การผลิตเปปไทด์ที่มีคุณสมบัติในการยับยั้งเอนไซม์เปลี่ยนรูปแองจิโอเทนซินจากหนังปลาสวายโมง

นางสาวมนัสวี ขันธสม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2557

PRODUCTION OF ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES DERIVED FROM THAI PANGA SKIN

Manatsawee Khuntasom

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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PRODUCTION OF ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES DERIVED FROM THAI PANGA SKIN

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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มนัสวี ขันธสม : การผลิตเปปไทด์ที่มีคุณสมบัติในการยับยั้งเอนไซม์เปลี่ยนรูปแองจิโอเทนซินจากหนังปลาสวายโมง (PRODUCTION OF ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES DERIVED FROM THAI PANGA SKIN) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 120 หน้า.

หนังปลาสวายโมงจัดเป็นวัสดุเหลือทิ้งจากกระบวนการผลิตเนื้อปลาสวายโมงแล่แช่แข็งซึ่งปกติถูกทิ้งหรือแปรรูปเป็นอาหารสัตว์ที่มีมูลค่าต่ำ การผลิตเปปไทด์ที่มีคุณสมบัติยับยั้งเอนไซม์เปลี่ยนรูปแองจิโอเทนซินเป็นแนวทางหนึ่งในการนำหนังปลาสวายโมงมาใช้ประโยชน์อย่างมีประสิทธิภาพ อีกทั้งยังเป็นการเพิ่มมูลค่าให้กับหนังปลาสวายโมง การนำหนังปลามาใช้เป็นสารตั้งต้นยังไม่มีการศึกษามากนัก วัตถุประสงค์ของการศึกษานี้คือ เพื่อเปรียบเทียบคุณสมบัติในการยับยั้งกิจกรรมเอนไซม์เปลี่ยนรูปแองจิโอเทนซินของโปรตีนไฮโดรไลเสทที่ผลิตจากหนังปลาสวายโมงในรูปแบบต่าง ๆ 3 รูปแบบ ได้แก่ หนังปลาที่ผ่านการกำจัดไขมัน หนังปลาที่ผ่านการแช่ด้วยสารละลายด่างและกรด และเจลาติน โดยถูกย่อยด้วยเอนไซม์โปรติเอสทั้งหมด 6 ชนิด ได้แก่ แอลคาเลส เพปซิน ทริปซิน โปรตาเมกซ์ และโปรติเอสจากเชื้อ *Virgibacillus halodenitrificans* SK 1-3-7 วัดระดับการย่อยสลายและการยับยั้งกิจกรรมเอนไซม์เปลี่ยนรูปแองจิโอเทนซินที่ 12 ชั่วโมง ผลการศึกษาพบว่าชนิดของโปรติเอส และรูปแบบของสารตั้งต้นมีอิทธิพลต่อระดับการย่อยสลายและการยับยั้งกิจกรรมเอนไซม์เปลี่ยนรูปแองจิโอเทนซินของโปรตีนไฮโดรไลเสท (P<0.05) ค่าการยับยั้งกิจกรรมเอนไซม์เปลี่ยนรูปแองจิโอเทนซินจำเพาะสูงที่สุด คือ ร้อยละ 4.9 ต่อไมโครกรัมไกลซีนสมมูล ในตัวอย่างหนังปลาที่แช่ด้วยสารละลายด่างและกรดที่ย่อยด้วยเพปซิน ค่ากิจกรรมการยับยั้งเอนไซม์เปลี่ยนรูปแองจิโอเทนซินเพิ่มขึ้นตามระยะเวลาในการย่อยสลาย และค่ากิจกรรมการยับยั้งมีค่าสูงที่สุดเมื่อย่อยเป็นระยะเวลา 12 ชั่วโมง อย่างไรก็ตามค่าดังกล่าวใกล้เคียงกับที่ย่อยเป็นเวลา 2 ชั่วโมง (P>0.05) โปรตีนไฮโดรไลเสทที่ย่อยด้วยเพปซินเป็นเวลา 2 ชั่วโมงจึงถูกนำมาแยกด้วยเทคนิคอัลตราฟิลเตรชัน โดยใช้เยื่อกรองขนาด 30 5 และ 1 กิโลดาลตันตามลำดับ โปรตีนไฮโดรไลเสทที่มีขนาดเล็กกว่าเยื่อกรองขนาด 1 กิโลดาลตันแสดงค่าการยับยั้งกิจกรรมเอนไซม์เปลี่ยนรูปแองจิโอเทนซินสูงที่สุด คือ ร้อยละ 49.8 ที่ความเข้มข้นเปปไทด์ 25 ไมโครกรัมสมมูลไกลซีนต่อมิลลิลิตร และเมื่อนำส่วนดังกล่าวมาแยกต่อด้วยเทคนิคโครมาโทกราฟฟีแบบแลกเปลี่ยนประจุลบและแบบแยกตามขนาด ตามลำดับ เปปไทด์หลังจากทำบริสุทธิ์บางส่วนแสดงค่าการยับยั้งกิจกรรมเอนไซม์เปลี่ยนรูปแองจิโอเทนซินคิดเป็นร้อยละ 53.8 ที่ความเข้มข้นเปปไทด์ 25 ไมโครกรัมสมมูลไกลซีนต่อมิลลิลิตร และความเข้มข้นของเปปไทด์ที่สามารถยับยั้งกิจกรรมเอนไซม์เปลี่ยนรูปแองจิโอเทนซินร้อยละ 50 (IC_{50}) มีค่าเท่ากับ 19 ไมโครกรัมสมมูลไกลซีนต่อ

มิลลิลิตร ผลการวิเคราะห์ LC-MS/MS พบว่าตัวอย่างหลังจากการแยกด้วยโครมาโทกราฟฟี่ตามขนาดประกอบด้วยเปปไทด์ที่มีกรดอะมิโนชนิดที่ไม่มีขั้ว (ลิวซีนและวาลีน) กรดอะมิโนชนิดที่มีวงแหวน (ทริปโตแฟน และไทโรซีน) หรืออาร์จินีน ที่ตำแหน่งสุดท้ายของสายเปปไทด์ เปปไทด์เหล่านี้มีขนาดมวลโมเลกุลอยู่ระหว่าง 0.7-1.5 กิโลดาลตัน การยับยั้งกิจกรรมเอนไซม์เปลี่ยนรูปแองจิโอเทนซินลดลงร้อยละ 30 หลังผ่านการย่อยในระบบทางเดินอาหารแบบจำลอง ผลการทดลองบ่งชี้ว่าโปรตีนไฮโดรไลเสทที่มีสมบัติการยับยั้งกิจกรรมเอนไซม์เปลี่ยนรูปแองจิโอเทนซินสามารถผลิตได้จากหนังปลาสวายโมงที่ผ่านการแช่ด้วยสารละลายด่างและกรด และย่อยด้วยเพปซิน

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2557 ลายมือชื่ออาจารย์ที่ปรึกษา_____

MANATSAWEE KHUNTASOM : PRODUCTION OF ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES DERIVED FROM THAI PANGA SKIN. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 120 PP.

THAI PANGA SKIN/ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDE/PROTEIN HYDROLYSATE

Thai Panga (*Pangasius hypopthalmus* × *P. bocourti*) (*Pangasius hypopthalmus* × *P. bocourti*) skin is a by-product of the frozen fillet industry which is normally discarded or converted into low-value animal feed. The production of ACE inhibitory peptides may be an efficient utilization that can increase the value of Thai Panga skin. The use of whole skin as a substrate has not yet been fully investigated. The objective of this study was to compare the ACE inhibitory activity of protein hydrolysate produced from different forms of Thai Panga skin. Three forms of Thai Panga skin, namely defatted, alkaline-acid pretreated and gelatin, were used as a substrate for hydrolysis by 6 proteases including Alcalase, pepsin, trypsin, papain, Protamex, and protease from *Virgibacillus halodenitrificans* SK 1-3-7. The degree of hydrolysis (DH) and ACE inhibitory activity at 12 h were measured. It was shown that the type of protease applied and substrate influenced DH and ACE inhibition ($P<0.05$). The highest specific ACE inhibitory activity of 4.9%/μg Gly equivalent (eq.) was obtained from the pepsin-hydrolyzed alkaline-acid pretreated skin. ACE inhibitory activity increased with hydrolysis time and showed the highest ACE inhibition at 12 h. However, it was comparable to that obtained at 2-h hydrolysis. The hydrolysate of pepsin at 2 h was subjected to sequential ultrafiltration membrane fractionation using

30-kDa, 5-kDa and 1-kDa molecular weight cut-off, respectively. Permeate obtained from the 1-kDa membrane showed the highest ACE inhibition of 49.8% at 25 µg Gly eq./mL. This active fraction was further fractionated using anion exchange chromatography and size-exclusion chromatography, respectively. The partially purified peptides showed ACE inhibitory activity of 53.8% at 25 µg Gly eq./mL with an IC_{50} of 19 µg Gly eq./mL. LC-MS/MS analysis revealed that potent fraction from size-exclusion chromatography contained peptides with hydrophobic (Leu or Val), aromatic (Trp or Tyr) residues or Arg at the ultimate position of peptides. The peptides exhibited molecular mass between 0.7 and 1.5 kDa. The ACE inhibitory activity of the partially-purified peptides decreased approximately 30% after *in vitro* simulated pepsin-pancreatin digestion. The results suggested that hydrolysate possessing ACE inhibitory activity can be produced using alkaline-acid pretreated Thai Panga skin hydrolyzed by pepsin.

School of Food Technology Student's Signature_____

Academic Year 2014 Advisor's Signature_____

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Manatsawee Khuntasom

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LIST OF ABBREVIATIONS

Arg	=	Arginine
^{o}C	=	Degree celcius
CFU	=	Colony forming unit
DH	=	Degree of hydrolysis
EDTA	=	Ethylenediaminetetraacetic acid
EC	=	Enzyme Commission
Glu/Gln	=	Glutamic acid/Glutamine
GC/MS	=	Gas chromatography coupled to a mass spectrometry
GI	=	Gastrointestinal
Gly eq.	=	Glycine equivalent
kDa	=	kilo Dalton (10^3 Dalton)
kg	=	kilogram (10^3 gram)
KKS	=	Kallikrein-kinin system
h	=	Hour
Hypro	=	Hydroxyproline
HPLC	=	High performance liquid chromatography
HA	=	Hippuric acid
HHL	=	Hippuryl-L histidyl-L-leucine
Leu	=	Leucine
Lys	=	Lysine

LIST OF ABBREVIATIONS (Continued)

LC-MS/MS	=	Liquid chromatography with tandem mass spectrometry
mg	=	Milligram (10^{-3} gram)
mmHg	=	Millimeter of mercury
mL	=	Milliliter (10^{-3} Liter)
mM	=	Millimolar (10^{-3} Molar)
OD	=	Optical density
Pro	=	Proline
Phe	=	Phenylalanine
RAS	=	Renin-angiotensin system
Ser	=	Serine
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TNBS	=	2,4,6-trinitrobenzenesulfonic acid
Trp	=	Tryptophan
Tyr	=	Tyrosine
U.S.FDA	=	The United States Food and Drug Administration
Val	=	Valine

CHAPTER I

INTRODUCTION

1.1 Introduction

Hypertension is a symptom that systolic and diastolic blood pressure increase over 140 and 90 mmHg, respectively. It is a worldwide epidemic problem and also a key risk factor of heart and heart-related diseases (World Health Organization, 2013). Recent index revealed that 30% world population died by cardiovascular diseases, 45% of heart disease and 51% of stroke mortality, which is related with hypertension (World Health Organization, 2013). The proportion of hypertensive patients is predicted to reach about 1.56 billion in 2025 (Kearney et al., 2005)

Hypertension is associated with abnormal of two physiological systems, which are renin-angiotensin system (RAS) and kallikrein-kinin system (KKS) (Regoli and Jr., 2015; Sayer and Bhat, 2014). These are regulated by a dipeptidyl carboxy metallopeptidase or angiotensin I converting enzyme (ACE, EC 3.4.15.1) (Fuchs et al., 2008; Natesh, Schwager, Sturrock, and Acharya, 2003). In RAS, ACE induces hypertension by generating the active vasoconstrictor, angiotensin II. Angiotensin II leads to vasoconstriction, sodium and fluid retention, resulting in hypertension. In KKS, ACE rapidly degrades vasodilatory agents, bradykinin and kallidin. Therefore, the inhibition of ACE is the major target to reduce blood pressure (Mentz et al., 2013). Although synthetic drugs provide beneficial effect, their adverse side effects including cough, taste disturbance, skin rash, and others are evident (Sica, 2010). This leads a

hypertensive patient (which naturally no symptom) to be difficult to comply with the therapy (Silverman and Holladay, 2015). ACE inhibitory peptides with non-toxicity, thus, have become an alternative compound for preventing hypertension (Hernández-Ledesma, Contreras, and Recio, 2011).

