the N-terminus (Li et al., 2004). The tripeptides of LKP from dried bonito (Fujita and Yoshikawa, 1999), VKP from freshwater clams (Tsai, Lin, Chen, and Pan, 2006), VPP from sour milk (Nakamura, Yamamoto, Sakai, Okubo, Yamazaki, and Takano, 1995), RIY from rapeseed (Marczak et al., 2003), VYP from cheese (Abubakar, Saito, Kitazawa, and Itoh, 1998), and VAP from grass carp (Chen et al., 2012) were also all reported to possess ACE inhibitory activity. Our results demonstrate that the tripeptides LLP and PQP show common structural features that are shared with previously reported potent ACE inhibitors.

Table 3.4 Amino acid sequence of peptides that permeated Caco-2 cell monolayers using a *de novo* peptide sequencing algorithm.

Amino acid sequence	**Mass (Da)**	**IC_{50} (μM)**	**Parent protein[a]**	**ACE inhibitory sequences[b]**
LLP	341.80	0.41	Titin fragment (chicken, *Gallus gallus*)	LLP
AHL	345.50	1.23	Myosin heavy chain (rainbow trout, *Oncorhynchus mykiss*)	AH, HL
PQP	341.50	0.47	Collagen alpha 1(I) chain precursor (chicken, *Gallus gallus*)	PQ
MCS	340.39	0.29	Calpain inhibitor (calpastatin) (*Bos taurus*)	-
GTY	340.29	9.77	Titin fragment (chicken, *Gallus gallus*)	GT
ALSC	393.35	10.99	Beta-2 adrenergic receptor (rainbow trout, *Oncorhynchus mykiss*)	-

[a] Parent protein was obtained from BIOPEP database and NCBI database via BLAST program; [b] ACE inhibitory sequences were reported in the literatures from BIOPEP database.

3.5 Conclusions

The whole mince fraction of tilapia hydrolyzed by *Virgibacillus halodenitrificans* SK1-3-7 proteinases yielded the hydrolysate with the highest ACE inhibitory activity compared to the myofibrillar and sarcoplasmic fractions. Although *in vitro* gastrointestinal digestion decreased the potency of the ACE inhibition of the protein hydrolysate, it enhanced the permeability of the peptides across Caco-2 cell monolayers. More importantly, the permeated peptides still exerted ACE inhibitory activity. A tripeptide that permeated Caco-2 cells and exhibited the most potent ACE inhibition was identified to be MCS, which has not previously been described with these properties. Therefore, *V. halodenitrificans* SK1-3-7 proteinases-hydrolyzed tilapia mince could be a functional food ingredient containing ACE inhibitor peptides after *in vitro* GI digestion and permeation across intestinal epithelial (Caco-2) cells.

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CHAPTER IV

CHARACTERIZATION AND IDENTIFICATION OF ANGIOTENSIN I-CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES FROM TILAPIA PROTEIN HYDROLYSATE

4.1 Abstract

Angiotensin I-converting enzyme (ACE) inhibitory activity of tilapia mince (TM) hydrolyzed by *Virgibacillus halodenitrificans* SK1-3-7 proteinases with a 48% degree of hydrolysis showed the highest ACE inhibitory activity with an IC_{50} value of 0.54 mg/mL. After ultrafiltration (UF) and chromatographic separation via anion exchange, cation exchange and size exclusion chromatography, the fraction with the highest ACE inhibitory activity with an IC_{50} value of 0.15 mg/mL was obtained. The peptides showed uncompetitive inhibition characteristics with an inhibition constant (K_i) of 0.18 mg/mL. The peptides showed high thermostability at 100 and 121°C, as well as pH stability at a wide pH range of 2-10, and also maintained ACE inhibitory activity after *in vitro* gastrointestinal digestion. The most potent novel ACE inhibitory peptide identified was MILLLFR with an IC_{50} value of 0.12 µM. TM prepared by *V. halodenitrificans* SK1-3-7 proteinases could serve as a source of peptides with ACE inhibitory activity.

Keywords: ACE inhibitory peptides, tilapia muscle, purification, stability

4.2 Introduction

Hypertension has been considered as the most common serious chronic health problem. Angiotensin I-converting enzyme (ACE) is the key enzyme that is involved in the regulation of hypertension. The inhibition of ACE activity can lead to an overall antihypertensive effect (Khantaphant, Benjakul, and Kishimura, 2011). More recently, many ACE inhibitory peptides have been isolated from enzymatic hydrolysis of both animal and plant protein sources (Guang and Phillips, 2009; Vercruysse, Camp, and Smagghe, 2005). Among them, fish protein is an excellent source of ACE inhibitory peptides (Charoenphun, Youravong, and Cheirsilp, 2013; Chen, Wang, Zhang, Wu, and Xia, 2012; Raghavan and Kristinsson, 2009; Wijesekara, Qian, Ryu, Ngo, and Kim, 2011).

Biological activities of protein hydrolysates are related to the amino acid composition and sequence, size, and configuration of peptides (Matsui, Yukiyoshi, Doi, Sugimoto, Yamada, and Matsumoto, 2002). The separation and the identification of peptides and amino acids have to be studied in order to obtain better knowledge about the composition of protein hydrolysates (Silvestre, 1997). Amino sequencing and characterization of individual peptides could also be important to elucidate mechanism of an ACE inhibitory peptide (Raghavan, Kristinsson, and Leeuwenburgh, 2008). To date, the attempt to identify and characterize ACE inhibitory peptides derived from various protein hydrolysates to access the structure-activity relationship has increased. However, the structure-activity relationship of ACE inhibitory peptides has not yet been established due to a large variety of ACE inhibitory peptides with different amino acid sequences have been identified.

Functional, nutritional and biological properties of peptides in the food matrix are highly influenced by molecular structure, interactions with other components and the conditions of processing (Hwang, 2010). Gastrointestinal (GI) digestion is of particular importance in the bioavailability of ACE inhibitory peptides. After oral ingestion, gastrointestinal enzymes may break up peptides, thereby increasing or decreasing their activity. Study of the stability of bioactive peptides during processing and after GI digestion pertaining to the effects on their ACE inhibitory activity would be of critical for application point of view.

Previous study revealed that tilapia muscle hydrolysate with ACE inhibitory activity can be obtained through the use of *Virgibacillus halodenitrificans* SK1-3-7 proteinases. The objective of this study was to purify ACE inhibitory peptides from tilapia muscle protein hydrolysate prepared by *V. halodenitrificans* SK1-3-7 proteinases. In addition, stability of purified peptides was evaluated with respect to temperatures, pHs and simulated digestion by gastrointestinal enzymes.

4.3 Materials and methods

4.3.1 Materials

Fresh tilapia (*Oreochromis niloticus*) were purchased from a local market in Nakhon Ratchasima, Thailand. Fish were stored in ice and transported to laboratory at Suranaree University of Technology within 30 min and processed immediately upon arrival. *V. halodenitrificans* SK1-3-7 isolated from Thai fish sauce fermentation was used as the source of proteinase. Details of *V. halodenitrificans* SK1-3-7 production were described in Chapter III (section 3.3.2). *V. halodenitrificans* SK1-3-7 proteinases activity was measured at 450 nm using azocasein as a substrate. One unit (U) of

proteinase activity was defined as the amount of enzyme that produced an absorbance change of 0.01 per min at 450 nm under the condition of 60°C and pH 9.0 for 60 min. Angiotensin I-converting enzyme (ACE) from rabbit lungs, hippuryl-histidyl-leucine (HHL), hippuric acid (HA), pepsin from porcine stomach mucosa, pancreatin from porcine pancreas and trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile and trifluoroacetic acid (TFA) were of HPLC grade. All other reagents and chemicals used were of analytical grade.

4.3.2 Preparation of protein hydrolysate

Whole fish were filleted, deskinned, and minced using a meat grinder (Model 8-22, Marblehead, Ohio, USA) with a screen size of 1.5 mm perforation plate. Tilapia mince (TM) was defatted using isopropanol at a sample to solvent ratio of 1:4 at 40°C for 20 min. Fat extraction was performed for three times. Defatted TM was dried for 3 h in a vacuum oven at 60 °C, then ground into powder and passed through an 18-mesh sieve. The defatted TM powder was vacuum-packed and kept at -20°C until use.

Defatted TM powder was suspended in deionized water (1% w/v) and adjusted to pH 9.0 using 1 M NaOH. The mixture was incubated at 60°C for 5 min, and lyophilized crude proteinases were added to the mixture at concentration of 5 U/g TM powder. Hydrolysis was carried out at 60°C for 8 h in a shaking water bath. The hydrolysate was heated at 90°C for 10 min to terminate proteinase activity and centrifuged at 10,000 × g at 4°C for 20 min. The supernatant was referred to as TM hydrolysate. Degree of hydrolysis was determined according to the TNBS method (Adler-Nissen, 1979) as described in Chapter III (section 3.3.5.1).

4.3.3 Determination of ACE inhibitory activity

The ACE inhibitory activity analysis was performed using an HPLC according to Cushman and Cheung (1971) with some modifications. Hippuryl-Histidyl-Leucine (HHL) was dissolved in 50 mM sodium borate buffer (pH 8.3) containing 0.5 M NaCl to achieve a final concentration of 8.3 mM. A reaction mixture containing 50 µL of ACE solution (25 mU/mL) and 50 µL of sample were pre-incubated at 37°C for 5 min. Subsequently, 150 µL HHL was added and the mixture was incubated at 37°C for 60 min. The reaction was terminated by the addition of 250 µL of 1 M HCl. The released hippuric acid (HA) was extracted by the addition of 1.5 mL ethyl acetate followed by centrifugation at 800 × g and 4°C for 15 min. The upper layer (1 mL) was transferred into a new tube and evaporated at 80°C until dry, then 1 mL of deionized water was added to the HA. The amount of HA was determined via RP-HPLC on a Zorbax Eclipse XDB C18 column (4.6 mm i.d. × 150 mm, Agilent, Palo Alto, CA, USA) at 25°C. The column was connected to an Agilent 1260 HPLC system. The dissolved samples were filtered through 0.45 µm syringe filters and 20 µL were injected. The column was eluted with mobile phase (A) of 0.05% TFA in water and (B) of 0.05% TFA in acetonitrile at a flow rate of 0.5 ml/min. The separation was carried out using 20-60% of mobile phase B for the first 13 min, maintained at 60% of mobile phase B for 2 min, and then returned to 20% of mobile phase B for 2 min. The elution was monitored at 228 nm. A series of standard HA solutions was prepared to construct a calibration curve. The degree of ACE inhibition was calculated as follows:

$$\text{Degree of inhibition (\%)} = \frac{[(A-B)-(C-D)]}{(A-B)} \times 100$$

where A is the HA concentration of a reaction containing ACE without peptide, B is the HA concentration of a reaction containing ACE previously inactivated by adding HCl in the absence of peptide, C is the HA concentration of a reaction in the presence of ACE and peptide, and D is the HA concentration of a reaction containing ACE previously inactivated by adding HCl in the presence of peptide. The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity. Specific inhibitory activity was calculated from ACE inhibition (%) divided by total peptide content (mg).

4.3.4 Purification of ACE inhibitory peptides

The TM hydrolysate exhibiting the highest ACE inhibitory activity, after 8 h of hydrolysis (48% DH), was fractionated by ultrafiltration (Pall Corporation, Ann Arbor, MI, USA) using a 30-kDa molecular-weight-cut-off (MWCO) membrane, followed by a 5-kDa MWCO membrane to obtain 3 fractions corresponding to molecular weight (MW) below 5 kDa, between 5 and 30 kDa, and above 30 kDa. Each fraction was assayed for ACE inhibitory activity. Yield (%) was calculated based on peptide content. The fraction with the highest specific inhibitory activity was lyophilized and stored at -20°C for further purification. Unless otherwise indicated, the peptide content was determined by measuring the absorbance at 215 nm using the lyophilized 5-kDa permeate fraction as a standard. Based on the absorbance at 215 nm, the purity of the standard was more than 98% as compared with the synthesized peptide.

The 5-kDa permeate exhibiting the highest specific inhibitory activity was purified using fast protein liquid chromatography (FPLC; AKTA, GE Healthcare Bioscience Co., Uppsala, Sweden). The lyophilized fraction was dissolved in 50 mM Tris-HCl buffer (pH 7.5), loaded onto a diethylaminoethyl (DEAE)-Sephacel ion

exchange column (2.6 × 6.5 cm, GE Healthcare Biosciences AB, Uppsala, Sweden) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) and then eluted with a linear gradient of NaCl (0-1.0 M) in the same buffer at a flow rate of 1.0 mL/min. Each fraction (5 mL) was monitored at 215 nm. The fractions were pooled and analyzed for ACE inhibitory activity. The strongest specific inhibitory activity fraction was lyophilized and used for further purification. The active fraction was dissolved in 10 mM sodium acetate buffer (pH 4.0) and loaded onto a carboxymethyl (CM)-Sepharose column (2.6 × 6.5 cm, GE Healthcare Biosciences AB) equilibrated with 10 mM sodium acetate buffer (pH 4.0) and eluted with a linear gradient of NaCl (0-1.0 M) in the same buffer at a flow rate of 1.0 mL/min. The pooled fraction possessing the highest specific inhibitory activity was lyophilized and dissolved in deionized water before applying onto a Superdex peptide 10/300 GL column (10 × 300 mm, GE Healthcare Biosciences AB). The column was equilibrated and eluted with deionized water in an isocratic mode at a flow rate of 0.3 mL/min. The eluate was collected in 0.5 mL fractions. The fraction showing the highest specific inhibitory activity was further analyzed by LC-MS/MS.

4.3.5 LC-MS/MS

The amino acid sequence of the fraction was determined using the Ultimate 3000 LC System (Dionex Ltd., Surrey, UK) coupled to an ESI-Ion Trap MS (HCT Ultra PTM Discovery System, Bruker, Germany) with electrospray. Peptides were separated on a nanocolumn (Acclaim PepMap 100 C18, 3 µm, 100A, 75 µm id × 150 mm). Eluent A was 0.1% formic acid in water. Eluent B was 80% acetonitrile in water containing 0.1% formic acid. Elution was performed using a linear gradient from 0 to 70% of eluent B at a flow rate of 300 nL/min for 13 min. The *de novo* amino acid sequence was determined by Pepnovo, which can be accessed via the URL

http://proteomics.ucsd.edu/LiveSearch/. The identified peptides were subjected to *in silico* ACE inhibitory activity analysis using the BIOPEP database (http://www.uwm.edu.pl/biochemia). The BLAST program was used for homology searches between the obtained sequences against non-redundant protein sequences (nr) database from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.3.6 Peptide synthesis

Based on their Pepnovo scores, six out of 10 peptides identified from LC-MS/MS were chosen and chemically synthesized using a solid phase peptide synthesis method (GL Biochem Ltd., Shanghai, China). The purity of the synthesized peptides was greater than 98% as determined via HPLC analysis. The molecular mass of the synthesized peptides was confirmed by the manufacturer using liquid chromatography coupled to a mass spectrometer (LC-MS/ESI). The ACE inhibitory activity of each synthesized peptide was determined as previously described.

4.3.7 Stability of ACE inhibitory peptides

4.3.7.1 Effect of temperature

The purified peptides (0.75 mg/mL) were incubated separately at 20, 40, 60, 80, and 100°C for 2 h and at 121 °C for 15 min. Before the ACE inhibitory activity was determined, the samples were allowed to cool to room temperature (25°C) and the pH value was adjusted to 8.3.

4.3.7.2 Effect of pH

The purified peptides (0.75 mg/mL) were incubated at 40°C under various pH values of 2, 4, 6, 8 and 10 for 2 h. Subsequently, pH values were adjusted to 8.3 before ACE inhibitory activity was determined.

4.3.7.3 Effect of *in vitro* gastrointestinal digestion

The stability of the ACE inhibitory peptides when challenged with *in vitro* gastrointestinal digestion was assessed by mixing a peptide solution (10 mg/mL) in 0.1 M KCl-HCl (pH 2.0) buffer with 1% (w/w) pepsin. The mixture was incubated in a 37°C water bath for 4 h and then neutralized to pH 7.0 with addition of 2 M NaOH solution. The neutralized suspension (1 mL) was centrifuged at 10,000 × g for 20 min and the supernatant was assayed for ACE inhibitory activity. The remaining neutralized suspension was further digested by 1% (w/w) pancreatin at 37°C for 4 h. The pancreatic digestion was terminated by boiling the reaction mixture for 15 min followed by centrifugation at 10,000 × g for 20 min. The supernatant was used for ACE inhibitory activity assays. Controls were prepared by mixing the inactivated pepsin and pancreatin (by boiling enzymes for 15 min) with the peptide solution.

4.3.8 Kinetics of ACE inhibition

The kinetics of ACE inhibition of the potent peptides were investigated. Various substrate concentrations (0.5, 1.0, 3.0, 5.0 and 7.0 mM) were incubated with ACE in the absence or presence of 0.0193 and 0.0772 mg/mL of the purified peptides. Each reaction mixture was assayed as described above. The K_m and V_{max} values were determined using the Lineweaver-Burk plot. The inhibitor constant (K_i) was determined from the plot between of $1/V_{max}^{app}$ and the inhibitor concentration.

4.3.9 Statistical analysis

All experiments were carried out in triplicate and the means with standard deviations were reported. The data were analyzed using analysis of variance (ANOVA) and means comparison was performed using Duncan's multiple range test (DMRT), at

a 95% confidence level using a SPSS package (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA).

4.4 Results and discussion

4.4.1 Purification of ACE inhibitory peptides

TM hydrolysate at 8 h of hydrolysis (48% DH) exerted high ACE inhibitory activity with an IC_{50} value of 0.54 mg/mL. To isolate the active peptides, ultrafiltration was firstly used. The fraction corresponding to MW < 5 kDa had the highest ACE inhibitory activity with specific inhibitory activity of 626.71%/mg peptide, whereas the fraction with MW > 30 kDa showed the lowest ACE inhibitory activity ($p < 0.05$, Table 4.1). These results confirmed that small peptides exhibit better ACE inhibition (Darewicz, Borawska, Vegarud, Minkiewicz, and Iwaniak, 2014; Power, Fernández, Norris, Riera, and FitzGerald, 2014). The longer chain peptides are typically more difficult to bind with the active site of ACE, resulting in a decrease in inhibitory activity (Natesh, Schwager, Sturrock, and Acharya, 2003). This might partly explain low inhibitory activity of the fraction with MW > 30 kDa. Although the fraction with MW < 5 kDa showed lower peptide yield than others, its specific inhibitory activity increased by almost 4-fold compared to the crude hydrolysate. Therefore, sequential ultrafiltration appeared to be an effective method for concentrating ACE inhibitory peptides in the TM hydrolysate.

Table 4.1 ACE inhibitory activity of TM hydrolysate and fractions obtained by ultrafiltration.

Fraction	ACE inhibition (%)	Peptide content (mg)	Specific inhibitory activity (%/mg)	Yield (%)
TM hydrolysate	85.27 ± 1.11	0.51	167.20	100
> 30 kDa	51.10 ± 3.11	0.22	232.27	43
5 - 30 kDa	65.06 ± 1.97	0.17	382.71	33
< 5 kDa	87.74 ± 1.59	0.14	626.71	27

Therefore, the fraction with MW < 5 kDa was selected for further purification. After DEAE-Sephacel anion exchange chromatography, Fr1 and Fr2 were obtained (Figure 4.1a). The Fr1 unbound fraction possessed the strongest ACE inhibitory activity with specific inhibitory activity of 868.73%/mg peptide. The active Fr1 was subjected to a CM-Sepharose cation exchange column, yielding two fractions, Fr1-I and Fr1-II (Figure 4.1b). The Fr1-II fraction exhibited the strongest ACE inhibitory activity. The Fr1-II fraction was further separated into four fractions using size exclusion chromatography (Figure 4.1c). The P1 fraction showed the most potent ACE inhibitory activity with an IC_{50} of 0.15 mg/mL. Purification of ACE inhibitory peptides increased by 15-fold based on the four-step purification scheme used (Table 4.2).

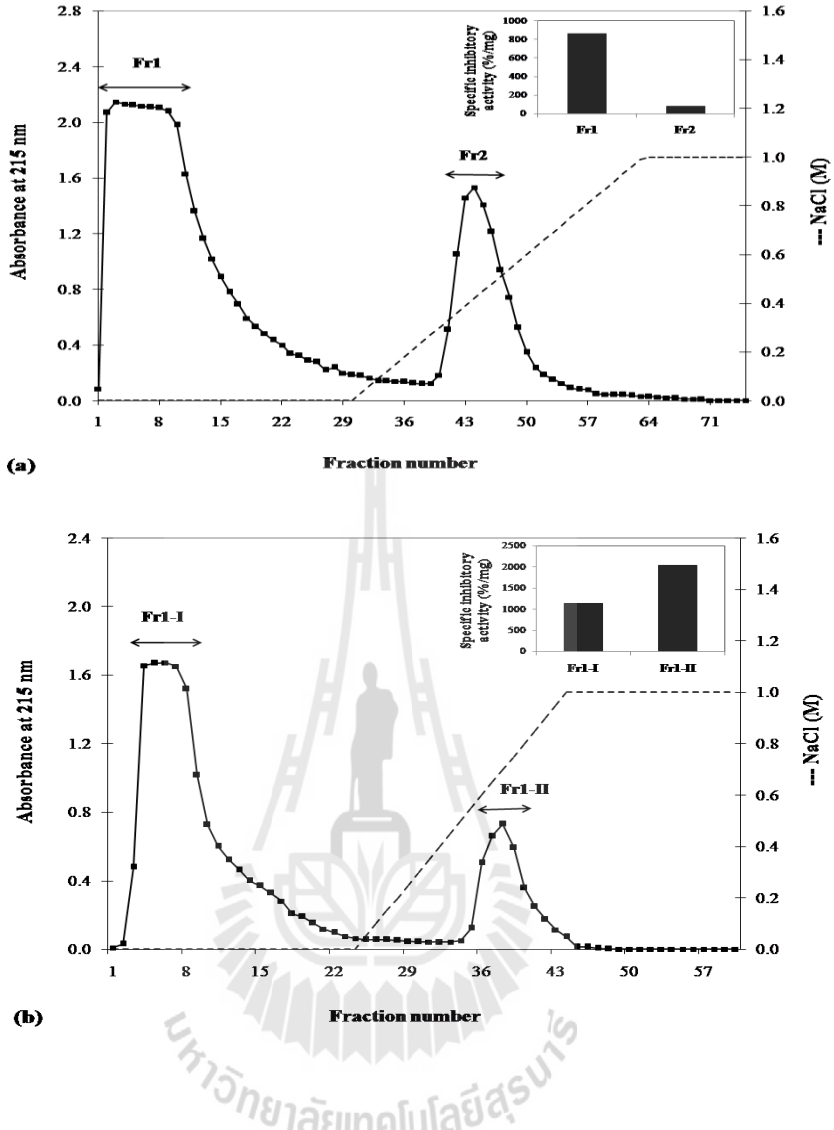
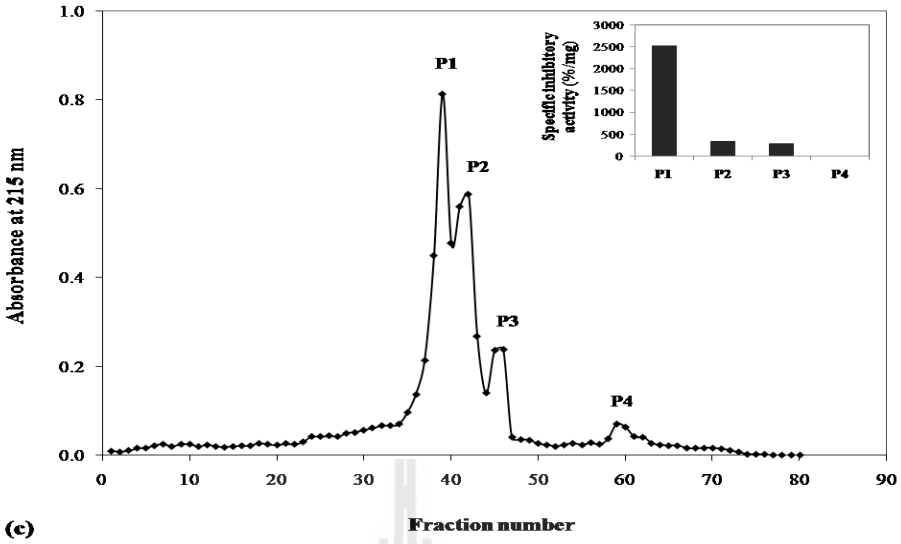


Figure 4.1 Chromatograms and specific ACE inhibitory activity (insert panel) at various purification steps. (a) DEAE-Sephacel ion exchange chromatogram of the ultrafiltrated fraction of 5-kDa MWCO membrane eluted with a linear gradient of NaCl (0-1.0 M) in 50 mM Tris-HCl buffer at pH 7.5, (b) CM-Sepharose ion exchange chromatogram of the Fr1 fraction eluted with a linear gradient of NaCl (0-1.0 M) in 10 mM sodium acetate buffer at pH 4.0 and (c) Superdex peptide 10/300 GL size exclusion chromatogram of the Fr1-II fraction eluted with deionized water.