ACE inhibitory protein hydrolysate/peptides are commonly produced by enzymatic hydrolysis (Kim and Wijesekara, 2010; Wang, Mao, Cheng, Xiong, and Ren, 2010). By this method, protease, protein substrate, and hydrolysis condition highly influence the release of ACE inhibitory peptides (Kim and Wijesekara, 2010). Commercial enzymes used to produce protein hydrolysates with ACE inhibitory activity include Alcalase, pepsin, trypsin, papain, and Protamex (Gómez-Guillén, Giménez, López-Caballero, and Montero, 2011; Hernández-Ledesma, Contreras, and Recio, 2011). In addition, the use of new sources of enzyme has frequently been reported. Protease from *Virgibacillus halodenitrificans* SK 1-3-7 isolated from Thai fish sauce fermentation (Montriwong, Kaewphuak, Rodtong, Roytrakul, and Yongsawatdigul, 2012) might potentially be used to produce protein hydrolysate exerting ACE inhibitory activity since it has been reported to produce ACE inhibitory peptides from tilapia (Toopcham, Roytrakul, and Yongsawatdigul, 2015).

Both plants and animals are sources to generate ACE inhibitory peptides. Among them, collagenous materials seem to be valuable sources of ACE inhibitory peptides production because strong ACE inhibitory activity evidently associated with Pro residue in the peptide, particularly at the C-terminal position (Korhonen and Pihlanto, 2006).

Thai Panga (*Pangasius hypopthalmus* × *P. bocourti*) is a hybrid freshwater fish extensively bred at Northeast Thailand. It has a white flesh and mild flavor and has

become an economically important fishery product of Thailand. In 2014, approximately 340,000 tonnes of frozen pangasius fillet entered markets in more than 70 countries (Food and Agriculture Organization of the United Nation, 2014). Fish skins which are discarded from processing plant may be utilized in production of protein hydrolysates with ACE inhibitory activity.

Fish skin mainly contains covalently cross-links of triple helical tropocollagen (Shoulders and Raines, 2009). This strong structure is difficult for enzymatic cleavage (Zhang, Olsen, Grossi, and Otte, 2013). Some previous studies about production of ACE inhibitory peptides from collagenous materials were carried out by disrupting the triple helical structure through boiling into uncoiled structure known as gelatin before use (Byun and Kim, 2001; Himay, Ngo, Ryu, and Kim, 2012; Lee, Jeon, and Byun, 2011). In gelatin preparation, skins are commonly pretreated by soaking in diluted alkaline and acid solution before thermal extraction (Jongjareonrak et al., 2010; Karim and Bhat, 2009). This chemical pretreatment contributes to non-covalent bond disruption so as to partially uncoil the helical structure to ease gelatin solubilization (Karim and Bhat, 2009). Pretreatment of fish skin by alkaline-acid solution might be sufficient to increase the accessibility of enzyme for releasing ACE inhibitory peptides.

In general, crude protein hydrolysate usually contains variant oligopeptides and some impurities (Clemente, 2000). Ultrafiltration in combination with liquid chromatography can isolate and enrich ACE inhibitory peptides with desirable size, charge and hydrophocity (Li-Chan, 2015). Since ACE inhibitory activity is strongly influenced by the first three residues from C-terminus, amino acid sequencing would indicate the interaction between ACE inhibitory peptide and ACE active site

(Asoodeh et al., 2014; Byun and Kim, 2002; Li et al., 2014; Rohit, Sathisha, and Aparna, 2012; Vercruysse et al., 2010).

ACE inhibitory peptides must undergo digestion by pepsin and pancreatic enzymes (Barrett, Ghishan, Merchant, Said, and Wood, 2006). This might affect the modification of peptides, leading to an increase or decrease in their biological activity prior to reach the target site *in vivo* (Alemán, Gómez-Guillén, and Montero, 2013; Barrett, Ghishan, Merchant, Said, and Wood, 2006). A simulated *in vitro* peptic-pancreatic digestion is a promising way to predict the fate of ACE inhibitory peptide in the digestive system (Hur, Lim, Decker, and McClements, 2011). It provides a convenient and reproducible process with lower cost to evaluate the efficacy of the peptides of interest (Hartmann and Meisel, 2007).

The purpose of this study was to evaluate ACE inhibitory activity of protein hydrolysates produced from Thai Panga skin, which was prepared by different treatments, namely defatted skin, alkaline-acid pretreated skin and gelatin. The skins were hydrolyzed by commercial proteases including Alcalase, pepsin, trypsin, papain, Protamex and protease from *Virgibacillus halodenitrificans* SK 1-3-7. Fractionation was carried out using consecutive ultrafiltration, anion exchanger and size-exclusion chromatography. Amino acid sequencing of partially-purified peptide was performed by LC-MS/MS. The partially-purified peptides were subsequently subjected into a simulated *in vitro* peptic-pancreatic digestion to predict its stability in gastrointestinal tract system.

1.2 Research objectives

The objectives of this study were:

1. To produce and compare ACE inhibitory potency of protein hydrolysates produced from skins of Thai Panga in different forms, namely defatted skin, alkaline-acid pretreated skin and gelatin, and hydrolyzed by commercial and *Virgibacillus halodenitrificans* SK 1-3-7 proteases

2. To select the suitable form of substrate, protease and hydrolysis time to produce protein hydrolysates with ACE inhibitory activity.

3. To partially-purify the potent ACE inhibitory peptides and analyze their amino sequences.

4. To determine the stability of ACE inhibitory peptides against simulated pepsin-pancreatin digestion.

1.3 Research hypotheses

Thai Panga skin has potential as a raw material for the production of ACE inhibitory protein hydrolysates. Form of substrate and proteases provide the different ACE inhibitory activity among protein hydrolysates. Partial purification can improve crude protein hydrolysates. Gastrointestinal proteases could affect ACE inhibitory activity of the peptides.

1.4 Scope of the study

The defatted skin, alkaline-acid pretreated skin and gelatin of Thai Panga were hydrolyzed by commercial proteases, including Alcalase, pepsin, trypsin, papain Protamex; and protease from *Virgibacillus halodenitrificans* SK 1-3-7. The degree of

hydrolysis and ACE inhibitory activity of protein hydrolysates were determined. The suitable substrate and protease were selected to produce protein hydrolysates for ACE inhibition. The optimal hydrolysis time for ACE inhibitory peptide production was evaluated. Crude protein hydrolysate was further partially purified by consecutive ultrafiltration, anion exchange chromatography and size exclusion chromatography. The peptide sequences were analyzed by LC-MS/MS. The stability against simulated pepsin-pancreatin digestion of partially-purified peptides was elucidated.

1.5 Expected results

This research would lead to an efficient utilization of Thai Panga skin. Production and ACE inhibitory peptides characteristics would be critical information for bioactive peptide research from Thai Panga skin. The peptides would be a prospect as a nutraceutical product with the potential to prevent hypertension.

CHAPTER II

LITERATURE REVIEW

2.1 Thai Panga

Thai Panga (*Pangasius hypopthalmus* × *P. bocourti*) or common Thai name "Pla Sawai Mong" is a freshwater fish in family *Pangasiidae*. It is a hybrid between female *Pangasius hypopthalmus* (trade name "Tra" or Thai name "Pla Sawai") and male *Pangasius bocourti* (trade name "Basa" or "Pla Mong" in Thai) (Hatachote, Sriphairoj, and Na-Nakorn, 2009). It has a rounded head and blunt snout similar to *P. bocourti*. The stout body without scale has a dark grey dorsum. Dorsal, pectoral and pelvic fins are also dark grey. In addition, it has a silvery side and white belly. Specifically, it has a greenish tint stripe from a dorsal fin to a caudal fin, the unique characteristic which distinguishes from their breeding fish (Figure 2.1).

Figure 2.1 Thai Panga (*Pangasius hypopthalmus* × *Pangasius bocourti*)

Source: Modified from http://www.komchadluek.net

The inland fisheries of Thai Panga located at Northeast Thailand along the Maekhong River. Reproduction of Thai Panga fish is achieved by inducing broodfish to spawn using hormone injections and incubating eggs in conical shape jars, fiberglass tanks or water recirculation system at 27-29^{o}C. The eggs later hatch to larvae within 24 hours and are transferred to a nursery concrete or earthen pond. Juvenile are commonly fed by artemias, rotifers, small crustaceans or boiled egg yolk while pelleted feed is suitable for a mature fish. Thai Panga can be harvested after 6-8 months with a weight of about 1 kg (Kaewla, Arpakulanu, Chumnongsittathum, and Nilsri, 2011; Manee and Tangprakhon, 2008).

Hybrid fish is a prolific spawner and produces relatively large numbers of larvae equal to its female broodfish, while it has a firm texture and mild flavor similar to *P. bocourti*. This advantage is currently promoted as a new economically important fishery product of Thailand competing with Vietnamese Pangasius (Thiparos, 2014; Tulyapongrak, Ratana-Arporn, and Runglerdkriangkrai, 2008). Recently approximately 340,000 tonnes of frozen pangasius fillet entered markets in more than 70 countries in 2014 where European Union, Latin America and Asia were the major markets (Food and Agriculture Organization of the United Nation, 2014).

Studies on Thai Panga fish are mostly concerned with farming practices (Kaewla, Arpakulanu, Chumnongsittathum, and Nilsri, 2011; Manee and Tangprakhon, 2008). The post-harvest studies of Thai Panga are involved with the frozen fillet products. Tulyapongrak, Ratana-Arporn, and Runglerdkriangkrai (2008) studied the physical, chemical and microbiological changes of Thai Panga fish during chilled storage and indicated that electrical properties as measured by Torymeter and K-value can be used as freshness index for Thai Panga fish. Thipbharos (2014) implied that geosmin content in frozen Thai Panga fish fillets could be reduced using

ozone, banana leaf ash or sodium chloride solutions based on SPME-GC/MS analysis and sensory evaluation. However, utilization of Thai Panga waste, particularly skin, has not been widely studied. Disposal of Thai Panga skins is presently accomplished by converting to an animal feed with low returns.

2.2 Fish skin protein

The dermis layer of fish skin comprises high concentration of structural proteins (elastin and collagen) that could be converted to high value products in food, pharmaceutical, cosmeceutical and biomedical products (Cho, Jin, Rha, Kim, and Hwang, 2014; Morimur et al., 2002). The collagen fibrous forming to a connective tissue of fish skin is assembled from many collagen fibrils, which each collagen fibril is formed by cross linking of individual tropocollagen (Figure 2.2) (Shoulders and Raines, 2009). Tropocollagen, a primary strucrure of collagen comprises three polypeptide left-hand α-chain helices. Each α-helix chains are twisted together into a right hand triple helix. Each helix chain contains a repeating of Gly-X-Y as a primary sequence, where X and Y are high possibility to be Pro and Hyp (Shoulders and Raines, 2009). The structure of tropocollagen is stabilized by hydrogen bonding between Gly and Pro of neighboring chains (Shoulders and Raines, 2009). Additionally, inter-chain hydrogen bonds can be formed between water molecule and hydroxyl group of amino acid at Y position. At the N- and C-terminal of helical structure has short non-helical domains namely, telopeptides. Telopeptides has a role in fibril formation and stabilization thereby the oxidation of Lys side chain in their molecules (Shoulders and Raines, 2009). Most of 29 collagen types, animal skin principally contain type I collagen, whose structure normally composed of two α_1 (I) and one α_2 (I) chains (Gorgieva and Kokol, 2011). Fish skin collagen contains a

variety of amino acids and it has less content of proline and hydroxylproline but higher in serine and threonine than that of land mammals (Karim and Bhat, 2009).

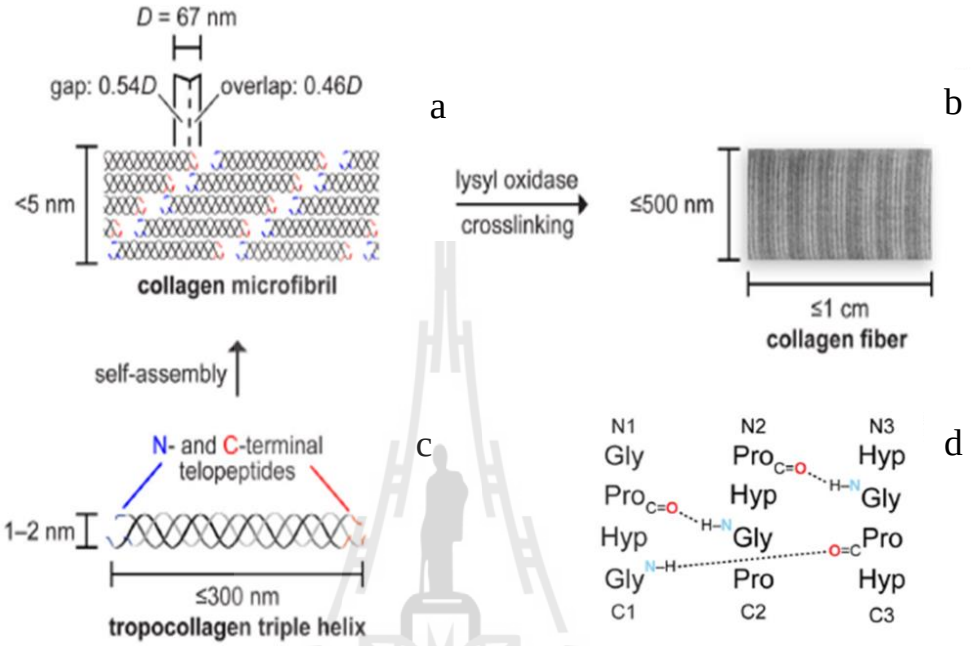

Figure 2.2 Tropocollagen is held by hydrogen bonds between Gly and Pro of neighboring chain (d). Tropocollagens covalently cross-link to form collagen fibril, and many collagen fibrils assemble into collagen fiber of the skin (c, a, b).