(c)

Figure 4.1 Chromatograms and specific ACE inhibitory activity (insert panel) at various purification steps. (a) DEAE-Sephacel ion exchange chromatogram of the ultrafiltrated fraction of 5-kDa MWCO membrane eluted with a linear gradient of NaCl (0-1.0 M) in 50 mM Tris-HCl buffer at pH 7.5, (b) CM-Sepharose ion exchange chromatogram of the Fr1 fraction eluted with a linear gradient of NaCl (0-1.0 M) in 10 mM sodium acetate buffer at pH 4.0 and (c) Superdex peptide 10/300 GL size exclusion chromatogram of the Fr1-II fraction eluted with deionized water (Continued).

Table 4.2 Summary of specific inhibitory activity (%/ mg peptide) and purity after series of purification of ACE inhibitory peptides of TM hydrolysate.

Purification step	Total peptide content (mg)	ACE inhibition (%)	Specific inhibitory activity (%/mg peptide)	Purity (fold)
TM hydrolysate	0.51	85.3	167.2	1.0
Ultrafiltration	0.14	87.7	626.7	3.3
DEAE-Sephacel	0.07	58.9	868.7	5.2
CM-Sepharose	0.02	37.8	2020.3	12.1
Superdex peptide 10/300 GL	0.01	35.3	2520.7	15.1

4.4.2 Amino acid sequencing of the purified peptides

Ten peptides in the active P1 fraction were identified from *de novo* sequencing (Table 4.3). A search of potential ACE inhibitory activity that can be derived from the identified peptides was carried out with the BIOPEP database in which the identified peptides were compared to previously reported sequences (Byun and Kim, 2002; Cheung, Wang, Ondetti, Sabo, and Cushman, 1980; Ichimura, Hu, Aita, and Maruyama, 2003; Meisel, 1998; Murray and FitzGerald, 2007; Van Platerink, Janssen, and Haverkamp, 2008). Most of the identified peptides contained potential ACE inhibitory peptides within their sequences. The majority of ACE inhibitory peptides derived from TM hydrolysate were relatively short sequences containing 6 to 13 amino acids. This is in agreement with the results of Natesh et al. (2003), which demonstrated that the active site of ACE cannot accommodate large peptide molecules based on their crystallography studies. However, the structure-activity relationship of ACE inhibitory peptides has yet to be fully established. Structure-activity correlations among different ACE inhibitor peptides indicate that the C-terminal tripeptide sequence strongly

influences the binding to ACE (Sharma, Singh, and Rana, 2011). In addition, the branched aliphatic amino acids at the N-terminal end were reported to be most effective for increasing the peptide binding activity of ACE (Byun and Kim, 2002). Although di- or tripeptides with high potent ACE inhibitory activity have been widely reported, longer peptides were also found to possess the strong ACE inhibitory activity. For instance, FFGRCVSP (IC_{50} = 0.40 µM) from ovalbumin, FKGRYYP (IC_{50} = 0.55 µM) from chicken muscle, NGTWFEPP (IC_{50} = 0.63 µM) from Huruan myofibrillar protein, and LKPNM (IC_{50} = 2.4 µM) from dried bonito muscle were discovered (Fujita and Yoshikawa, 1999; Fujita, Yokoyama, and Yoshikawa, 2000; Ghassem et al., 2011).

To gain better understanding on the structure-activity relationship, some of the identified peptides were chemically synthesized, including MILLLFR, LNLQDFR, KHQDFF, MILLLF, LNLQDF, and HQDF. The first three peptide sequences were selected based on a high Pepnovo score (more than 80%), indicating highly accurate sequence search. Others were obtained from a homology search against the NCBI database via the BLASTP algorithm, which revealed high homology with skeletal muscle proteins. The peptide MILLLFR exhibited the most potent ACE inhibitory activity with an IC_{50} of 0.12 µM (Table 4.4). Arg (R) at the C-terminus of this peptide might play an important role in ACE inhibitory activity. When Arg was removed, the peptide MILLLF showed a decrease in inhibitory activity. This was in agreement with the results of Murray and FitzGerald (2007). However, the effect of an Arg at the C-terminus on the inhibitory activity appears to vary depending on adjacent amino acids. When the Arg at the C-terminus of LNLQDFR was removed, the resulting peptide showed an increase of ACE inhibitory activity. Structure-activity results suggested that the positive charge of Arg or Lys at the C-terminus markedly influences

ACE inhibitory potency (Murray and FitzGerald, 2007; Guang and Phillips, 2009). A possible interaction may exist between the inhibitor and an anionic binding site of ACE that is distinct from the catalytic site (Guang and Phillips, 2009). However, hydrophilic amino acid residues in the peptide sequence could also affect inhibitory activity by limiting the access of the peptide to the active site of ACE (Kim and Byun, 2012). This might explain why LNLQDF exhibited weaker inhibitory activity than that of MILLLFR. It should be noted that MILLLFR is a novel peptide that showed stronger inhibition than VPAAPPK (IC_{50} = 0.45 µM) and NGTWFEPP (IC_{50} = 0.63 µM) derived from Haruan myofilbrillar protein (Ghassem et al., 2011).

Table 4.3 Amino acid sequence of peptides using *de novo* peptide sequencing algorithm.

Fraction	PepNovo score	Mass [M+H] (Da)	Amino acid sequence	ACE inhibitory sequences reported in the literature[a]
1	96.03	905.83	MILLLFR	LF, LLF, FR
2	89.18	905.66	LNLQDFR	LN, LQ, FR
3	80.39	821.41	KHQDFF	-
4	51.90	1526.64	KPLLLMQLLLLFR	KP, LLLF, PL, LF, FR
5	47.36	920.80	QNLLNYR	LN, NY
6	43.75	791.64	QDLLFR	LLF, LF, FR
7	37.94	903.49	NLGALLFR	LG, GA, LLF, LF, FR
8	36.87	904.60	NELLLFR	LLF, LF, FR
9	31.61	677.49	ELLGFV	LG, GF
10	7.35	679.49	AHLLLL	AH, HL, HLL

[a] BIOPEP database.

Table 4.4 ACE inhibitory activity of the selected synthetic peptides.

Synthesized peptide	Mass (Da)	IC_{50} (μM)
MILLLFR	905.22	0.12
LNLQDFR	905.03	0.85
KHQDFF	820.91	ND
MILLLF	749.03	ND
LNLQDF	748.84	0.51
HQDF	545.56	ND

ND, not detected.

4.4.3 Stability of ACE inhibitory peptides

The P1 fraction retained its ACE inhibitory activity even after severe thermal treatments at 100°C or 121°C (Figure 4.2a). In addition, inhibitory activity remained constant over a wide pH range of 2-10 (Figure 4.2b), indicating that peptides in P1 fraction were both thermo- and pH stable. The ACE inhibitory peptides from tilapia also showed resistance to *in vitro* gastrointestinal digestion ($p > 0.05$, Figure 4.2c), revealing that the tilapia peptides might be true ACE inhibitors because gastrointestinal proteinases did not affect their ACE inhibitory potency. This suggests a potential therapeutic use because it would be resistant to gastrointestinal digestion. Some food-protein derived ACE inhibitors acting as an alternative substrate failed to show hypotensive activity after oral administration *in vivo*. In addition, some served as substrates of gastrointestinal proteinases, decreasing their inhibitory activity upon digestion (Fujita et al., 2000; Tsai, Chen, and Pan, 2008).

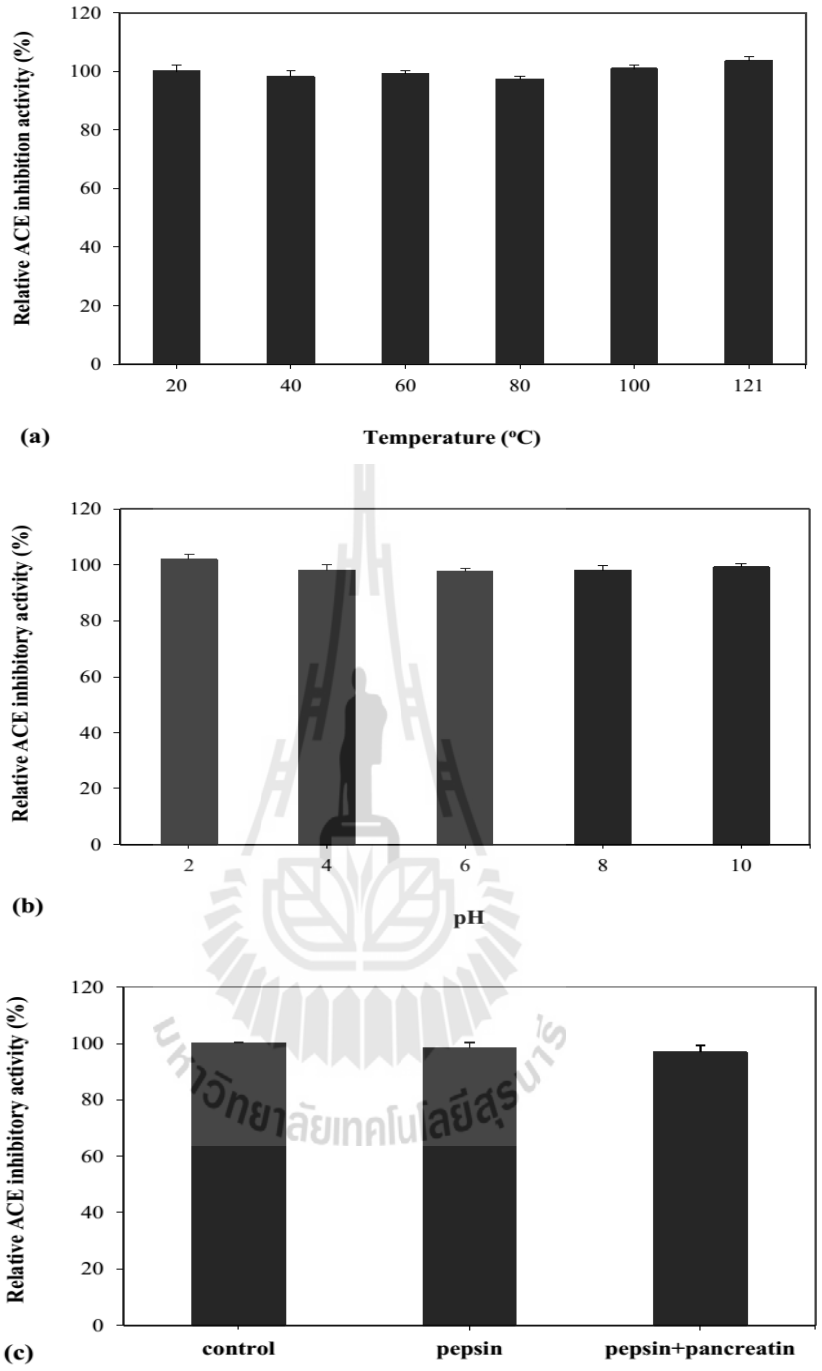


Figure 4.2 Stability of the P1 fraction after incubation at various (a) temperatures and (b) pHs, and (c) *in vitro* gastrointestinal digestion. The relative ACE inhibitory activity was calculated as the ratio of ACE inhibitory activity between the control and the treatments. Bars represent mean ± SD.

4.4.4 Mode of inhibition

In the presence of tilapia peptides, both K_m and V_{max} decreased as compared to the reaction without peptides, indicating the uncompetitive inhibition characteristic, with a K_i value of 0.18 mg/mL (Figure 4.3a and b). This implied that tilapia peptides bind to only the ACE-substrate complex but not the free enzyme. The inhibition pattern of the ACE peptide inhibitor is related to the peptide structure. The ACE inhibitory peptide derived from lysozyme hydrolysate and having an Arg residue at the C-terminus also showed uncompetitive inhibition characteristics (Asoodeh et al., 2012).

ACE inhibitory peptides from snake venom and most of the isolated peptides from food protein hydrolysates have shown competitive inhibition (Wu and Ding, 2002; Chen et al., 2012). Noncompetitive or uncompetitive ACE inhibitors have also been reported (Li, Le, Shi, and Shrestha, 2004; Wang et al., 2008; Sheih, Fang, and Wu, 2009; Asoodeh et al., 2012). Ondetti and Cushman (1982) proposed a binding model for interactions between the substrate and the active site of ACE. The C-terminal tripeptide residues may interact with the three subsites, S_1 (antepenultimate), S_1' (penultimate), and S_2' (ultimate), at the active site of ACE (Byun and Kim, 2002). ACE prefers a substrate or competitive inhibitor possessing hydrophobic (aromatic or branched side chains) amino acids at the three C-terminal residues (Murray and FitzGerald, 2007). However, the inhibition site of noncompetitive and uncompetitive inhibitors reported in food peptides has yet to be specified (Jao, Huang, and Hau, 2012).

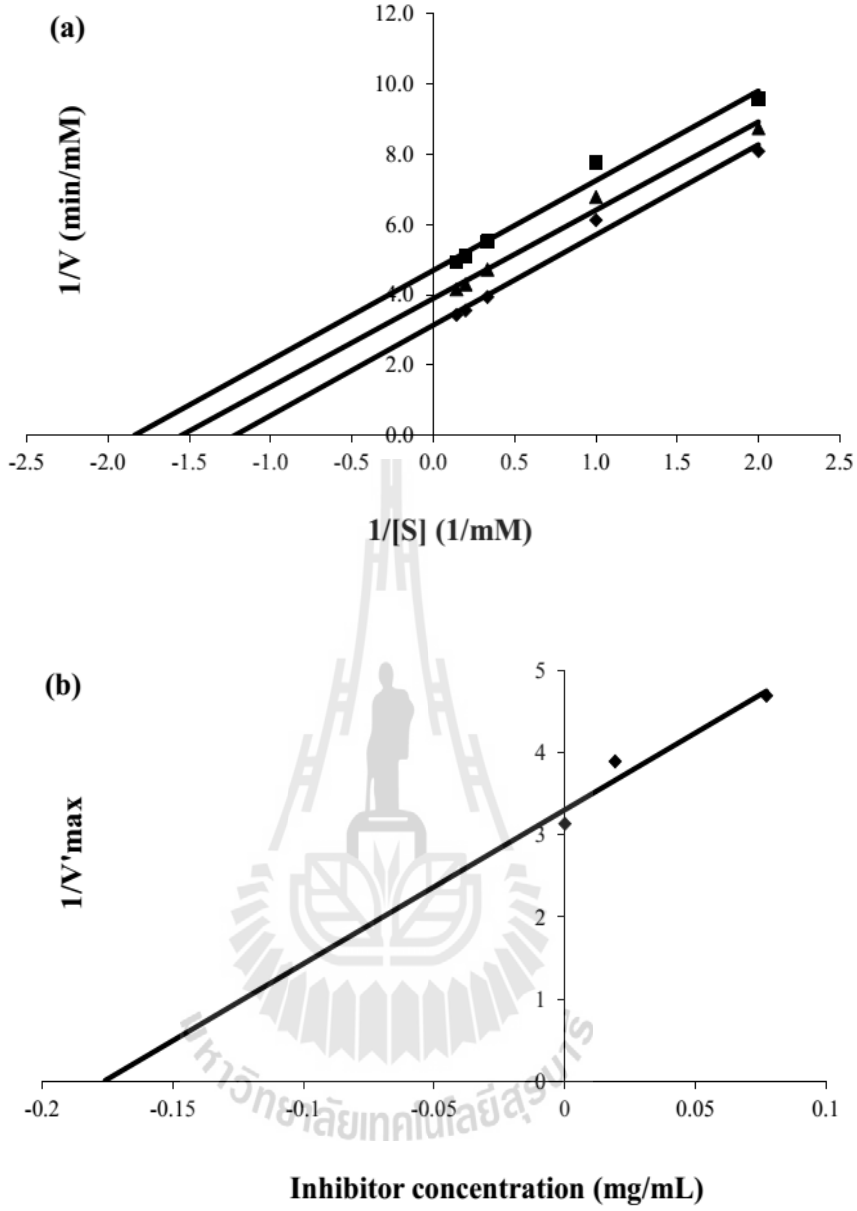


Figure 4.3 (a) Lineweaver-Burk plot of ACE inhibition by the P1 peptides from TM hydrolysate. The ACE activities were measured in the absence and presence of the P1 peptides (♦, control; ▲, 0.0193 mg/mL; ■, 0.0772 mg/mL). 1/V and 1/S represents the reciprocal of velocity and substrate, respectively. (b) The Dixon plot for measuring the inhibitor constant (K_i).

4.5 Conclusions

Tilapia muscle mince hydrolyzed by *V. halodenitrificans* SK1-3-7 proteinases yielded a protein hydrolysate with high ACE inhibitory activity. After a series of chromatographic separations, the most effective fraction exhibited an IC_{50} of 0.15 mg/mL and uncompetitive inhibition with a K_i of 0.18 mg/mL. Tilapia peptides showed high thermostability at 100 and 121°C, maintained inhibitory activity at a wide pH range of 2-10 and withstood *in vitro* gastrointestinal digestion. The novel peptide MILLLFR, containing hydrophobic amino acids (Met, Ile, Leu, and Phe) and an Arg residue at the C-terminus, possessed the most potent ACE inhibitory activity. Therefore, the peptides from *V. halodenitrificans* SK1-3-7 proteinases-hydrolyzed tilapia mince are potential functional food ingredients with ACE inhibitory activity.

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CHAPTER V

IMMUNOMODULATORY ACTIVITY OF PROTEIN HYDROLYSATES DERIVED FROM *VIRGIBACILLUS HALODENITRIFICANS* SK1-3-7 PROTEINASES

5.1 Abstract

Modulation of inflammation-related immune response on THP-1 macrophages of protein hydrolysates derived from tilapia mince, casein, and pea protein, were investigated. The protein substrates were hydrolyzed by partially-purified *Virgibacillus halodenitrificans* SK1-3-7 proteinase. Degree of hydrolysis (DH) of casein was found to be the highest throughout the course of hydrolysis. After THP-1 macrophages challenging, tilapia mince hydrolysate (TMH) enhanced innate immunity through induction of IL-1β and COX-2 expression. Anti-inflammatory activity was found in casein hydrolysate (CH) and pea protein hydrolysate (PPH) by attenuating of LPS-induced pro-inflammatory gene expression in THP-1 macrophages. CH suppressed IL-1β, IL-6, IL-8, TNF-α, and COX-2. While, PPH reduced LPS-induced IL-6 and TNF-α responses. In addition, CH and PPH showed stronger suppression of LPS-induced pro-inflammatory gene expression compared with non-hydrolyzed casein and pea protein. These results suggests that TMH, CH, and PPH prepared from *V. halodenitrificans* SK1-3-7 proteinase are potential functional food ingredients with immunomodulatory activity.

Keywords: protein hydrolysate, macrophages, immunomodulation, inflammation

5.2 Introduction

It has been postulated that consumption of certain foods may reduce susceptibility for the establishment and/or progression of immunological diseases (Sandré et al., 2001). Several researchers have demonstrated that immunomodulating peptides can be generated through enzymatic hydrolysis from various food proteins including whey (Gauthier, Pouliot, and Saint-Sauveur, 2006), casein (Stuknyte, Noni, Guglielmetti, Minuzzo, and Mora, 2011), fish (Yang et al., 2009), pea (Ndiaye, Vuong, Duarte, Aluko, and Matar, 2012) and soy (Kong, Guo, Hua, Cao, and Zhang, 2008). These protein hydrolysates can enhance immune cell functions, measured as lymphocyte proliferation, natural killer (NK) cell activity, antibody synthesis, cytokine regulation, and nitric oxide production. Moreover, they also showed anti-inflammatory activity by reducing inflammatory mediators.

The production of immunomodulating peptides can be achieved by several proteinases including Alcalase, thermolysin, pepsin, mixture of trypsin and chymotrypsin (Kong et al., 2008; Ndiaye et al., 2012; Saint-Sauveur, Gauthier, Boutin, and Montoni, 2008; Yang et al., 2009). However, the use of proteinases from novel sources in protein hydrolysate production has not been widely studied. *Virgibacillus halodenitrificans* SK1-3-7 isolated from fish sauce fermentation has been found to secreted serine proteinases with subtilisin-like characteristics (Montriwong, Kaewphuak, Rodtong, Roytrakul, and Yongsawatdigul, 2012). The previously results demonstrated that the proteinase can be effective processing-aid for production of tilapia hydrolysate with angiotensin I-converting enzyme (ACE) inhibitory activity. Other bioactivities, particularly immunomodulating activity of protein hydrolysates derived from *V. halodenitrificans* SK1-3-7 proteinase has never been explored.

Inflammation is a complex process that is modulated by an array of inflammatory mediators and cytokines (Zhou et al., 2008). A conventional approach in the study of inflammation *in vitro* is the use of macrophages that can be activated by Toll-like receptor ligands such as bacterial lipopolysaccharide (LPS) and interferon-γ, resulting in the production of different inflammatory mediators, such as the transcription factor nuclear factor (NF)-κB, pro-inflammatory cytokines (e.g. interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α), cyclooxygenase-2 (COX-2), among others (Murray and Wynn, 2011). Excessive production of these inflammatory mediators participate in a variety of metabolic disorders including obesity, tumourigenesis, autoimmune response and atherosclerosis (Dia, Bringe, and Mejia, 2014). Therefore, the control of excessive inflammation is important to maintain health and wellness.

The aims of this study were to produce protein hydrolysates derived from various protein sources including tilapia mince, casein, and pea protein, hydrolyzed by *V. halodenitrificans* SK1-3-7 proteinase. Effect of protein hydrolysates on modulation of inflammation-related immune response were tested in THP-1 macrophages. In addition, anti-inflammatory activity of casein and pea protein hydrolysates on LPS-induced THP-1 macrophages in order to mimic inflammatory condition was also determined.

5.3 Materials and methods

5.3.1 Materials

Fresh tilapia (*Oreochromis niloticus*) was purchased from a local market in Nakhon Ratchasima, Thailand. Fish were stored in ice and transported to a laboratory

at Suranaree University of Technology within 30 min. Tilapia mince was defatted, dried, and ground as described in Chapter IV (section 4.3.2). Pea protein isolate was obtained from Yantai Oriental Protein Tech Co., LTD (Zhaoyuan, Shandong, China). Casein was obtained from Dutch Protein and Services (Tiel, The Netherlands). Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents and chemicals used were of analytical grade.

5.3.2 Partial purification of *V. halodenitrificans* SK1-3-7 proteinase

The enzyme production by *V. halodenitrificans* SK1-3-7 were prepared as described in Chapter III (section 3.3.2). The crude enzyme was slowly added ammonium sulfate to a final concentration of 1 M with a gentle stirring at 4°C. The sample was centrifuged at 10,000×g at 4°C for 20 min and the supernatant was subsequently filtered through a 0.45 μm cellulose acetate membrane. The filtrate was applied to a Phenyl-Sepharose column (2.6 × 6.5 cm, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) equilibrated with 1 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl (pH 7.0). The elution was performed using an AKTA Purifier (GE Healthcare, Uppsala, Sweden) with a linear gradient of 1-0 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl (pH 7.0) at a flow rate of 1 mL/min. The eluate was monitored at 280 nm and collected in 5 mL fractions. All fractions were tested for proteinase activity using azocasein as a substrate. One unit (U) of proteinase activity was defined as the amount of enzyme that produced an absorbance change of 0.01 at 450 nm per min under the condition of 60°C and pH 9.0 for 60 min. Fractions containing proteinase activity were pooled and dialyzed against 50 mM Tris-HCl (pH 7.0). The dialysate was referred to as the partially-purified proteinase.