Source: Modified from Shoulders and Raines (2009).

The usage of collagen is mostly related to their functional property including gelling, emulsifying, film and foaming agent in food, cosmetic as well as biomedical industry (Gómez-Guillén, Giménez, López-Caballero, and Montero, 2011; Karim and Bhat, 2009). Many researchers attempted to optimize the extraction condition for improving the functional properties of fish skin collagen and gelatin in the past

decade. However, number studies showed that collagen and gelatin from fish skin has poor functional property when compared with that of commercial products derived from bovine and porcine organ. This might be due to lower imino acid content in the fish skin when compared with that of land mammal (Ahmad, Benjakul, and Nalinanon, 2010; Cheow, Norizah, Kyaw, and Howell, 2007; Karim and Bhat, 2009; Kasankal, Xue, Weilong, Hong, and He, 2007; Lassoued et al., 2014; Nagai and Suzuki, 2002; Nagai, Yamashita, Taniguchi, Kanamori, and Suzuki, 2001; Nikoo et al., 2014; Olsen, Toppe, and Karunasagar, 2014; Zeng et al., 2009).

Collagen and gelatin have also been reported as sources of biologically active peptides (Banerjee and Shanthi, 2012; Gómez-Guillén, Giménez, López-Caballero, and Montero, 2011). Bioactivities of collagen- and gelatin-derived peptides, such as antimicrobial activity, mineral binding capacity, lipid lowering effect, immunomodulatory activity, antihypertensive/ACE inhibitory activity and antioxidative activity have been reported (Gómez-Guillén, Giménez, López-Caballero, and Montero, 2011). Among them, the studies about collagen and gelatin derived peptides have greatly dealt with antihypertensive or ACE inhibitory properties. Since Pro residues in repeating Gly-Pro-Hyp of collagen have associated with high antihypertensive properties (Cheung, Wang, Ondetti, Sabo, and Cushman, 1980; Korhonen and Pihlanto, 2006).

2.3 Angiotensin I converting enzyme (ACE)

2.3.1 Structural and biochemical properties of angiotensin I-converting enzyme (ACE)

Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) or other designations i.e. kininase II, peptidyl-dipeptidase A, dipeptidyl carboxy peptidase I, carboxy

cathepsin, dipeptide hydrolase and peptidase P, is a zinc metallopeptidase belonging to gluzincin family of metalloproteases (Barrett, Rawlings, and Woessner, 2004). In human, there are two distinct isoforms which are encoded by the same gene including somatic ACE (sACE) and germinal or testis ACE (gACE or tACE, respectively) (Barrett, Rawlings, and Woessner, 2004). Both forms are membrane bound protein and can be released as a soluble enzyme into extracellular fluids via the proteolytic cleavage by secretase or sheddase (Guang, Phillips, Jiang, and Milani, 2012). Germinal ACE is a smaller isozyme found exclusively in testis and responsible in germinal cell differentiation (Bernstein et al., 2013). The enzyme contains a single catalytic active domain identical to C-domain of sACE, except for a unique 67-residue sequence constituting its NH_2 (Ehlers, Fox, Strydom, and Riordan, 1989). It comprises 732 amino acids and a molecular weight of around 90-110 kDa (Barrett, Rawlings, and Woessner, 2004). C-domain of tACE has been used as a representative of sACE to study the interaction between ACE inhibitor and ACE active site (Guy, Lambert, Warner, Hooper, and Turner, 2005).

The larger isozyme, sACE (molecular weight 150-180 kDa) contains 1306 amino acids and can be detected in a variety of tissues, particularly abundant on the endothelial surface of lung and kidney (Riordan, 2003; Turner and Hooper, 2002). Its integral membrane protein composes of an intracellular domain, hydrophobic transmembrane region and extracellular domain (Bernstein et al., 2013). The extracellular domain is divided into two highly homologous domains including C-domain and N-domain (Barrett, Rawlings, and Woessner, 2004). Structurally, both domains have predominance of α-helices and the overall shape is an ellipse. The active site groove is positioned at the central structure and has a lid-like structure covering the active site channel that restrict the access of large polypeptide to the

active site, which both domains contain a sequence His-Glu-Met-Gly-His, a zinc binding ligand for catalytic activity (Regulska, Stanisz, Regulski, and Murias, 2014; Sturrock, Natesh, Rooyen, and Acharya, 2004; Williams, Corvol, and Soubr, 1994).

By analogy with carboxypeptidase A and thermolysin, Cushman and Ondetti (1984) proposed a predictive model of ACE active site which thereafter became a template for ACE inhibitor designs. In this model, ACE active site comprises three binding subsites (S_1, S_1', and S_2') for interacting with the substrate ligand. The zinc ion between S_1 and S_1' is an enzyme cofactor that assists to reduce the activation energy for hydrolysis of peptide bond via increasing the electrophillicity to the carbonyl oxygen of the oligopeptide substrate and nucleophillicity to the water molecule. The position between S_1 and S_1'subsite is a hydrogen bond donor (H-X). A positive charge site is located next to S_2' for binding the terminal carboxyl group of substrates (Figure 2.3).

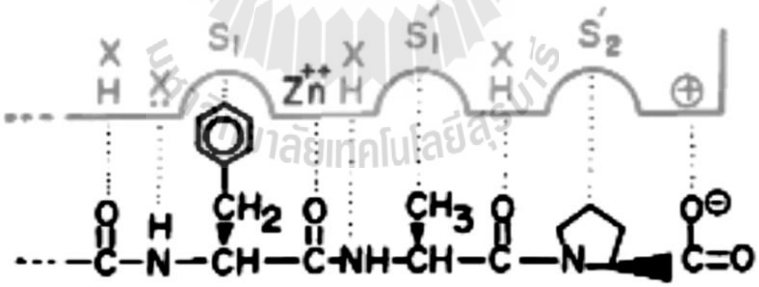

Figure 2.3 The predictive model of ACE active site binding with a venom peptide.
Source: Cushman and Ondetti (1984).

C-domain requires monovalent anions, notably chloride for the activation whereas N-domain can be completely activated without chloride (Sturrock, Natesh, Rooyen, and Acharya, 2004). ACE shows the optimal activity at 37^{o}C, pH 8.3 and

requires 300 mM NaCl for its activation (Barrett, Rawlings, and Woessner, 2013). The enzyme could be inhibited by EDTA and other metal chelating agents and can be reactivated by manganese, zinc and cobalt (Cushman, Cheung, Sabo, and Ondetti, 1977). It has the diversity of substrate specificity *in vitro* due to the individual amino acid sequence at the active site of each domain (Rioli et al., 2003). The role in blood pressure regulation, both domains responsibly degrade a vasodilator, bradykinin in kallikrein-kinin system, while only C-domain of sACE mainly produces a vasoconstrictor, angiotensin II in renin angiotensin system (Sayer and Bhat, 2014).

2.3.2 Renin-angiotensin and kallikrein-kinin system

Renin-angiotensin-aldosterone system (RAAS) or renin-angiotensin system (RAS) is the hormone system, responsible for the regulation of blood pressure, sodium and fluid homeostasis and tissue repair via inflammatory and proliferative mechanisms (Mentz et al., 2013). The RAS enzymatically cascade (the pathway which is involved in angiotensin production) begins when the liver-produced angiotensinogen (α2-globulin) is released into the circulation and renin (aspatyl protease, EC 3.4.23.15) is released from juxtaglomerular cells of the kidney (Guang, Phillips, Jiang, and Milani, 2012). Renin then generates the inactive decapeptide angiotensin I (Ang I, Ang 1-10, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) from anhiotensinogen as shown in Fig. 2.3. Ang I is cleaved at the C-terminal dipeptide, His-Leu, by angiotensin converting enzyme (ACE) at the endothelial surface of blood vessels, producing the critical active vasoconstrictor, angiotensin II (Ang II, Ang 1-8, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) (Mentz et al., 2013). Ang II could exert biological effects by interacting with the specific receptor including type-1 angiotensin (AT1) receptor and type-2 angiotensin (AT2) receptor (Kloet, Krause, and Woods, 2010). Although AT2 receptor is able to regulate the renal function and blood pressure

(Tikellis, Cooper, and Thomas, 2006), most of physiological actions are mainly induced by Ang II- AT1 receptor complex including: vasoconstriction, stimulation of the synthesis and release of aldosterone; sodium reabsorption from the renal tubule; cardiac growth; proliferation of vascular smooth muscle; increase in peripheral noradrenergic activity and central activity of sympathetic nervous system; stimulation of the release of vasopressin; and inhibition of renal renin (Contreras et al., 2002). The excessive activity of RAS is recognized as a key role for the development of pathological changes in the arterial wall which is a cardiovascular risk factor in subjects with essential hypertension (Contreras et al., 2002).

Those deleterious effects of RAS can be counteracted by kallikrein-kinin system (KKS) (Regoli and Jr., 2015). It is an enzymatic system implicated in blood pressure regulation and inflammation through bradykinin and related kinins, which are produced by two proteolysis pathways including plasma and tissue kallikrein (Saxena, Thompson, Udekem, and Konstantinov, 2011). Plasma kallikrein produces the vasoactive peptide, bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), by hydrolyzing high molecular weight kinigonen, while tissue kallikrein hydrolyzes low molecular weight kininogen liberating Lys-bradykinin or kallidin (Albert-Weißenbergera, Sirén, and Kleinschnitz, 2013). Both bradykinin and kallidin can be further converted into des-Arg9 bradykinin and des-Arg10 kallidin, respectively, by carboxypeptidase (kininase I) (Albert-Weißenbergera, Sirén, and Kleinschnitz, 2013). Lys-bradykinin can be transformed into bradykinin by proteolysis of aminopeptidase (Golias, Charalabopoulos, Stagikas, Charalabopoulos, and Batistatou, 2007). The two kinin derivatives functionally exert the inflammatory response by mediation of G-protein-coupled receptor, B1 receptor, while bradykinin and kallidin primarily bind to B2 receptor (Saxena, Thompson, Udekem, and Konstantinov, 2011). Mediation of B2

receptor is implicated in hypotension by vasodilation, which is involved reduction of systemic vascular resistance, diuresis and sodium excretion by a kidney (Regoli and Jr., 2015). However, these efficient vasodilatory agents have a short life; they are largely inactivated in the lung vasculature and limited amounts could reach the arterial blood (Regoli and Jr., 2015). Bradykinin and kallidin can be rapidly destroyed into inactive vasodilatory peptides by enzymatic hydrolysis of kininase II or ACE. Therefore, inhibition of ACE is a dual mechanism which could not only reduce the impact of RAS but also potentiate the activity of KKS (Regoli and Jr., 2015).

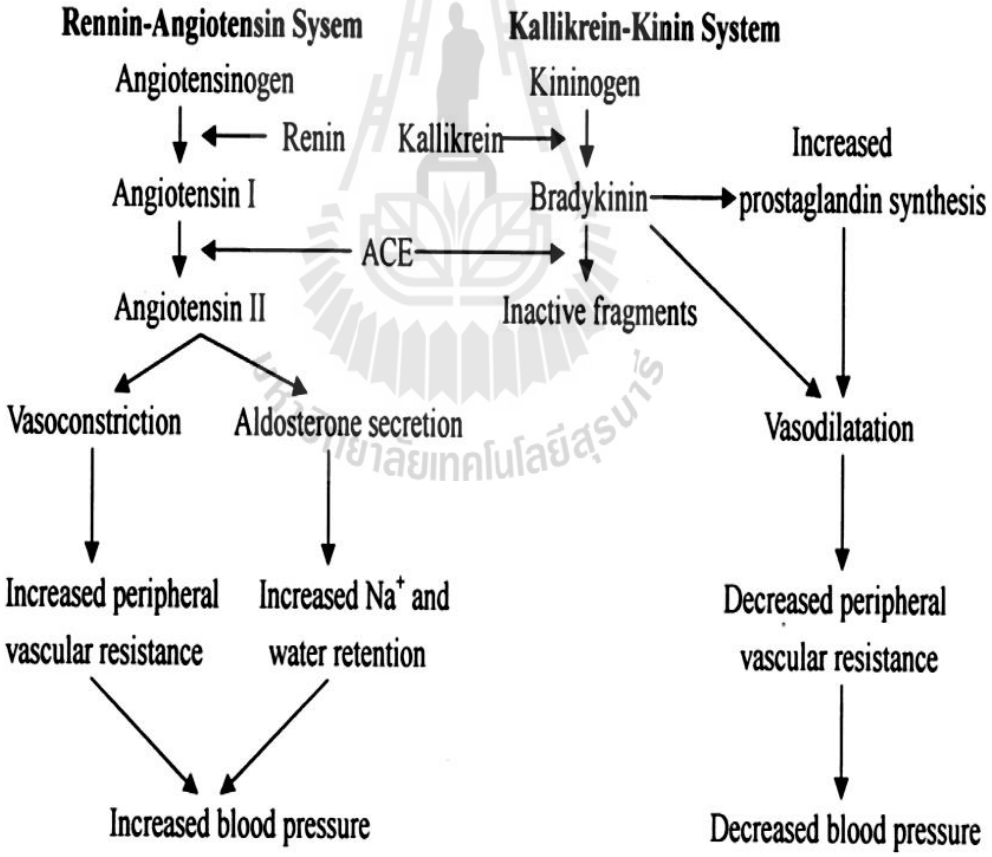

Figure 2.4 Role of ACE in blood pressure regulation.