Protein content was determined using the Lowry method using bovine serum albumin (BSA) as a standard (Lowry et al., 1951).

Protein patterns of *V. halodenitrificans* SK1-3-7 proteinase at each partial purification step were determined using SDS-PAGE (Laemmli, 1970). Protein solutions were mixed with sample buffer (62.5 mM Tris-HCl (pH6.8), 2% SDS, 25% glycerol, 0.01% bromophenol blue, and 5% β-mercaptoethanol) (Bio-Rad, Hercules, CA, USA) at a ratio of 1:1 and boiled for 3 min. The samples (10 µg) were loaded onto 12% Mini-PROTEAN® TGX™ precast polyacrylamide gel (Bio-Rad). Gel was run at a constant voltage of 100 V. After electrophoresis, the gel was stained with 0.125% Coomassie Brilliant Blue R-250 and destained in a solution containing 25% ethanol and 10% acetic acid.

5.3.3 Preparation of protein hydrolysates

Casein, pea protein, and defatted tilapia mince powder were suspended in Milli-Q water (1% w/v) and adjusted to pH 9.0 using 1 M NaOH. The mixtures were incubated at 60°C for 5 min. The partially-purified enzyme from *V. halodenitrificans* SK1-3-7 was added to the mixtures at final unit activity of 1.6 U. Hydrolysis was carried out at 60°C in a shaking water bath. At each time interval (0, 2, 4, 6, 8, 10, and 12 h), the mixtures were taken and heated at 90°C for 10 min to terminate proteinase activity. The heated samples were then centrifuged at 10,000×g for 20 min at 4°C. The supernatants were referred to as tilapia mince hydrolysate (TMH), casein hydrolysate (CH), and pea protein hydrolysate (PPH). Degree of hydrolysis was determined according to the TNBS method (Adler-Nissen, 1979) as described in Chapter III (section 3.3.5.1). Protein content was determined using the Lowry method using BSA as a standard (Lowry et al., 1951). Lipopolysaccharide (LPS) contamination was

monitored using chromogenic LAL endotoxin assay kit (L00350, GenScript, New Jersey, USA) according to the manufacturer's instructions. One endotoxin unit (EU) is equivalent to 0.2 ng of LPS (Munson, 1985).

5.3.4 Inflammatory modulating effect of protein hydrolysates

5.3.4.1 Cell culture

The human monolytic leukemia cell line THP-1 (American Type Culture Collection, Rockville, MD, USA) was grown in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS; Hyclone, Thermo Fisher Scientific, Northumberland, UK) and 1% penicillin/streptomycin (P/S; Gibco BRL) at 37 °C in an incubator humidified with 5% CO_2. Cells were sub-cultured twice a week. THP-1 cells were used at a passage number between 12-25.

5.3.4.2 Cytotoxicity assay

Cytoxicity was determined by MTT assay according to the method of Chanput, Mes, Vreeburg, Savelkoul, and Wichers (2010) with some modifications. The MTT assay determines the viability of cells by the reduction of yellow soluble MTT in the metabolically active cells. Before performing the assay, THP-1 monocytes were induced for differentiation into macrophages in a 96-well cell culture plate (8.2×10^5 cells/well) with 100 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma Chemical) for 48 h at 37°C in an incubator humidified with 5% CO_2. Differentiated, plastic-adherent cells were washed twice with RPMI 1640 medium containing 10% FBS and 1% P/S and rested for another 24 h in the culture medium. Protein hydrolysates were diluted with the culture medium at a ratio of 1:4, to attain final concentrations of 1 to 800 µg/mL. After removing the culture medium, THP-1 macrophages were exposed to the protein hydrolysates at the tested concentrations in the volume of 100 µL for 6 h at

37°C. Subsequently, the tested samples were removed and added 100 µL of fresh medium containing 0.5 mg/mL MTT into each well and incubated for 2 h at 37°C. After removing the MTT solution, 30 µL DMSO:ethanol (1:1) was added into each well and the plate was mildly shaken on a shaker for 5 min. The absorbance was measured at 570 nm using a microplate reader (Tecan Austria GmbH, Groedig, Austria). RPMI 1640 medium containing 10% FBS and 1% P/S was used as a control. The relative cell viability was expressed as a percentage relative to the control (non-treated cells).

5.3.4.3 Exposure of protein hydrolysates to THP-1 macrophages

THP-1 monocytes were seeded into 12-wells cell culture plates (Greiner, Germany) with 1 mL cell suspension (10^6 cells/well). The macrophage-like state was induced by treating THP-1 monocytes with 100 ng/mL PMA for 48 h as described above. THP-1 macrophages were exposed to TMH, CH, and PPH at various concentrations of 10, 100, and 800 µg/mL, which were found to be non-toxic, and incubated for 6 h at 37°C in an incubator humidified with 5% CO_2. To eliminate the effect of LPS associated with the protein hydrolysates, which can contribute to inflammation-related gene expression on macrophages, the equivalent LPS concentrations of each protein hydrolysate were also added to another set of THP-1 macrophages and treated in the same manner as protein hydrolysate-treated counterparts. The amount of LPS in TMH, CH, and PPH were 0.53, 0.68, and 2.1 pg/µg sample, respectively. The control used in all measurements was THP-1 macrophages exposed with the culture medium containing 10% FBS and 1% P/S. After the incubation period, the culture medium containing the tested samples was removed and the treated cells were harvested and stored at -80°C for RNA isolation, cDNA synthesis, and Real

Time-PCR analysis. The experiments were performed by two independent biological replicates.

5.3.4.4 Inflammation-related gene expression

Inflammation related gene expression was carried out according to the method of Chanput et al. (2010). Total RNA was isolated using RNeasy mini kit (Qiagen, Valancia, USA) with RNase-free DNase (Qiagen) treatment for 15 min according to the manufacturer's instructions. RNA quality was monitored on a 1% agarose gel and RNA quantity was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA). Complementary DNA (cDNA) was synthesized from isolated RNA with iScript cDNA synthesis kit (Bio-Rad). The synthesized cDNA of 200 ng was mixed with 10 µL of IQ^{TM} SYBR Green Supermix (Bio-Rad) and primer pairs in a 20 µL reaction volume and preheated at 95°C for 90 s, followed by PCR for 40 cycles, denaturing temperature of 95°C for 10 s, annealing temperature of 58°C for 10 s, and elongation temperature of 72°C for 15 s, and finally elongation temperature of 72°C for 2 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to normalize the expression of the target genes. The primers used are presented in Table 5.1. Relative fold change was calculated using the $2^{\wedge-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Real Time-PCR of each sample was performed in duplicate.

Table 5.1 Sequences of Real Time-PCR primers.

Gene	Accession number	Primer working concentration (µM)	Sequence (5′ → 3′)
IL-1β	NM_000576.2	0.1	F-GTGGCAATGAGGATGACTTGTTC
			R-TAGTGGTGGTCGGAGATTCGTA
IL-6	NM_000600.3	0.1	F-AGCCACTCACCTCTTCAGAAC
			R- GCCTCTTTGCTGCTTTCACAC
IL-8	NM_000584.2	0.1	F- CTGATTTCTGCAGCTCTGTG
			R- GGGTGGAAAGGTTTGGAGTATG
IL-10	NM_000572.2	0.4	F- GTGATGCCCCAAGCTGAGA
			R- CACGGCCTTGCTCTTGTTTT
TNF-α	NM_000594.2	0.1	F- CTGCTGCACTTTGGAGTGAT
			R- AGATGATCTGACTGCCTGGG
COX-2	NM_000963.2	0.1	F- CAGCACTTCACGCATCAGTT
			R- CGCAGTTTACGCTGTCTAGC
NF-κB	NM_003998.2	0.4	F- TGAGTCCTGCTCCTTCCA
			R- GCTTCGGTGTAGCCCATT
GAPDH	NM_002046.3	0.1	F- TGCACCACCAACTGCTTAGC
			R- GGCATGGACTGTGGTCATGAG

5.3.4.5 Effect of protein and their hydrolysates on LPS-stimulated THP-1 macrophages

THP-1 macrophages (1×10^6 cells/mL) in 12 well-cell culture plates were treated with 1 mL culture medium containing 100 µg/mL samples (either casein, pea protein, CH, or PPH) and 0.5 ng/mL LPS for 6 h at 37°C in an incubator humidified with 5% CO_2. The culture medium containing 0.5 ng/mL LPS was used as a control. Subsequently, the cells were harvested and stored at -80°C for RNA isolation, cDNA synthesis, and pro-inflammatory gene expression (IL-1β, Il-6, IL-8, TNF-α, COX-2, and NF-κB). Two biological replicates were carried out.

5.3.5 Statistical analysis

Data from Real Time-PCR were performed by two biological and two technical replicates, while other experiments were carried out in triplicate. Data were expressed as means with standard deviations. Data were analyzed using analysis of variance (ANOVA) and means comparison was performed using Duncan's multiple range test (DMRT), at 95% confidence level using a SPSS package (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA).

5.4 Results and discussion

5.4.1 Partial purification of *V. halodenitrificans* SK1-3-7 proteinase

Crude *V. halodenitrificans* SK1-3-7 proteinase in 1 M $(NH_4)_2SO_4$ were separated into two protein peaks after applying on a phenyl-Sepharose hydrophobic interaction column and only bound fractions with elution at 0 M $(NH_4)_2SO_4$ possessed proteolytic activity (Figure 5.1), indicating that the active fractions contain proteins with highly hydrophobic. Two bands of contaminant proteins were removed after partial purification and protein bands with molecular mass of 45, 46, and 20 kDa remained (Figure 5.2, Lane 3). Proteinases from *Virgibacillus* sp. SK33 and SK37 showed proteolytic activity at molecular mass of 19, 32, 34, and 44 kDa (Phommao, Radtong, and Yongsawatdigul, 2010; Sinsuwan, Rodtong, and Yongsawatdigul, 2010). Montriwong et al. (2012) found that fibrinolytic enzymes from *V. halodenitrificans* SK1-3-7 showed molecular mass of 60, 44, 36, and 20 kDa, after phenyl-Sepharose chromatography. Therefore, three protein bands in the present study were likely to be proteinase. Based on the purification scheme, purity of proteinase from *V. halodenitrificans* SK1-3-7 increased by 5.4-folds with 47% yield (Table 5.2).

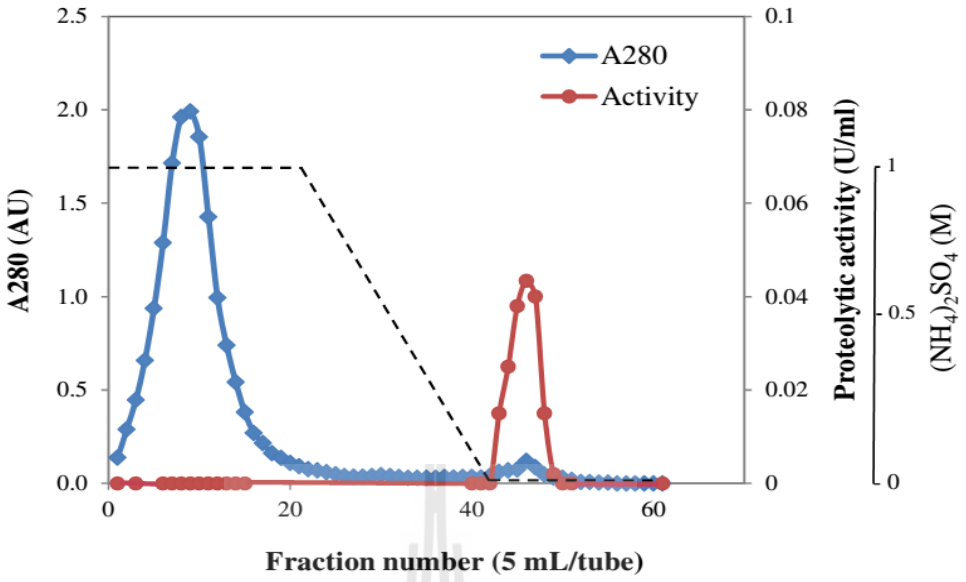

Figure 5.1 Chromatogram of crude *V. halodenitrificans* SK1-3-7 proteinase on a phenyl-Sepharose column. Elution was carried out with a linear gradient of 1-0 M $(NH_4)_2SO_4$. (----) denotes $(NH_4)_2SO_4$ concentration.