Source: Li, Leu, Shi, and Shresthaa (2004).

2.3.3 Pharmaceutical ACE inhibitors

ACE inhibitors are one class of antihypertensive drugs which are regularly used in the treatment of hypertension and related cardiovascular diseases (Mentz et al., 2013). The venom extract of *Bothrop jararaca* (nonapeptide teprotide, SQ 20,881, BPP_{9a}, Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) was found as the first agent possessing ACE inhibitory effect and bradykinin potentiating activity (Ferreira, Bartelt, and Greene, 1970; Ng and Vane, 1970). Owing to the unavailability by oral administration, it was unsuccessfully used as an antihypertensive drug (Bhuyan and Mugesh, 2011). Later the first orally active ACE inhibitor, captopril (captoten, SQ 14,225) was approved by U.S.FDA. It has been the breakthrough for exploitation of other ACE inhibitors (Cushman et al., 1997). The inhibitors which are commonly prescribed for hypertensive patients can be categorized into three subgroups including: 1) sulfhydryl (-SH) containing inhibitors e.g. captopril, zofenopril; 2) dicarboxylate (-COOH) containing inhibitors e.g. lisinopril, enalapril, benazepril, quinapril, ramipril, trandolapril; and 3) phosphate ($-PO_2$) containing inhibitor i.e. fosinopril (Figure 2.5) (Atlas, 2007). These inhibitors are divided based on the zinc-chelating group presenting in their structure (Bhuyan and Mugesh, 2011). The sulfhydryl-containing drugs bind to zinc ion through the SH group, leading to the formation of zinc (II)-thiolate (Figure 2.5). The carboxyl-containing drugs interact through carboxylate group and fosinopril interacts through the phosphate group. Other functional groups are required for ACE inhibitors including terminal carboxyl group for ionic interaction with a positively charge residue and a central carbonyl oxygen is required to act as hydrogen bond acceptor (Regulska, Stanisz, Regulski, and Murias, 2014). In addition, N-terminal phenyl residue is preferably obligated by S_1subsite and proline is necessary for the interaction with S_2' subsite. ACE inhibitory drugs block the action of

ACE as a competitive inhibitor. Captopril and lisinopril are an active drug while other inhibitors are ester-containing prodrugs which require *in vivo* hepatic and intestinal wall esterolysis to generate the active form to attack ACE (Regoli and Jr., 2015).

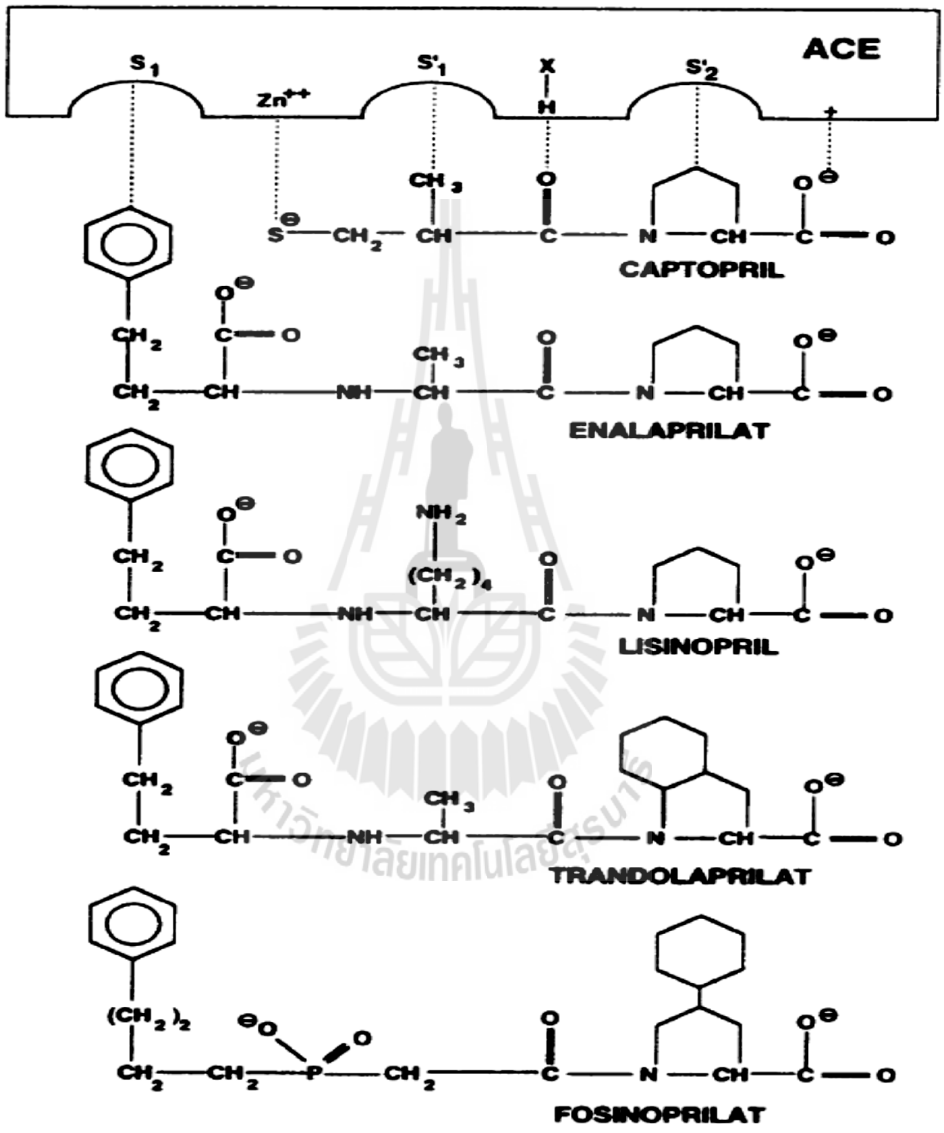

Figure 2.5 Structures of ACE inhibitor drugs coordinating with the active site of ACE via their carboxyl groups, whereas captopril and fosinopryl coordinate via sulfhydryl and phosphoryl groups, respectively.

Source: Williums et al. (1996).

ACE inhibitors produce positive outcomes and have achieved widespread usage in the treatment of hypertension, cardiovascular diseases, and renal disease (Sica, 2010). Nonetheless, both physiological and nonphysiological adverse effects have been the basis for ACE inhibitors therapy (Sica, 2010). Dry cough is a frequent undesirable effect initiated by ACE inhibitors, accounting for 3.9% of patients (Regoli and Jr., 2015). Another serious side effect is angioedema. The inhibitors increase risk of foetopathy for the usage during the third trimester of pregnancy and they cause malformation for the exposure to ACE inhibitors during the first trimester of pregnancy (Guang, Phillips, Jiang, and Milani, 2012). The physiological consequences include hypotension, deterioration of renal function, and hyperkylemia (Atlas, 2007). Furthermore, toxic effects associated mainly with captopril including taste disturbance (metallic and salty taste), skin rash, neutropenia, hepatic toxicity, and proteinuria (membranous nephropathy) (Atlas, 2007).

2.4 ACE inhibitory peptides

Unacceptable side effects of synthetic drugs provoke the search of natural ACE inhibitors, since they provide a safer, milder and lower cost alternative for preventing high blood pressure (Erdmann, Cheung, and Schröder, 2008; Gu, Majumder, and Wu, 2011; Hernández-Ledesma, Contreras, and Recio, 2011). Bioactive peptides exhibiting diverse physiological functions have been isolated from various sources. ACE inhibitory peptides showed the inhibition mechanisms distinctive from that of synthetic ACE inhibitors. The synthetic drugs deactivate ACE by competitively chelating Zn^{2+} buried at the active site of ACE via any of sulfhydryl, phosphoryl, and carboxylate group (Atlas, 2007). Unlike the synthetic drug, ACE

inhibitory peptides use their C-terminal amino acid residues to inhibit ACE by some of hydrogen bonding, van der Waals interaction, electrostatic interaction or hydrophobic interaction with the amino acid residues located at either the catalytic site or other binding site of ACE as a competitive, non-competitive, uncompetitive and mixed ACE inhibitor depending on molecular characteristics of the peptide (Asoodeh et al., 2014; Banerjee and Shanthi, 2012; Jimsheena and Gowda, 2011; Li et al., 2014; Vercruysse, Camp, Morel, Rouge´, Herregods, and Smagghe, 2010). Many studies concurred that ACE inhibitory activity of peptides is considerably dependent on their C-terminal amino acid residues (Gómez-Guillén, Giménez, López-Caballero, and Montero, 2011; Harnedy and FitzGerald, 2012; Hernández-Ledesma, Contreras, and Recio, 2011; Lafarga and Hayes, 2014).

Typically, peptides are not active within the original protein structure even though they can be released from their parent proteins by gastrointestinal digestion or during food processing (Hernández-Ledesma, Contreras, and Recio, 2011). Those peptides may also be produced by other procedures such as enzymatic hydrolysis, acid or alkaline hydrolysis, and microbial fermentation, resulting in a crude protein hydrolysate (He, Liu, and Ma, 2013; Kim and Wijesekara, 2010). Among them, enzymatic hydrolysis is considered as an effective method for ACE inhibitory protein hydrolysates and peptide production (Harnedy and FitzGerald, 2012; Li-Chan, 2015; Wang, Mao, Cheng, Xiong, and Ren, 2010).

2.4.1 Production of ACE inhibitory peptides by enzymatic hydrolysis

Enzymatic hydrolysis is a common process used to produce ACE inhibitory hydrolysates and peptides (Kim and Wijesekara, 2010; Wang, Mao, Cheng, Ren, and Xiong, 2010). It is carried out under mild condition with an ability to control product quality without residual organic solvent or toxic chemicals in the products when

compared with acid and alkaline hydrolysis (Agyei and Danquah, 2011; Clemente, 2000; He, Franco, and Zhang, 2013). By this method, protease and protein substrate are crucial factors governing structural features of peptides (molecular size, amino acid composition, peptide chain length, peptide sequence and amount of peptides), which, in turn, influence ACE inhibitory potency of peptides (Kim and Wijesekara, 2010). Furthermore, hydrolysis time and substrate pretreatment also contributed to the improvement of ACE inhibitory activity of protein hydrolysates (Skierka and Sadowska, 2007).

2.4.1.1 Proteases for ACE inhibitory peptide production

Proteolytic enzymes using for ACE inhibitory peptides production are generally obtained from plants, microbes and animals (Hernández-Ledesma, Contreras, and Recio, 2011). Since the ability of enzymes to hydrolyze protein substrates is highly variable, the selection of suitable enzyme to produce peptides with desired bioactive properties is essential (Clemente, 2000). The commercial food-grade proteases which have been widely used for preparing ACE inhibitory peptide include Alcalase®, pepsin, trypsin, papain, and Protamex™ (Gómez-Guillén, Giménez, López-Caballero, and Montero, 2011; Hernández-Ledesma, Contreras, and Recio, 2011).

Alcalase® (EC 3.4.21.62), a serine endoprotease secreted by *Bacillus licheniformis*, has a broad specificity and high specificity toward aromatic amino acids, Glu, Met, Leu, Ala, Ser and residues in peptide chains (Wong, 1995). It is the most efficient in the hydrolysis of food proteins (He, Liu, and Ma, 2013). The high degree of hydrolysis can be achieved in a relatively short time (Gómez-Guillén, Giménez, López-Caballero, and Montero, 2011). The enzyme has a potential to

produce potent ACE inhibitory peptides with low molecular weight in various plant and animal proteins (Ahhmed and Muguruma, 2010; Erdmann, Cheung, and Schröder, 2008; He, Liu, and Ma, 2013). Furthermore, some studies reported that Alcalase hydrolsates exhibited the highest ACE inhibitory activity compared to other enzymes (Ahn, Jeon, Kim, and Je, 2012; Alemána et al., 2011; Lee, Qian, and Kim, 2010; Ngo, Ryu, and Kim, 2014; Zhang, Olsen, Grossi, and Otte, 2013)

Pepsin (EC 3.4.23.1) is an aspatic protease family generally found in the gastric juices of vertebrates (Barrette, Rawlings, and Woessner, 2004). It preferably cleaves peptide bonds at aromatic amino acid residues (Phe, Tyr, Trp), Leu and Asp at carboxyl side of a peptide bond (Whitaker, 1994). Previous studies indicated that pepsin showed highly ACE inhibitory activity *in vitro*, those peptides contain hydrophobic amino acid residues at C-terminal which can interact with ACE active site (Dub et al., 2013; Muguruma et al., 2009).