Table 5.2 Purification table of proteinase from *V. halodenitrificans* SK1-3-7.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold	Yield (%)
Crude proteinase	20.87	1.15	0.05	1.0	100
Crude proteinase in 1 M $(NH_4)_2SO_4$	12.09	1.01	0.08	1.6	88
phenyl-Sepharose	2.01	0.54	0.27	5.4	47

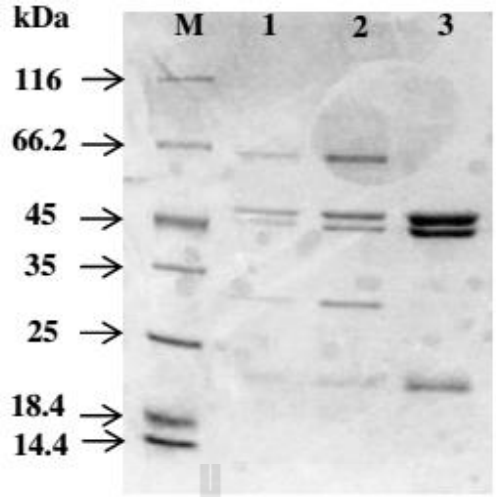

Figure 5.2 SDS-PAGE patterns of crude proteinase (1), crude proteinase in 1 M $(NH_4)_2SO_4$ (2), active fraction obtained from phenyl-Sepharose (3), M denotes standard markers.

5.4.2 Protein hydrolysis by *V. halodenitrificans* SK1-3-7 proteinase

Tilapia mince, casein, and pea protein could be effectively hydrolyzed by the *V. halodenitrificans* SK1-3-7 proteinase. Degree of hydrolysis (DH) of all samples rapidly increased in the first 2 h and reached plateau after 8 h of hydrolysis (Figure 5.3). A reduction in hydrolysis rate may be due to a decrease in concentration of peptide bonds available for hydrolysis, enzyme inhibition and/or enzyme inactivation (Guerard et al., 2002). The highest DH was found in CH, followed by TMH, and PPH throughout the course of hydrolysis ($p < 0.05$). Structural differences among each substrate might limit accessibility of the enzyme, resulting in varied DH values. Higher flexibility of casein may make it more susceptible to proteolysis and resulting in a higher DH (Otte, Shalaby, Zakora, Pripp, and El-Shabrawy, 2007). Since DH of each protein appeared to attain the maximum at 8 h, hydrolysates obtained from this condition were selected for testing inflammation-related immune responses.

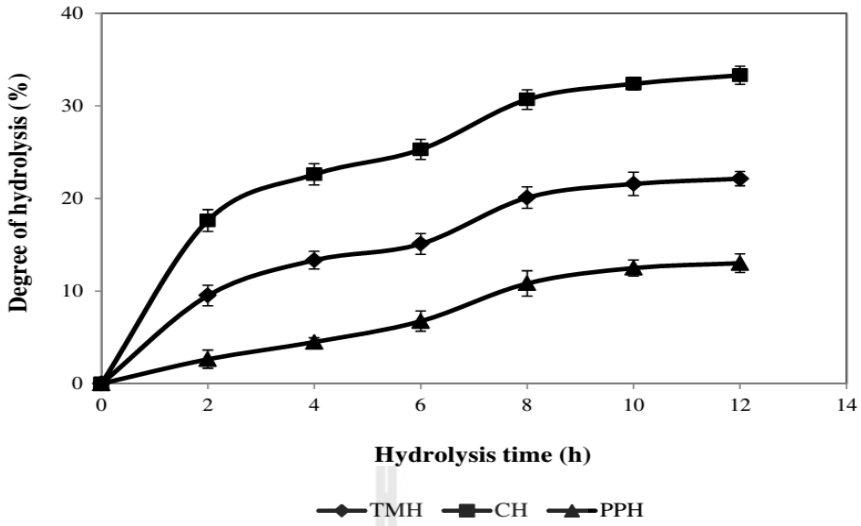

Figure 5.3 Degree of hydrolysis of tilapia mince hydrolysate (TMH), casein hydrolysate (CH), and pea protein hydrolysate (PPH) at different hydrolysis time. Values are expressed as the means ± S.D. (n = 3).

5.4.3 Immunomodulation of protein hydrolysates on THP-1 macrophages

Cytotoxicity of TMH, CH, and PPH was firstly tested on THP-1 macrophages to determine non-cytotoxic concentration for the experiment of inflammatory modulation. Overall cell viability after 6 h exposure to hydrolysates at concentrations ranging of 1-800 µg/mL were greater than 95% ($p > 0.05$), indicating that all hydrolysates at the tested concentrations were nontoxic to THP-1 macrophages. Based on the results, all the hydrolysates at the nontoxic concentrations of 10, 100, and 800 µg/mL were used for further investigation of inflammatory modulation effect.

Inflammation was monitored using gene expression of different markers, including transcription factor NF-κB, pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α), pro-inflammatory enzyme (COX-2), and anti-inflammatory cytokine (IL-10). It was realized that hydrolysates at the tested concentrations contained LPS at

> 1 pg/mL. This concentration is known to activate expression of pro-inflammatory genes in THP-1 macrophages (Chanput et al., 2012). LPS are derived from cell membrane of Gram-negative bacteria and continuously liberated into the environment. The release does not occur only with cell death but also during growth and division. LPS are commonly found almost everywhere and it is possible to find LPS during production processes or in the finished products (Magalhães et al., 2007). Although many researchers have attempted to search for the method for removing LPS, general method for LPS removal from protein solutions is still not available to date. Thus, the expression of inflammatory genes stimulated by LPS at the same concentration found in the hydrolysates would be critical to differentiate "true response" derived from proteins/hydrolysates themselves. The *V. halodenitrificans* SK1-3-7 proteinase was also observed inflammatory responses on THP-1 macrophages before using for hydrolysis and found that the proteinase did not exhibited any responses on inflammation-related gene expression.

Challenging THP-1 macrophages with TMH resulted in an induction of the overall expression of inflammation-related genes in a dose-dependent manner (Figure 5.4), however the up-regulation of these expression levels were likely due to LPS in the hydrolysate as responses from hydrolysates were comparable with those treated with equivalent LPS concentrations. Only the expression of IL-1β and COX-2 genes of TMH was higher than that of LPS treatments ($p < 0.05$), indicating that TMH stimulated immune response of THP-1 macrophages. TMH appeared to enhance innate immunity through induction of IL-1β and COX-2 gene expression. Stimulation of pro-inflammatory mediators of some food compounds have been well-documented, such as rice hull polysaccharides (Yang, Hsieh, and Lin, 2015), glucans derived from fungi and plants (Ramberg, Nelson, and Sinnott, 2010). Reinforcement of the pro-inflammatory

cytokine responses can improve host defense against infection in immunodeficiency patients (Netea, Kullberg, and Van der Meer, 2004).

Interestingly, exposure of THP-1 macrophages to CH resulted in a large reduction of the expression of pro-inflammatory genes (IL-1β, IL-6, IL-8, TNF-α, COX-2) and NF-κB gene compared to LPS treatments ($p < 0.05$, Figure 5.5). The expression of IL-10 gene was found to be similar with LPS treatments ($p > 0.05$). The results suggested that CH might have anti-inflammatory activity by suppressing the expression of pro-inflammatory genes in LPS-stimulated THP-1 macrophages. Treatment of THP-1 macrophages with PPH showed that the expression of IL-1β, IL-8, IL-10, COX-2, and NF-κB genes were up-regulated in the similar manner as LPS treatments ($p > 0.05$, Figure 5.6), while the expression of IL-6 and TNF-α genes were lower than those of LPS treatments ($p < 0.05$). The results indicated that PPH might be able to inhibit LPS-induced IL-6 and TNF-α responses on THP-1 macrophages. Based on these results, CH and PPH were further exposed to LPS-stimulated THP-1 macrophages in order to assure their suppressive effects.

Stimulation of TMH, CH, and PPH on THP-1 macrophages showed different inflammation-related gene expression patterns. Amino acid composition in the peptide sequences of different protein sources may be an important factor manifesting the immunomodulatory activity of protein hydrolysates. Moreover, peptide size has also been reported to affect immunomodulatory activity. For instance, peptide fraction with molecular weight above 5 kDa derived from almond protein hydrolysate showed the inhibition of relative gene expressions of pro-inflammatory cytokines, including IL-6, IL-1β, and TNF-α in the activated macrophages (Udenigwe et al., 2013). Stuknyte et al. (2011) found that 3 kDa-fraction of casein hydrolysate produced by *Lactococcus lactis*

subsp. *lactis* GR5 proteinases showed anti-inflammatory activity by decreasing the basal NF-κB activity in recombinant Caco-2 cell layers. In addition, Lee et al. (2009) found that egg white peptides with molecular weight 1.3 kDa attenuated the symptoms of inflammatory bowel disease.

LPS resulted in an increase of the expression level of the transcription factor NF-κB, pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) and enzyme (COX-2) in a dose-dependent manner. It is well known that bacterial LPS strongly induced the immune cells especially through monocytes and macrophages (Feng, Ling, and Duan, 2010). LPS firstly binds to lipopolysaccharide binding protein (LBP) to form LPS-LBP, which subsequently forms a complex with a membrane protein called CD14. The LPS-LBP-CD14 complexes further interact with Toll-like receptor 4 (TLR4) on the cell surface of the macrophages, which exclusively transduce the LPS signal across the membrane (Hsu and Fu, 2004). Activation of TLR4 initiates a signaling pathway, leading to the activation of the transcription factor NF-κB and triggers production of pro-inflammatory cytokines, including IL-1, IL-6, IL-8, and TNF-α (Chow et al., 1999). Moreover, the expression of COX-2 is induced by reactive oxygen species, bacterial LPS, TNF-α and IL-1β during inflammation through the NF-κB signaling pathways (Udenigwe et al., 2013). The suppression of NF-κB by CH may result in a reduced expression of IL-1β, IL-6, IL-8, TNF-α and COX-2 genes. The down-regulated TNF-α gene induced by PPH treatment may result in a decrease of IL-6 since TNF-α regulates the inflammatory response by inducing IL-6 (Begue et al., 2006). These results implied that CH and PPH tended to have anti-inflammatory activity by inhibiting of the expression of pro-inflammatory genes.