Trypsin (EC 3.4.21.4) is a serine endopeptidase produced in pancreas and preferably cleaves peptide bond at the carboxyl side of peptide bond containing Arg and Lys (Barrette, Rawlings, and Woessner, 2004). Tryptic peptides exerting high ACE inhibitory activity have been found in plants, animals and animal products (Asoodeh et al., 2014; Hou et al., 2012; Lee, Jeon, and Byun, 2014; Power, Fernández, Norris, Riera, and FitzGerald, 2014; Tanzadehpanah, Asoodeh, Saberi, and Chamani, 2013).

Papain (EC 3.4.22.2) is a cysteine protease found in the latex of tropical papaya fruit (*Carica papaya*) (Barrette, Rawlings, and Woessner, 2004). The enzyme has a fairly broad substrate specificity (Whitaker, 1994). Using papain individually or in combination with Alcalase and trypsin could increase ACE inhibitory activity of various animal and plant peptides (Asoodeh, Yazdi, and

Chamani, 2012; Gu, Li, Liu, Yi, and Cai, 2011; Ngoa, Ryu, Voa, Himaya, and Kim, 2011; Rui, Boye, Simpson, and Prasher, 2013).

Protamex™ is a food grade commercial endoprotease produced from *Bacillus subtilis* with a wide specificity for peptide bonds (Wang et al., 2010). It is an inexpensive protease and could be used to produce ACE inhibitory peptides from marine wastes (Thi et al., 2011; Tsai, Chen, and Pan, 2008; Tsai, Lin, Chen, and Pan, 2006). It has been reported that fish waste (mackerel and cod bone and skin) hydrolysate prepared from Protamex showed the highest ACE inhibitory activity compared to Alcalase and Flavourzyme (He et al., 2007).

Protease from *Virgibacillus halodenitrificans* SK 1-3-7, a subtilisin-like serine protease, was isolated from Thai fish sauce fermentation. The enzyme is stable between pH 4 -10 and below 60^{o}C and showed high catalytic activity toward fibrin (Montriwong, Kaewphuak, Rodtong, Roytrakul, and Yongsawatdigul, 2012). Proteases secreted from *Virgibacillus sp.* have a broad specificity and high catalytic acivity toward fish protein (Sinsuwan, Rodtong, and Yongsawatdigul, 2010). The enzyme has been applied to produce protein hydrolysates with bioactive properties from both plant and animal origins (Arunachalam, Amirtham, and Appadorai, 2013; Lapsongphon and Yongsawatdigul, 2013; Wiriyaphan, Xiao, Decker, and Yongsawatdigul, 2015).

2.4.1.2 Protein sources for ACE inhibitory peptides production

Protein substrates are another vital factor affecting ACE inhibitory activity. The unique peptide sequence with diverse amino acid compositions were directly affected by protein substrates. Protein hydrolysates or peptides exhibiting ACE inhibitory activity or antihypertensive property found in previous studies could be derived from various food proteins including plants, animals and animal products.

Plant-derived ACE inhibitory peptides found in previous studies were mostly isolated from protein hydrolysates of cereals and cereal by-products. The cereals were used as substrates in enzymatic hydrolysis including rapeseed (He et al., 2013; Kinenen, Johannson, Gerd, Pihlava, and Pihlanto, 2012; Marczak et al., 2003; Yu et al., 2013), canola (Wu, Aluko, and Muir, 2008, 2009), peanut (Quist, Phillips, and Saalia, 2009), sunflower (Megı´as et al., 2009), lima bean (Torruco-Uco, Chel-Guerrero, Martı´nez-Ayala, Da´vila-Ortı´z, and Betancur-Ancona, 2009), jamapa bean (Torruco-Uco et al., 2009), amaranth (Tovar-Pérez, Guerrero-Legarreta, Farrés-González, and Soriano-Santos, 2009), wheat (Qua, Ma, and Zhao, 2013), chickpea (Barbana and Boye, 2010), yellow pea (Barbana and Boye, 2010), lentil (Barbana and Boye, 2011; Boye, Roufik, Pesta, and Barbana, 2010), cowpea (Segura-Campos, Chel-Guerrero, and Betancur-Ancona, 2011), arachin (Jimsheena and Gowda, 2010, 2011), chia (Segura-Campos, Salazar-Vega, Chel-Guerrero, and Betancur-Ancona, 2013), soybean (Gu and Wu, 2013), rice (Chen, Liu, Ye, Cai, Jic, and Wu, 2013), hemp seed (Girgiha et al., 2014), red bean (Durak, Baraniak, Jakubczyk, and wieca, 2013; Rui, Boye, Simpson, and Prasheret, 2013), *Parkia speciosa* seed (Siow and Gan, 2013), lupin (Boschin, Scigliuolo, Resta, and Arnoldi, 2014), pistachio (Li et al., 2014), and brewers' spent grain (Connolly, O'Keeffe, Piggott, Nongonierma, and FitzGerald, 2015). Among those sources, peptides derived from rice (VNP, VWP at 5 mg/kg body weight), rapeseed (LY, RALP at 0.15 g/kg body weight), and hemp seed (WVYY, PSLPA at 30 mg/kg body weight) showed antihypertensive activity in spontaneously hypertensive rats (SHR) after oral administration (Chen et al., 2013; Girgiha et al., 2014; He et al., 2013).

Protein hydrolysates exerting ACE inhibitory activity derived from muscle protein including myofibrillar, sarcoplasmic, and connective tissue of aquatic

as well as terrestrial animals were extensively studied. Myofibrillar proteins used to generate ACE inhibitory peptides could be obtained from porcine skeletal (Arihara, Nakashima, Mukai, Ishikawa, and Itoh, 2001; Castellano, Aristoy, Sentandreu, Vignolo, and Toldráb, 2013; Muguruma et al., 2009), travelly (Salampessy, Reddy, Kailasapathy, and Phillips, 2015), lizard (Wu, Feng, Lan, Xu, and Liao, 2015), goby (Nasria et al., 2013) , grass carp (Chen, Wang, Zhong, Wu, and Xia, 2012), haruan (Ghassem, Arihara, Babji, Said, and Ibrahim, 2011), seaweed pipefish (Wijesekara, Qian, Ryu, Ngo, and Kim, 2011), tilapia (Raghavan and Kristinsson, 2009), atlantic salmon (Nakajima, Yoshie-Starka, and Ogushi, 2009), coho salmon (Nakajima, Yoshie-Starka, and Ogushi, 2009), Alaska Pollack (Nakajima, Yoshie-Starka, and Ogushi, 2009), southern blue whiting (Nakajima, Yoshie-Starka, and Ogushi, 2009), shark meat (Wu et al., 2008), bull frog muscle (Qian, Jung, Lee, Byun, and Kim, 2007), cod (Jensen, Eysturskarð, Madetoja, and Eilertsen, 2014), and bonito (Fujita, Yamagami, and Ohshima, 2001). Peptides isolated from porcine myosin B (KRVIQY, VKAGF), chicken muscle (IKW, LAP, LKP), bull frog (GAAQLPCSADWW) as well as cod protein hydrolysates could show hypotensive effect in SHR (Muguruma et al, 2009; Qian, Jung, Lee, Byun, and Kim, 2007). Furthermore, peptide soup containing LKPNM derived from dried bonito was approved as Foods for Specified Health Use in Japan and has already been commercialized in a variety trade names (Vasotensin 120T, PeptACE, or Peptides 90) (Fujita, Yamagami, and Ohshima, 2001; Hartmann and Meisel, 2007). ACE inhibitory peptides can also be derived from sarcoplasmic protein hydrolysates. Bovine brisket (Bernardini et al., 2012), porcine globin (Yu et al., 2006), beef rump (Jang and Lee, 2005), and sipunculid worm (Dub et al., 2013) were example of water soluble protein resources which have been found in the literatures. Peptides, AWLHPGAPKVF derived from sipunculid worm could reduce

systolic blood pressure of SHR when orally administered at a dose of 10 mg/kg rat body weight. Connective tissues, particularly skins, were recommended as a potential substrate for producing ACE inhibitory peptides since they are a rich source of collagen containing a unique sequence (Gly-Pro-Hyp) through its triple helix structure (Gómez-Guillén, Giménez, López-Caballero, and Montero, 2011). The oligopeptides containing Pro especially at C-terminal end were reported to inhibit ACE activity effectively (Korhonen and Pihlanto, 2006). Mostly, the substrate used to release ACE inhibitory peptides from connective tissues were in the form of collagen or gelatin. Collagenous materials which have been employed to produce collagen hydrolysates for ACE inhibition include squid (Alemán, Gómez-Guillén, and Montero, 2013), sea bream scales (Fahmi et al., 2004), jelly fish (Liu, X., Zhang, M., Zhang, C., and Liu, C., 2012) and bovine Achilles tendon (Banerjee and Shanthi, 2012; Yuhao Zhang, Olsen, Grossi, and Otte, 2013), Alaska pollack skin (Byun and Kim, 2001, 2002), sea cucumber (Zhao et al., 2007), Pacific cod skin (Himay, Ngo, Ryu, and Kim, 2012; Ngoa, Ryu, Voa, Himaya, and Kim, 2011), squid (Alemán, Giménez, Pérez-Santin, Gómez-Guillén, and Montero, 2011; Lin, Lv, and Li, 2012), Atlantic salmon skin (Gu, Li, Liu, Yi, and Cai, 2011), duck skin (Lee et al., 2012), blacktip shark (Kittiphattanabawon, Benjakul, Visessanguan, and Shahidi, 2013), and thornback ray (Lassoued et al., 2015), skate skin (Lee, Jeon, and Byun, 2011; Ngo, Ryu, and Kim, 2014), chicken skin (Onuh, Girgih, Aluko, and Aliani, 2013), chum salmon skin (Lee, Jeon, and Byun, 2014), Alaska pollock frame (Hou et al., 2012), yellowfin sole frame (Jung et al., 2006), tuna frame (Lee, Qian, and Kim, 2010), and salmon pectoral fin (Ahn, Jeon, Kim, and Je, 2012). Collagen resources which were potentially produced antihypertensive peptides included jelly fish collagen (QPGPT, GDIGT), sea cucumber gelatin (MEGAQE QGD), freshwater hard clam (VKP, VKK), oyster

(VVYPWTQRF), tuna frame (GDLGKTTTVSNWSPPKYKDTP), and yellow fin sole frame (MIFPGAGGPQL). Some invertebrate animals, whole body of them were hydrolyzed into protein hydrolysates for elucidating their ACE inhibitory activity. These include oyster (Wang et al., 2008), cuttlefish (Balti et al., 2015; Balti, Nedjar-Arroume, Bougatef, Guillochon, and Nasri, 2010), hard clam shellfish (Tsai, Chen, and Pan, 2008), golden fresh water clam (Tsai, Lin, Chen, and Pan, 2006), cotton leafworm (Vercruysse, Smagghe, Matsui, and Camp, 2008), and silkworm pupa (Jia, Wu, Yan, and Gui, 2015; Wu, Ji, Yan, Du, and Gui, 2014).