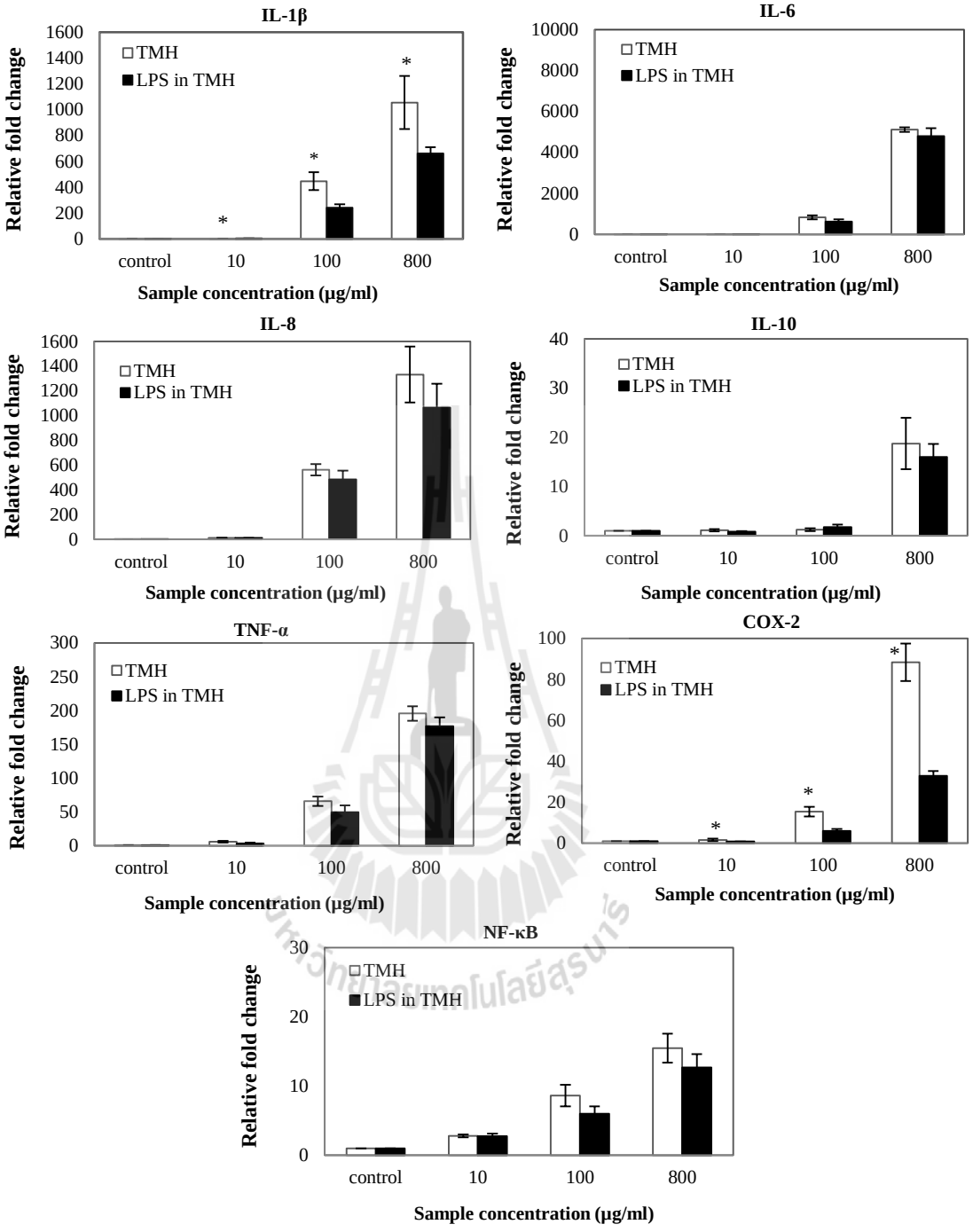

Figure 5.4 Differential inflammation-related gene expression of 6-h incubated THP-1 macrophages with tilapia mince hydrolysate (TMH) compared to stimulation of LPS contaminated in TMH. Data shown are the mean ± standard deviation (SD bars). *$p < 0.05$ compared with LPS treatments.

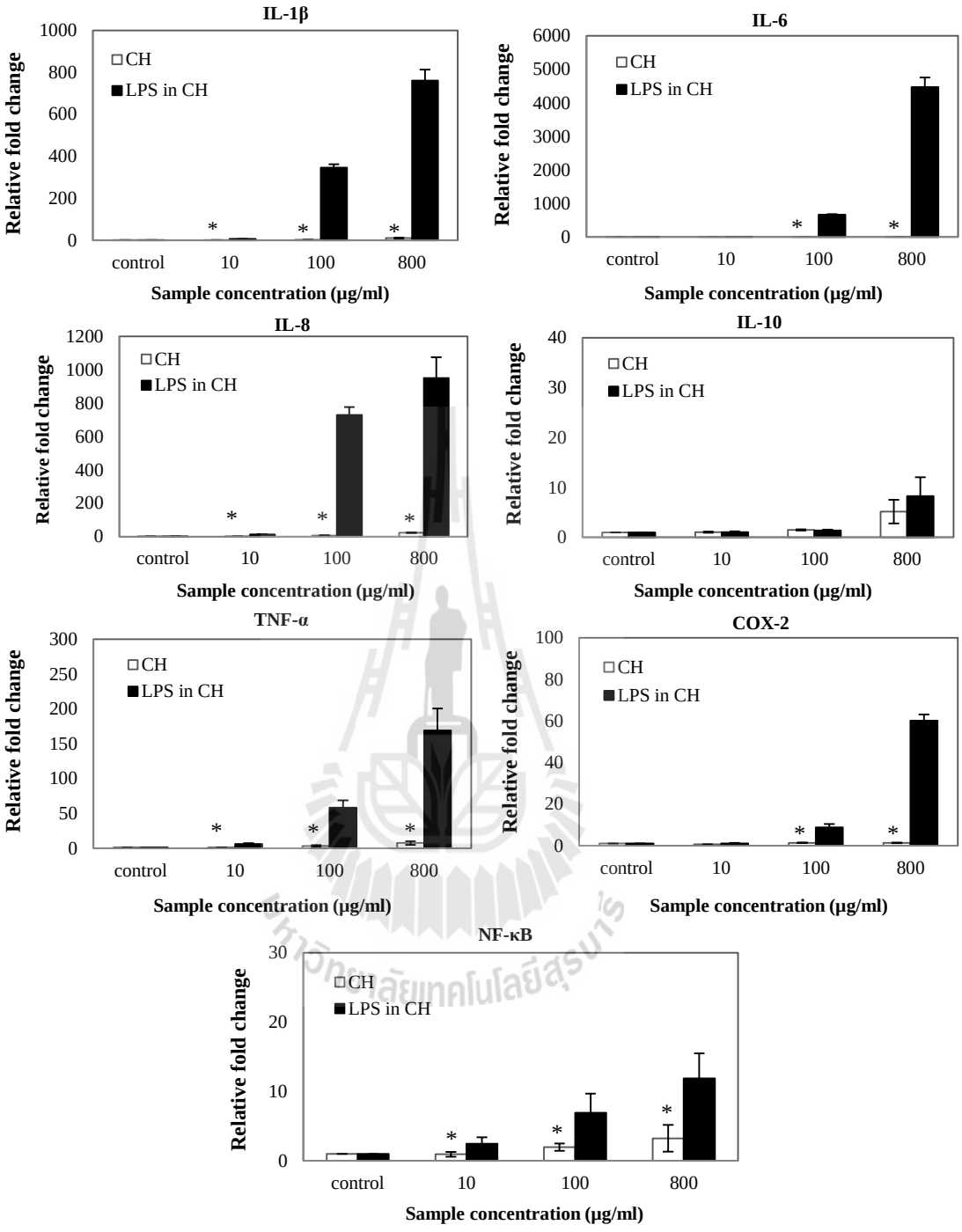

Figure 5.5 Differential inflammation-related gene expression of 6 h incubated THP-1 macrophages with casein hydrolysate (CH) compared to stimulation of LPS contaminated in CH. Data shown are the mean ± standard deviation (SD bars). *$p < 0.05$ compared with LPS treatments.

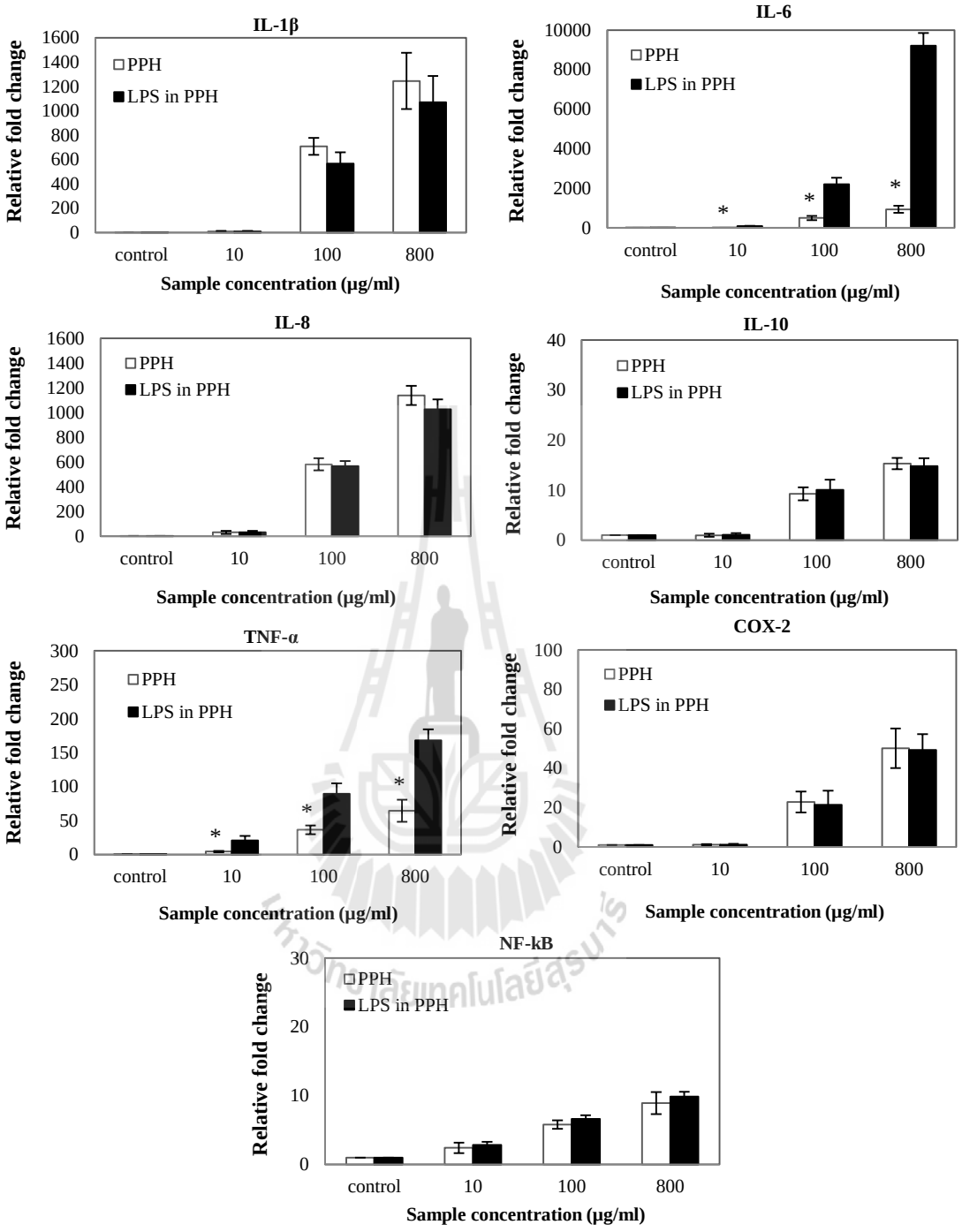

Figure 5.6 Differential inflammation-related gene expression of 6 h incubated THP-1 macrophages with pea protein hydrolysate (PPH) compared to stimulation of LPS contaminated in PPH. Data shown are the mean ± standard deviation (SD bars). *$p < 0.05$ compared with LPS treatments.

5.4.4 Suppression of LPS-induced inflammatory immune responses

To verify the anti-inflammatory effect of CH and PPH, THP-1 macrophages were co-incubated with 100 μg/mL the hydrolysates and 0.5 ng/mL LPS for 6 h exposure to mimic inflammatory condition. In addition, the intact casein and pea protein were also tested in order to clarify the effect of enzymatic hydrolysis on the anti-inflammatory effect. Relative fold change of inflammation-related genes from LPS-only stimulation was set as a reference for inflammatory status. Thus, the expression of genes above or below the LPS reference was designated as inductive or suppressive inflammatory effects, respectively. It is well known that inflammatory mediators including IL-1β, IL-6, IL-8, and TNF-α, COX-2, and NF-κB are induced by LPS. NF-κB is activated at site of inflammation in a diverse set of diseases and can induce the transcription of pro-inflammatory mediators and cytokines (Khan et al., 2011). TNF-α is an important mediator in the pathogenesis of lethal systemic inflammation in septic shock and also induces apoptosis in other cells and tissue damage (Cohen, 2002). It is mainly released from macrophages after LPS treatment (Ahn, Cho, and Je, 2014). TNF-α regulates the inflammatory response through a number of actions, such as inducing IL-1β and IL-6, and the increased expression of adhesion molecules (Begue et al., 2006). IL-6 is multifunctional cytokine, which is up-regulated by LPS and IL-1β, and high serum IL-6 levels are observed in inflammation, trauma, and autoimmune diseases (Yoon, Moon, Park, Im, and Kim, 2007). IL-8 plays critical roles in the development of pain and its expression increases in the brain after peripheral inflammation (Cui, An, Zhang, Zhao, Liu, and Yi, 2012). COX-2 leads to the conversion of arachidonic acid to prostaglandin (PG)-H_2, a precursor of PGE_2, which contributes to pathogenesis during inflammatory diseases (Udenigwe et al., 2013).