Egg and milk were also employed to produce ACE inhibitory peptides. Milk proteins are a primary source for ACE inhibitory peptides production (Korhonen and Pihlanto, 2006; Millsa, Ross, Hilla, Fitzgerald, and Stanton, 2011). Milk-derivedACE inhibitory peptides from bovine, buffalo, goat, sheep, yak as well as donkey have been reported (Bidasolo, Ramos, and Gomez-Ruiz, 2012; Brandelli, Daroit, and Corrêa, 2015; Espejo-Carpio, Gobba, Guadix, Guadix, and Otte, 2013; Mao, Ni, Sun, Hao, and Fan, 2007; Rohit, Sathisha, and Aparna, 2012). ACE inhibitory peptides derived from bovine milk have been identified in both milk protein hydrolysates and fermented dairy products (Korhonen and Pihlanto, 2006). Casein (α-casein, β-casein, κ-casein), whey (β-lactoglobulin and α-lactalbumin) and lactoferrin have been reported as good sources of ACE inhibitory peptides produced by enzymatic hydrolysis (Brandelli, Daroit, and Corrêa, 2015; Butylina, Luque, and Nystrom, 2006; Contreras, Carrón, Montero, Ramos, and Recio, 2009; Ferreira, et al., 2007; FitzGerald and Meisel, 2000; Jiang, Tian, Brodkor, and Huo, 2010; Millsa, Ross, Hill, Fitzgerald, and Stanton, 2011; Ortiz-Chao et al., 2009; Otte, Shalaby, Zakora, Prippa, and El-Shabrawy, 2007; Pan, Cao, Guo, and Zhao, 2012; Power, Fernández, Norris, Riera, and FitzGerald, 2014; Ruiz-Giménez et al., 2012; Stressler,

Eisele, and Fischer, 2013; Yamada et al., 2013). Some examples of ACE inhibitory peptides, such as RYLGY, AYFYPEL derived from bovine casein hydrolysates and LIWKL, RPYL, LNNSRAP isolated from bovine lactoferrin hydrolysates could exert antihypertensive effect in SHR after oral administration (Contreras et al., 2009; Jiang, Tian, Brodkor, and Huo, 2010; Ruiz-Giménez et al., 2012). Hen egg yolk and egg white have frequently been reported as substrates for producing ACE inhibitory peptides. These also include isolated egg white proteins such as ovotransferrin, ovalbumin and lysozyme (Asoodeh, Yazdi, and Chamani, 2012; Eckert et al., 2014; Fujita, Eiichiyokoyama, and Yoshikawa, 2000; Liu et al., 2010; Majumder and Wu, 2011; Pokora et al., 2014; Rao et al., 2012). In addition, the use of turtle and ostrich egg white protein for ACE inhibitory peptides production has been investigated (Rawendra et al., 2013; Tanzadehpanah, Asoodeh, Saberi, and Chamani, 2013). ACE inhibitory peptides derived from ovalbumin (LW), egg white hydrolysates (IQW, LKP) could reduce blood pressure of SHR after orally administered at doses of 10 mg/kg rat body weight (Fujita et al., 2000; Majumder and Wu, 2011).

ACE inhibitory protein hydrolysates derived from other alternative resources have been studied, such as shrimp head and scales (He et al., 2008), insect (Vercruysse, Smagghe, Matsui, and Camp, 2008), skip jack roe (Intarasirisawat, Benjakul, Wu, and Visessanguan, 2013), head and viscera of sardinella (Bougatef et al., 2008), rotifer (Lee, Hong, Jeon, Kim, and Byun, 2009), button mushroom (Lau, Abdullah, Shui, and Aminudin, 2014), marine algae (Koa et al., 2012; Lin et al., 2012; Qu et al., 2010; Sheih, Fang, and Wu, 2009), and pumpkin oil cake (Vaštag, Popovic´, Popovic´, Krimer, and Pericˇin, 2011).

2.4.1.3 Relationship between hydrolysis time and ACE inhibitory activity

Degree of hydrolysis (DH) is defined as the percentage of the total number of peptide bonds cleaved during hydrolysis. DH is a common indicator to monitor the extent of protein degradation (Adler-Nissen, 1979). The optimal DH of protein hydrolysate yields functional as well as biological property (Ferreiraa, et al., 2007; Nasria et al., 2013; Spellman, McEvoy, O'Cuinn, and FitzGerald, 2003). DH of protein hydrolysates is directly related to ACE inhibitory activity (Balti et al., 2015; Jiang, Tian, Brodkor, and Huo, 2010; Nasria et al., 2013; Qu et al., 2010) and can be controlled through hydrolysis time (Otte, Shalaby, Zakora, Prippa, and El-Shabrawy, 2007). It has been demonstrated that ACE inhibitory activity relied on DH only the first period of protein hydrolysis (Mao, Ni, Sun, Hao, and Fan, 2007; Megı´as et al., 2009; Tovar-Pérez, Guerrero-Legarreta, Farrés-González, and Soriano-Santos, 2009). During the first proteolysis progression, numbers of smaller peptides were released from their parent protein (Nagai, Yamashita, Taniguchi, Kanamori, and Suzuki, 2001; Torruco-Uco, Chel-Guerrero, Martı´nez-Ayala, Da´vila-Ortı´z, and Betancur-Ancona, 2009). Some of them have a suitable chain length and C- and N-terminal amino acid residues could exert inhibitory activity towards ACE (Nasria et al., 2013; Salampessy, Reddy, Kailasapathy, and Phillips, 2015).

2.4.1.4 Influence of substrate pretreatment on ACE inhibitory activity

Thermal and non-thermal treatments are known to lead to the degradation and aggregation of protein (Hammond and Jez, 2011; Verhoeckx et al., 2015). Several investigations have applied these treatments to pre-treat protein substrate prior to enzymatic hydrolysis in order to enhance ACE inhibitory activity.

The process which have been employed in the pretreatment process including ultrasonic (Jia et al., 2010; Jia, Wu, Yan, and Gui, 2015; Ma, Huang, Peng, Wang, and Yang, 2015; Zhou, Ma, Yu, Liu, Yagou, and Pan, 2013), heating (Zhang, Olsen, Grossi, and Otte, 2013; Rui et al., 2012) and high pressure (Banerjee and Shanthi, 2012). The studies showed that ultrasonic pretreatment increased ACE inhibitory activity of protein hydrolysate (Jia et al., 2010; Jia et al., 2015; Ma et al., 2015; Zhou et al., 2013). The optimal power and time could induce molecular unfolding and increase surface hydrophobicity, which facilitated the release of ACE inhibitory peptides from the protein (Jia et al., 2010). On the contrary, exceeding ultrasonic time and power could induce re-association or aggreagation to form more stable structure, hindering the release of ACE inhibitory peptides (Jia, Wu, Yan, and Gui, 2015). Zhou, Ma, Yu, Liu, Yagoub, and Pan, (2012) revealed that pre-heat treatment of bean protein isolates by boiling for 15 min yielded higher ACE inhibitory activities for the navy and small red beans hydrolysates compared to the unheated sample. This was likely due to unfolding of the compact structure of the parent proteins by heat. However, no improvement was found for pre-thermal treatment of black bean hydrolysates during Alcalase and Flavourzyme hydrolysis. SDS-PAGE pattern showed that this might be related to the large protein aggregation after heating of protein isolates, limiting the liberation of ACE inhibitory peptides. Zhang, Olsen, Grossi, and Otte, (2013) demonstrated that boiling collagen for 5 min before hydrolysis could rise ACE inhibitory activity of collagen hydrolysate compared with untreated collagen. They suggested that boiling induced the cleavage of hydrogen and covalent bonds, resulting in helix to coil transition, making the inner site more accessible to the enzyme. In this study, ACE inhibitory activity of collagen hydrolysate was not increased when pre-treated with high pressure (600 MPa, 15 min). This might be that under high pressure,

some intramolecular hydrogen bonds were disrupted and some new hydrogen bond with water molecule formed, leading to more stabilization of the triple helix structure. Consequently, the Pro-rich parts of collagen, which give rise ACE inhibitory activity, were not exposed after high pressure pre-treatment.

Apart from protease, protein substrate, suitable hydrolysis time as well as substrate pretreatment; enzyme and substrate ratio (Boschin, Scigliuolo, Resta, and Arnoldi, 2014; Lassoued et al., 2015; Onuh, Girgih, Aluko, and Aliani, 2013; Rawendraa et al., 2013; Roufik, Gauthier, and Turgeon, 2006; Tomatsu, Shimakage, Shinbo, Yamada, and Takahashi, 2013), pH (Roufik, Gauthier, and Turgeon, 2006), and temperature (Cheison, Schmitt, Lee, Letzel, and Kulozik, 2010; Rawendra et al., 2013) were also key factors affecting protein hydrolysis.

2.4.2 Fractionation of ACE inhibitory protein hydrolysates

Crude protein hydrolysate might not exhibit good biological activity since they contain variant oligopeptides and some impurities (Clemente, 2000). Membrane and liquid chromatography are therefore used as a means for fractionation and isolation of bioactive peptides from the crude hydrolysates (Li-Chan, 2015). Ultrafiltration has been more preferably applied in the large scale food production due to its fast and comparatively inexpensive as compared to a chromatographic technique (Agyei and Danquah, 2011; Raghavan and Kristinsson, 2009). In the laboratory, membrane filtration is commonly used in the initial step before chromatographic purification (Hatti-Kaul and Mattiasson, 2003). The target proteins or peptides are fractionated based mainly on the molecular mass (Janson, 2011). In ACE inhibitory peptide fractionation, the different MWCO membrane between 1-30 kDa was generally used and the strongest ACE inhibitory peptides were normally obtained from the permeate of the lowest MWCO-membrane, which showed ACE inhibitory activity higher than

that of crude hydrolysate (Chen et al., 2013; Chen, Wang, Zhong, Wu, and Xia, 2012; Dub et al., 2013; Eckert et al., 2014; Ghassem, Arihara, Babji, Said, and Ibrahim, 2011; Jiang, Tian, Brodkor, and Huo, 2010; Jung et al., 2006; Koa et al., 2012; Lee, Qian, and Kim, 2010; Lin, Lv, and Li, 2012; Mao, Ni, Sun, Hao, and Fan, 2007; Salampessy, Reddy, Kailasapathy, and Phillips, 2015; Segura-Campos, Chel-Guerrero, and Betancur-Ancona, 2011; Wu, Jia, Yan, Du, and Gui, 2014; Zhao et al., 2007). This corresponds to the fact that small peptides contribute to the ACE inhibitory activity of protein hydrolysates and short peptide sequences are good candidates for *in vivo* physiological antihypertensive agent (Hernández-Ledesma, Contreras, and Recio, 2011).

To isolate the potent active peptides, fractionated protein hydrolysate should be further subjected to the column chromatography [size exclusion (SEC), ion exchange chromatography (IC), or reverse-phase chromatography (RPC)] (Lafarga and Hayes, 2014). However, they are restrictive for the large scale application due to the high production cost (Agyei and Danquah, 2011). Nonetheless, they seemed to be effective in the laboratory scale, particularly for further identification and characterization (Korhonen and Pihlanto, 2006). The characteristics such as molecular mass, amino acid composition, peptide length, and peptide sequence are certainly useful in elucidating the ACE inhibition behavior of peptide as well as the interaction between the inhibitory peptide and ACE active site (Asoodeh et al., 2014; Byun and Kim, 2002; Li et al., 2014; Rohit, Sathisha, and Aparna, 2012; Vercruysse Vercruysse, Camp, Morel, Rouge´, Herregods, and Smagghe, 2010). Moreover, they are also beneficial to predict the digestibility (Boye, Roufik, Pesta, and Barbana, 2010; Contreras, Sanchez, Sevilla, Recio, and Amigo, 2013; Majumder and Wu, 2011; Ruiz-Giménez et al., 2012) and stability of an ACE inhibitory peptide undergoing extreme

food processing condition (Miguel, Alonso, Salaices, Aleixandre, and Lo´pez-Fandin˜o, 2007; Wang et al., 2008). This knowledge will pave the way for further production of antihypertensive peptides in aspect of protein and enzyme selection, hydrolysis optimization, and purifying condition.

2.4.3 Structural characteristics of potent ACE inhibitory peptides

The certain ACE inhibition model of food-derived peptides is still not established since the potent food-derived ACE inhibitory peptides have a diversity of amino acid composition, sequence, length and inhibition mechanism (competitive, non-competitive, uncompetitive or mixed-inhibitor) (Jao, Huang, and Hsu, 2012). The potency of ACE inhibitory peptides is dependent on peptide chain length and strongly influenced by hydrophobicity of amino acid residues at the ultimate position of peptides (Hong et al., 2008). Potent ACE inhibitory peptides normally contain 3-13 amino acids and have molecular weight of 500-1500 Da (Li, Leemail, Shi, and Shrestha, 2004). ACE inhibitory activity inversely correlated to the peptide length since the large peptides are hindered by the small cavity of ACE active site (Natesh, Schwager, Sturrock, and Acharya, 2003). The potent peptides usually contain Pro, Lys, Arg, and hydrophobic amino acids at the ultimate position of peptides (Byun and Kim, 2002; Cheung, Wang, Ondetti, Sabo, and Cushman, 1980; Li, Leemail, Shi, and Shrestha, 2004; Wu, Aluko, and Nakai, 2006b). The structure-activity relationship revealed that potent ACE inhibitory dipeptide should have a hydrophobic amino acid (Phe, Trp, Ala, Val, Leu, Ile, Met) at the C-terminus and a non-polar amino acid or positively charged amino acid (Lys, Arg) at the N-terminus (Wu, Aluko, and Nakai, 2006b). In case of tripeptides, the most favorable residues for the C-terminus are aromatic amino acids (Tyr, Phe, Trp), while branched chain hydrophobic amino acids (Val, Ile, Leu) are preferred for the N-terminus and positively charged amino acids

(Lys, Arg) are preferred for the middle position (Wu, Aluko, and Nakai, 2006b). C-terminal tetrapeptide residues of long-chain peptides (4-10 amino acid residues) were more important to their ACE-inhibitory activity than C-terminal tripeptide residues. The most preferred amino acid residues starting from C-terminus are Tyr and Cys for the first position; His, Trp and Met for the second position; with Ile, Leu, Val and Met for the third position; and Trp for the fourth position (Wu, Aluko, and Nakai, 2006a). Ondetti and Cushman (1982) proposed that hydrophobic amino acids and Pro at the C-terminal tripeptide contribute to strong binding with sub-active sites (S', S_1' and S_2') of ACE. Meisel (1993) speculated that the positive charge on the guanidine or ε-amino group of the C-terminal Arg and Lys side chain interacted with other anionic binding site of ACE apart from its catalytic site (FitzGerald and Meisel, 2000). Table 2.1 shows primary sequence of potent ACE inhibitory peptides derived from various food protein sources.