Excessive production of these inflammatory mediators can contribute to several acute and chronic inflammatory diseases. The pharmacological inhibition of these inflammatory mediators is an important target in the treatment of endotoxemia with bacterial infection.

CH suppressed the expression of LPS-induced IL-1β, IL-6, IL-8, TNF-α, COX-2, and NF-κB compared to LPS stimulation and non-hydrolyzed casein treatment ($p < 0.05$, Figure 5.7). However, the intact casein also decreased IL-6, IL-8, and COX-2 gene expression ($p < 0.05$) with lower extent as compared to the hydrolysate counterpart. This results suggested that peptides in CH exhibited greater anti-inflammatory responses than the intact casein. Several studies have demonstrated the anti-inflammatory activity of casein hydrolysate in both macrophages and intestinal epithelial cells. Casein hydrolysate produced by a combination of pepsin and colorase down-regulated transcription levels of TGF-β1, NF-κB, and COX-2 in intestinal epithelial cells, leading to reduction of gastrointestinal inflammation (Nielsen, Theil, Larsen, and Purup, 2012). In addition, casein hydrolysate from yak milk prepared by Alcalase showed significant down-regulation of nitric oxide (NO) production, as well as pro-inflammatory cytokines, IL-6, TNF-α, and IL-1β secretion in a dose-dependent manner in LPS-stimulated murine peritoneal macrophages. Moreover, yak milk casein hydrolysate exhibited significantly higher pro-inflammatory cytokine-attenuating activity compared with intact yak casein (Mao, Cheng, Wang, and Wu, 2011).

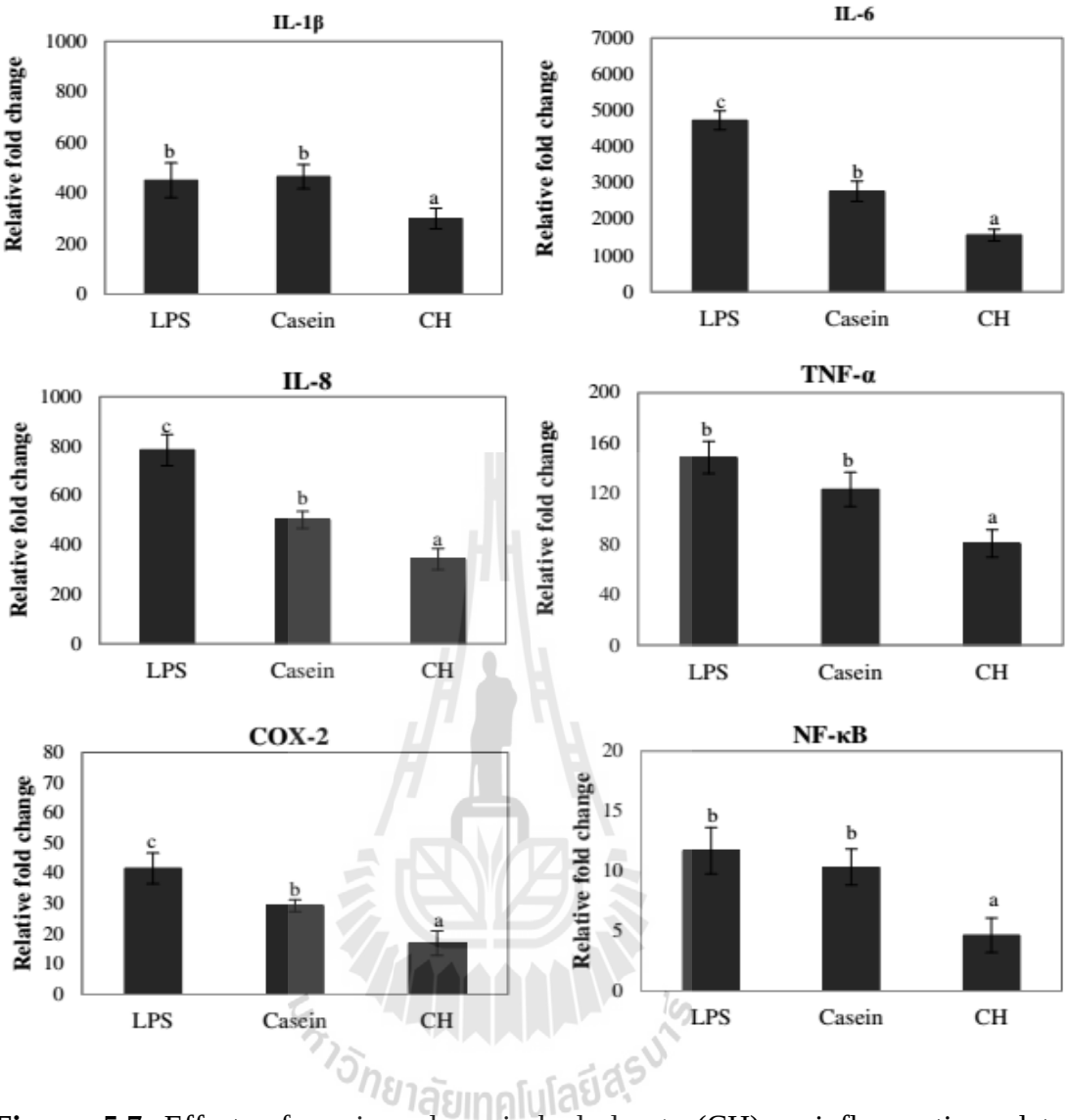

Figure 5.7 Effects of casein and casein hydrolysate (CH) on inflammation-related gene expression of LPS-stimulated THP-1 macrophages. Cells were co-incubated with 100 µg/mL of samples and 0.5 ng/mL of LPS for 6 h. Data shown are the mean ± standard deviation (SD bars). Different letters indicate significant differences ($p < 0.05$).

Both pea protein and PPH resulted in a down-regulation of IL-6 and TNF-α genes of LPS-stimulated THP-1 macrophages ($p < 0.05$, Figure 5.8). The suppressive effect of PPH was found to be stronger than that from pea protein ($p < 0.05$), implying

that peptides in PPH possessed greater anti-inflammatory activity than the non-hydrolyzed pea protein. Ndiaye et al. (2012) found that pea protein hydrolysate produced from thermolysin inhibited secretion of pro-inflammatory cytokines (TNF-α and IL-6) and exhibited significant inhibition of NO production by activated macrophages. Moreover, lupine protein hydrolysate prepared by a combination of Izyme AL and Alcalase decreased expression of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) in THP-1 macrophages (Millán-Linares, Bermúdez, Yust, Millán, and Pedroche, 2014). This is the first report of the inflammatory modulating activity of casein, pea, and fish protein hydrolysates generated by *V. halodenitrificans* SK1-3-7 proteinase. These protein hydrolysates may be potential functional ingredients modulating immune system.

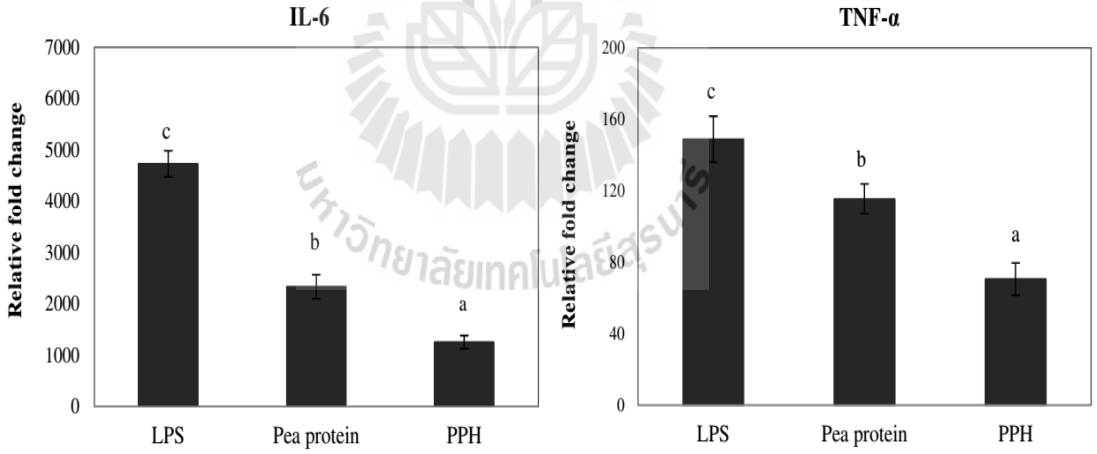

Figure 5.8 Effects of pea protein and pea protein hydrolysate (PPH) on inflammation-related gene expression of LPS-stimulated THP-1 macrophages. Cells were co-incubated with 100 µg/mL of samples and 0.5 ng/mL of LPS for 6 h. Data shown are the mean ± standard deviation (SD bars). Different letters indicate significant differences ($p < 0.05$).

5.5 Conclusions

Tilapia mince, casein, and pea protein were hydrolyzed by *V. halodenitrificans* SK1-3-7 proteinase, yielding hydrolysates with immunomodulating activity. TMH enhanced innate immunity through induction of IL-1β and COX-2. The CH and PPH exerted anti-inflammatory activity by suppressing pro-inflammatory mediators. Protein hydrolysates with immunomodulating activity can be produced using *V. halodenitrificans* SK1-3-7 proteinase.

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CHAPTER VI

SUMMARY

Whole muscle of tilapia hydrolyzed by *Virgibacillus halodenitrificans* SK1-3-7 proteinases showed the highest ACE inhibitory activity when compared to hydrolysates prepared from washed mince and sarcoplasmic proteins. Tilapia hydrolysate showed a mixed-type inhibition characteristic with an IC_{50} value of 0.54 mg/mL. Although *in vitro* gastrointestinal digestion decreased the ACE inhibitory activity of the hydrolysate, it enhanced permeability of peptides across Caco-2 cell monolayers. The permeated peptides exerted ACE inhibitory activity and were identified to be short peptides containing 3-4 amino acids. The novel ACE inhibitory peptide with the highest inhibition was identified to be MCS, with an IC_{50} value of 0.29 μM.

After series of chromatographic separation of tilapia hydrolysate, the most effective fraction for ACE inhibition showed an uncompetitive inhibitor characteristic with an IC_{50} value of 0.15 mg/mL. Tilapia peptides showed high thermostability at 100 and 121°C, maintained inhibitory activity at a wide pH range of 2-10 and resisted *in vitro* gastrointestinal digestion. The presence of hydrophobic amino acids (Met, Ile, Leu, and Phe) in the peptide sequence with Arg residue at the C-terminus possessed the most potent ACE inhibitory activity.

Tilapia muscle, casein, and pea protein were hydrolyzed by partially-purified proteinase from *V. halodenitrificans* SK1-3-7, yielding hydrolysates with immunomodulatory activity. Protein hydrolysates prepared from different protein

sources exerted different inflammation-related gene expression patterns. Tilapia mince hydrolysate enhanced innate immunity through induction of inflammation-related genes. While casein and pea protein hydrolysates showed anti-inflammatory activity by suppression pro-inflammatory mediators.

Therefore, the peptides from *V. halodenitrificans* SK1-3-7 proteinases-hydrolyzed tilapia mince has the potential to be ACE inhibitor and could be used in food systems. Moreover, protein hydrolysates derived from tilapia mince, casein, and pea protein with immunomodulatory activity can be produced by this proteinase as well.

BIOGRAPHY

Tidarat Toopcham was born in December 28th, 1985 at Nakhon Ratchasima, Thailand. She studied for her high school diploma at Saint Mary's College Nakhon Ratchasima (2001-2003). In 2008, she recieved the degree of Bachelor of Science (Food Technology) with second class honor from Suranaree University of Technology, Nakhon Ratchasima, Thailand. In 2008-2013, she received the Royal Golden Jubilee Scholarship from Thailand Research Fund to study for the degree of Doctor of Philosophy (Food Technology) at Suranaree University of Technology. During her graduate study, she presented parts of her research work at RGJ-Ph.D. Congress XV (Chonburi, Thailand, May 28-30, 2014) and the 16th Food Innovation Asia Conference 2014 (Bangkok, Thailand, June 12-13, 2014).