2.4.4 *In vitro* ACE inhibitory activity assay

In vitro assay has been applied to screen potent food-derived antihypertensive peptides (Hernández-Ledesma, Contreras, and Recio, 2011). Several methods with different substrates and analytical techniques have been found (Hernández-Ledesma, Contreras, and Recio, 2011). However, the method developed by Cushman and Cheung (1971) is the most common generally employed by numerous researchers. The assay is based on the hydrolysis of synthetic tripeptide, Hip-His-Leu by ACE (with and without the inhibition by bioactive peptides) under the specific condition (300 mM NaCl in 0.1 M borate buffer, pH 8.3 at 37^{o}C) to generate His-Leu and hippuric acid residue (Barrett, Rawlings, and Woessner, 2013; Cushman and Ondetti, 1984). The activity of ACE could obtain by the measurement of hippuric acid after extraction by ethyl acetate. The chromatographic technique based on HPLC was

recognized for quantifying the releasing hippuric acid at 228 nm. It provides more accurate and reproducible results (Boschin, Scigliuolo, Resta, and Arnoldi, 2014; Shalaby, Zakora, and Otte, 2006). The inhibitory potency is usually expressed as the IC_{50} value, which is a concentration needed to inhibit 50% of the enzyme activity (Hernández-Ledesma, Contreras, and Recio, 2011).

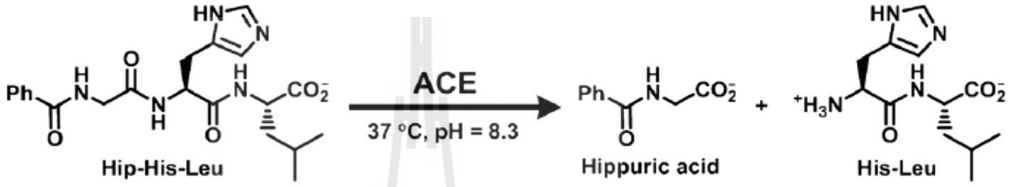

Figure 2.6 Hydrolysis of Hip-His-Leu tripeptides by ACE.

From: Bhuyan and Mugesh (2011).

2.4.5 *In vitro* gastrointestinal digestion of ACE inhibitory peptides

Dietary proteins and peptides after ingested must undergo the digestion firstly by pepsin in the stomach and are further hydrolyzed by several pancreatic digestive enzymes when enter the small intestine (Barrett, Rawlings, and Woessner, 2006). The action of pepsin together with pancreatic enzymes [trypsin which acts on peptide bonds with cationic amino acids (Arg, Lys, His); α-chymotrypsin which prefers to hydrolyze peptide bond at aromatic amino acids (Phe, Tyr, Trp); elastase which preferably cleaves at Ala, Ser, and Gly; carboxypeptidase A which habitually liberates amino acids at the C-terminal excepting Lys, Arg, and Pro; and carboxypeptidase B which favors the peptides towards C-terminal Arg and Lys] might affect the modification of bioactive peptides that finally increase or decrease their biological

activity prior to reach the target function in the body (Alemán, Gómez-Guillén, and Montero, 2013; Barrett, Rawlings, and Woessner, 2006). Although *in vivo* studies can provide a reliable result, it is costly and time-consuming process, *in vitro* screening assays are more preferably employed (Hartmann and Meisel, 2007). Peptic-pancreatic incubation seems to be a promising *in vitro* model to predict whether ACE inhibitory activity of peptides in the digestive system (Hur, Lim, Decker, and McClements, 2011). Several studies have adopted this approach (Escudero, Mora, and Toldrá, 2014; Hur, Lim, Decker, and McClements, 2011; Hwang, 2010; Lau, Abdullah, Shuib, and Aminudin, 2014; Majumder and Wu, 2011; Miguel, Alonso, Salaices, Aleixandre, and Lo´pez-Fandin˜o, 2007; Tsai, Chen, and Pan, 2008; Wu, Jia, Yan, Du, and Gui, 2014). Peptide chain length and amino acid composition of peptide are important factors determining ACE inhibitory activity. Long chain peptides are highly susceptible to proteolytic enzymes in the gastrointestinal tract, while short-chain peptides (2-4 amino acids) have more advantageous that they are less susceptible to hydrolysis by gastrointestinal tract enzymes (Quiros, Contreras, Ramos, Amigo, and Recio, 2009). Peptides containing Pro in their sequence tend to be resistant to gastric proteases hydrolysis, thus ACE inhibitory activity remains. Fujita, Yokoyama, and Yoshikawa (2000) have classified ACE inhibitory peptides according to their modified characteristics after *in vitro* gastrointestinal incubation into three groups including true inhibitor, substrate type inhibitor, and pro-drug type inhibitor. True inhibitory peptides could preserve their ACE inhibitory activity after incubation by gastrointestinal proteases. The true inhibitors found in previous reports were mostly short chain peptides which could escape the gastrointestinal enzymes digestion such as peptide KHV which was derived from silkworm (*Bombyx mori*) (Wu, Jia, Yan, Du, and Gui, 2014) and IQW, IRW, LKP from ovotransferrin hydrolysates (Majumder and Wu,

2011). In addition, long chain peptide containing Pro, such as VQLYP, LHLPLP, VQCYGPNRPQF which were derived from cuttlefish, β-casein and algae protein waste, respectively remained inhibitory activity after gastrointestinal digestion (Balti, Nedjar-Arroume, Bougatef, Guillochon, and Nasri, 2010; Quiros, Contreras, Ramos, Amigo, and Recio, 2009; Sheih, Fang, and Wu, 2009). For substrate-type inhibitors, their ACE inhibitory activity decrease after the gastrointestinal incubation. The peptides in this group were mostly long chain which after the cleavage of gastrointestinal enzymes, the degraded peptides lost their overall ACE inhibitory activity. The substrate type peptides previously reported such as very long peptides derived from bovine tendon collagen, AKGANGAPGIAGAPGFPGARGPSGPQ-GPSGPP and PAGNPGDGQPGAKGANGAP. Their ACE inhibitory activity decreased from 72.04% and 59.7% to 60.1% and 51.4%, respectively after gastrointestinal digestion (Banerjee and Shanthi, 2012). In addition, DKVGINY (IC_{50} = 84.2 µg/mL), KGYGGVSL (IC_{50} =253.6 µg/mL), DKVGINYW (IC_{50} = 25.2 µg/mL) derived from whey protein concentrate were lost their ACE inhibitory activity with an increase in IC_{50} to 130.8, 336.3 and 254.5 µg/mL, respectively after partially hydrolyzed by gastric proteases (Ferreira et al., 2007). For pro-drug type inhibitors, when the peptides are hydrolyzed, they release true inhibitory fragment, resulting in an increase in ACE inhibitory activity. Pro-drug peptide inhibitor such as peptide MEGAQEAQGD from sea cucumber (*Acaudina molpadioidea*) showed ACE inhibitory activity after gastrointestinal incubation (IC_{50} = 5.3 µM), which was less than before incubation (IC_{50} = 4.5 µM) (Zhao et al., 2009). Peptides RIGLF, AHEPVK, and PSSNK from button mushroom (*Agaricus bisporus*) exhibited an increase in ACE inhibitiory activity to 90% from approximately 63-80%, after gastric digestion (Lau, Abdullah, Shui, and Aminudin, 2014). Typically, true inhibitors and

pro-drugs are recommended as they possibly exhibit the antihypertensive effect *in vivo*, while the substrate type is likely to be less effective (Ahhmed and Muguruma, 2010).

Table 2.1 Angiotensin I converting enzyme inhibitory peptides derived from food proteins.

Protein sources	Enzymes	Peptide sequences	IC_{50}	References
Plants				
Rapeseed meal	Subtilisin	RIY	28 μM	Marczak et al. (2003)
		VWIS	30 μM	
	Alcalase	LY	0.11 mM	He et al. (2013)
Rapeseed albumin	Alcalase	PFDSYFVC	0.2 mg/mL	Yu et al. (2013)
Canola meal	Alcalase	VSV	0.2 μM	Wu, Aluko, and Muir (2008)
		FL	1.3 μM	
Rice	Alcalase	VNP	6.4 μM	Chen et al. (2013)
		VWP	4.5 μM	
Soy bean	Thermolysin	IVF	< 10 μM	Gu and Wu (2013)
		LLF	< 10 μM	
		LNF	< 10 μM	
		LSW	< 10 μM	
		LEF	< 10 μM	
Red bean	Alcalase → papain	PVNNPQIH	206.7 μM	Rui, Boye, Simpson, and Prasher (2013)

Table 2.1 Angiotensin I converting enzyme inhibitory peptides derived from food proteins (Continued).

Protein sources	Enzymes	Peptide sequences	IC_{50}	References
Plants (continued)				
Wheat gluten	Trypsin	IPALLKR	43 μM	Asoodeh et al. (2014)
		AQQLAAQLPAMCR	68 μM	
Pistachio	Trypsin → pepsin	ACKEP	126 μM	Li et al. (2014)
Brewers' spent grain	Alcalase	IVY	80.4 μM	Connolly, O'Keeffe, Piggott, Nongonierma, and FitGerald (2015)
		ILDL	96.4 μM	
Soya milk	PROTIN SD-NY10	FFVY	1.9 μM	Tomatsu et al. (2013)
		WHP	4.8 μM	
		FVP	10.1 μM	
		LHPGDAQR	10.3 μM	
		IAV	27.0 μM	
		VNP	32.5 μM	
		LEPP	100.1 μM	
		WNPR	880 μM	

Table 2.1 Angiotensin I converting enzyme inhibitory peptides derived from food proteins (Continued).

Protein sources	Enzymes	Peptide sequences	IC_{50}	References
Animals				
Porcine myosin heavy chain	Thermolysin	MNPPK	945.5 µM	Arihara, Nakashima, Mukai,
		ITTNP	549.0 µM	Ishikawa, and Itoh (2001)
Porcine myosin B	Pepsin	KRVIQY	6.1 µM	Muguruma et al. (2009)
		VKAGF	20.3 µM	
Chicken muscle	Thermolysin	LKA	8.5 µM	Fujita, Eiichiyokoyama, and
		LKP	0.3 µM	Yoshikawa (2000)
		LAP	3.5 µM	
		FQKPKR	14 µM	
		IVGRPRHQG	2.4 µM	
		FKGRYYP	0.6 µM	
		IKW	0.2 µM	
Bullfrog	Alcalase	GAAQLPCSADWW	1.0 µM	Qian, Jung, Lee, Byun, and Kim (2007)
Porcine hemoglobin	Pepsin	LGFPTTKTYFPHF	4.9 µM	Yu et al. (2006)
		VVYPWT	6.0 µM	
Yak milk β-casein	Alcalase	PLPLL	0.3 µM	Mao, Ni, Sun, Hao, and Fan (2007)

Table 2.1 Angiotensin I converting enzyme inhibitory peptides derived from food proteins (Continued).

Protein sources	Enzymes	Peptide sequences	IC_{50}	References
Animal products				
β -Casein of donkey's milk	Pepsin → pancreatin	VAPFPQPVVP	273 μM	Bidasolo, Ramos, and Gomez-Ruiz (2012)
β-Lactoglobulin	Trypsin	IIAEK	63.7 μM	Power, Fernández, Norris, Riera, and FitzGerald (2014)
		IPAVFK	144.8 μM	
		ALPMHIR	42.6 μM	Ferreiraa et al. (2007)
	Protease N Amano	SAPLRVY	8 μM	Ortiz-Chao et al. (2009)
Buffalo colostrum	Trypsin	IIAMK	498 μM	Rohit, Sathisha, and Aparna (2012)
Bovine lactoferrin	Pepsin	LIWKL	0.5 μM	Ruiz-Giménez et al. (2012)
		RPYL	56.5 μM	
		LNNSRAP	105.3 μM	
κ-Casein	AS1.398 neutral protease	RYPSYG	54 μM	Jiang, Tian, Brodkorb, and Huo (2010)
Yak milk κ-casein	Alcalase	PPQIN	0.3 μM	
α-Casein	Subtilisin	MKP	0.3 μM	Yamada et al. (2013)
β-Casein	Protease from *Lactobacillus*	TP	440 μM	Stressler, Eisele, and Fischer (2013)

Table 2.1 Angiotensin I converting enzyme inhibitory peptides derived from food proteins (Continued).

Protein sources	Enzymes	Peptide sequences	IC_{50}	References
Animal products (continued)				
β-Casein	*helveticus* ATCC 15009	MP	515 μM	Stressler, Eisele, and
		LP	5.7 mM	Fischer (2013)
		DQRF	21 μM	
Egg white	Alcalase	TNGIIR	70 μM	Yu et al. (2012)
Egg white	Protease from fig-leaf	SWVE	33.9 μM	Pokora et al. (2014)
	gourd fruit	DILN	73.4 μM	
Overtransferrin	Thermolysin → pepsin	IQW	1.5 μM	Majumder and Wu (2011)
		IRW	0.6 μM	
		LKP	2.9 μM	
Ovalbumin	Pepsin	FGRCVSP	6.2 μM	Fujita et al. (2000)
		ERKIKVYL	1.2 μM	
		FFGRCVSP	0.4 μM	
		LW	6.8 μM	
		FCF	11 μM	
		NIFYCP	15 μM	
Hen egg white lysozyme	Gastrointestinal proteases	MKR	25.7 μM	Rao et al. (2012)

Table 2.1 Angiotensin I converting enzyme inhibitory peptides derived from food proteins (Continued).

Protein sources	Enzymes	Peptide sequences	IC_{50}	References
Animal products (continued)				
Hen egg white lysozyme	Gastrointestinal proteases	RGY	61.9 μM	Rao et al. (2012)
		VAW	2.9 μM	
	Papain → trypsin	FQSNFNTQATNR	30 μg/mL	Asoodeh, Yazdi, and Chamani (2012)
Egg yolk	Asian pumpkin protease	LAPSLPGKPKPD	2.0 μM	Eckert et al. (2014)
Ostrich egg white	Trypsin	AFKDQDTQQVPFR	80.2 μM	Tanzadehpanah, Asoodeh, Saberi, and Chamani (2013)
Shark meat	Protease SM98011	CF	2.0 μM	Wu et al. (2008)
		QT	2.7 μM	
		MF	1.5 μM	
Seaweed pipefish	Alcalase	TFPHGP	0.6 μM	Wijesekara, Qian, Ryu, Ngo, and Kim (2011)
		HWTTQR	1.4 μM	
Haruan myofibrillar	Thermolysin	VPAAPPK	0.5 μM	Ghassem, Arihara, Babji, Said, and Ibrahim (2011)
		NGTWFEPP	0.6 μM	
Grass carp	Alcalase	VAP	5.3 μg/mL	Chen, Wang, Zhong, Wu, and Xia (2012)

Table 2.1 Angiotensin I converting enzyme inhibitory peptides derived from food proteins (Continued).

Protein sources	Enzymes	Peptide sequences	IC_{50}	References
Fish and fish products (Continued)				
Travelly	Bromelain	AR	570.8 μM	Salampessy, Reddy, Kailasapathy, and Phillips (2015)
		AV	956.3 μM	
		APER	530.2 μM	
Lizard	Neutral protease	RVCLP	175 μM	Wu, Feng, Lan, Xu, and Liao (2015)
Dried bonito	Thermolysin	LKPNM	2.4 μM	
Bigeye tuna muscle	Pepsin	WPQAAQLMMQVDP	21.6	Qian, Je, and Kim (2007)
Chum salmon skin	Trypsin	GLPLNLP	18.7 μM	Lee, Jeon, and Byun (2014)
Skate skin	α-Chymotrypsin	PGPLGLTGP	95 μM	Lee, Jeon, and Byun (2011)
		QLGFLGPR	148 μM	
Atlantic salmon collagen	Alcalase → papain	AP	60 μM	Gu, Li, Liu, Yi, and Cai (2011)
		VR	33.2 μM	
Alaska pollack skins gelatin	Alcalase → pronase E → collagenase	GPL	2.6 μM	Byun and Kim (2001)

Table 2.1 Angiotensin I converting enzyme inhibitory peptides derived from food proteins (Continued).

Protein sources	Enzymes	Peptide sequences	IC_{50}	References
Fish and fish products (Continued)				
Alaska pollack skins gelatin	Alcalase → pronase E → collagenase	GPM	17.1 μM	Byun and Kim (2001)
Cod skin gelatin	Papain	TCSP	81% /0.5 mg/mL	Ngo, Ryu, Vo, Himaya, and Kim (2011)
		TGGGNV	68% /0.5 mg/mL	
Pacific cod skin gelatin	Gastrointestinal proteases	LLMLDNDLPP	35.7 μM	Himaya, Ngo, Ryu, and Kim (2012)
Skate skin gelatin	Alcalase	MVGSAPGVL	3.1 μM	Ngo, Ryu, and Kim (2014)
		LGPLGHQ	4.2 μM	
Salmon pectoral fin	Alcalase	VWDPPKFD	9.1 μM	Ahn, Jeon, Kim, and Je (2012)
		FQDYVPLSCF	10.8 μM	
		FNVPLTQ	7.2 μM	
Alaska pollock frame	Trypsin	NGMTY	39.2%/20 μg/mL	Hou et al. (2012)
		NGLAP	33%/20 μg/mL	
		WT	31.4%/20 μg/mL	

Table 2.1 Angiotensin I converting enzyme inhibitory peptides derived from food proteins (Continued).

Protein sources	Enzymes	Peptide sequences	IC_{50}	References
Fish and fish products (Continued)				
Tuna frame	Pepsin	GDLGKTTTVSNWSPPKYLDTP	11.3 μM	Lee, Qian, and Kim (2010)
Skipjack roe	Alcalase	FVSACSVAG	3.5 μM	Intarasirisawat, Benjakul, Wu, and Visessanguan (2013)
		DWMKGQ	422.9 μM	
		LADGVAAPA	317.8 μM	
		YVNDAATLLPR	105.9 μM	
		DLDLRKDLYAN	67.4 μM	
		MCYPAST	58.7 μM	
		MLVFAV	3.1 μM	
Other sources				
Rotifer	Alcalase	DDTGHDFQDTGQAM	9.6 μM	Lee, Hong, Jeon, Kim, and Byun (2009)
Insect	Alcalase	AVP, VF	320 μg/mL	Vercruysse et al. (2010)
Cuttlefish	Digestive protease of cuttle fish	VTAP	6.1 μM	Balti, Nedjar-Arroume, Bougatef, Guillochon, and Nasri (2010)
		VIIF	8.7%/20 μg/mL	

Table 2.1 Angiotensin I converting enzyme inhibitory peptides derived from food proteins (Continued).

Protein sources	Enzymes	Peptide sequences	IC_{50}	References
Fish and fish products (Continued)				
Cuttlefish	Digestive protease of cuttle fish	MAW	16.3%/20 µg/mL	Balti, Nedjar-Arroume, Bougatef, Guillochon, and Nasri (2010)
Microalgae	Alcalase	VQGY	128.4 µM	Koa et al. (2012)
Algae protein waste	Pepsin	VQCYGPNRPQF	29.6 µM	Sheih, Fang, and Wu (2009)
Silkworm pupa	Alcalase	KHV	12.8 µM	Jia, Wu, Yan, and Gui (2015)
Sipunculid worm	Pepsin	AWLHPGAPKVF	135 M	Dub et al. (2013)
Cotton leafworm	Pepsin → trypsin → pancreatin	AVF	2123 µM	Vercruysse, Smagghe, Matsui, and Camp (2008)

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Thai Panga (*Pangasius hypopthalmus* × *P. bocourti*) skins were obtained from Nakhon Phanom Fisheries Office, Department of Fisheries, Thailand. Samples were kept in a polystyrene foam box filled with ice and transported to the laboratory at Suranaree University of Technology. Upon arrival, fish skins were cleaned and dried at 60^{o}C for 12 h and cut into pieces (0.5 × 0.5 cm). The dried samples were packed in the vacuum bag and kept at -20^{o}C until use. Alcalase 2.4L, Protamex was provided by Novozymes (Bagsvaerd, Denmark). Papain, pepsin, pancreatin, angiotensin-I-converting enzyme (ACE) from rabbit lung, hippuryl-L histidyl-L-leucine (HHL), and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hippuric acid (HA) was obtained from Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Acetonitrile with high performance liquid chromatograohy (HPLC) grade was purchased from Merck (Merck KGaA, Darmstadt, Germany). Trifluoroacetic acid (TFA) was purchased from Supelco (Bellefonte, PA, USA). Other chemicals and reagents used were of analytical grade.

3.2 *Virgibacillus halodenitrificans* SK 1-3-7 protease production

Virgibacillus halodenitrificans SK 1-3-7 protease was produced according to the method as described by Montriwong et al. (2012). A loopful of pure culture was

transferred from JCM 168 agar (0.5% yeast extract, 0.5% casamino acid, 0.1% sodium glutamate, 0.3% trisodium citrate, 0.02% KCl, 2% $MgSO_4$, 0.0036% $FeCl_2 4H_2O$, 0.0036% $MnCl_2 4H_2O$ and 5% NaCl) into Ym broth (1% yeast extract, 0.3% trisodium citrate, 0.2% potassium chloride, 2.5% magnesium sulfate) containing 2.5% NaCl and incubated at 35°C for 3 days. The inoculum was diluted to obtain optical density (OD) of 0.1 at 600 nm (approximately 10^7 CFU/mL). Fifty milliliters of diluted inoculum was transferred to a 2-L flask containing 450 mL of YM broth and incubated at 35°C for 5 days at a shaking speed of 100 rpm. Cell-free supernatants were collected by centrifugation at 10,000×g, 4°C for 20 min and lyophilized. The lyophilized powder was referred to as crude enzyme. Protease activity was assayed using azocasein as a substrate. One unit activity was defined as the amount of enzyme that increased OD_{450} of 0.01 per min at pH 9.0, 60°C for 60 min.

3.3 Protein substrate preparations

Fish skins were defatted by soaking skins (0.5 × 0.5 cm) in 10% butyl alcohol with the ratio of skin to solvent of 1 : 10 (w/v) at 4°C for 24 h with a continuous stirring and the solvent was changed every 3 h. Subsequently, skins were washed with tap water for 30 min followed by reverse osmosis (RO) water for 3 times. The washed skins were left in a hood at room temperature for 1 h. The defatted skins were lyophilized and ground at 4°C with a set speed of 20,000 rpm using IKA milling machine (IKA M20, IKA-Werke GmbHandCo, Staufen, Germany) and kept at -20°C until use.

Alkaline-acid pretreated skins were prepared according to the method of Jongjareonrak et al. (2010) with slight modifications. Skins were soaked in 0.1 N

NaOH with the ratio of 1 : 20 (w/v) for 24 h. The alkaline-acid treated skins were then washed with tap water until neutral pH was obtained. The skins were then soaked in 10% butyl alcohol at the ratio of 1 : 10 (w/v) for 12 h and continuously rinsed in tap water for 30 min. Subsequently, skins were soaked in 0.05 M acetic acid at a ratio of 1 : 20 (w/v) at 4^{o}C for 12 h and washed with tap water until pH of wash water was neutral, followed by washing with RO water for 3 times. All soaking steps were carried out at 4^{o}C with continuous stirring and changing the solution every 3 h. The skins were lyophilized, ground, and stored as described above.

Gelatin was extracted by gentle stirring the alkaline-acid pretreated skins in deionized (DI) water with skins/water ratio of 1 : 10 (w/v) at 45^{o}C for 12 h. The viscous solutions were centrifuged at 10,000×g, 4^{o}C for 30 min. The supernatants were collected as gelatin solution, lyophilized, and ground at 4^{o}C with a set speed of 20,000 rpm using IKA milling machine (IKA M20, IKA-Werke GmbHandCo, Staufen, Germany) and kept at -20^{o}C until used.

3.4 Chemical composition of protein substrates

3.4.1 Proximate analyses

Moisture, crude fat, crude protein and ash content of dried skin, dried defatted skin, dried pretreated skin and dried gelatin were determined according to AOAC (2000).

3.4.2 Amino acid composition

According to AOAC (2000), powders of all skin samples (0.05-0.1 g) were hydrolyzed with 6 N HCl-phenol at 110^{o}C for 24 h, while, cysteine and methionine contents were determined using performic acid oxidation with acid hydrolysis-sodium

metabisulfite method. The tryptophan content was determined following AOAC (2006) by alkaline hydrolysis. Quantitative analysis of total amino acids was achieved by a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge Science Park, England). Stepwise elution gradient was performed using lithium loading buffer pH 2.20, 2.80, 3.00, 3.15, 3.50, 3.55, and 6 (Biochrom Ltd., Cambridge Science Park, England). Peaks of amino acid residues were detected at 570 nm and at 440 nm for proline using amino acid standards for protein hydrolysates as an external standard (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The amino acid was expressed as g/100g protein.

3.4.3 SDS-PAGE pattern

All dried samples were dissolved in 5% SDS with skin to SDS ratio of 0.1 : 10 (w/v). The suspensions were homogenized using an IKA hand homogenizer (IKA Works Asia, Bhd, Malaysia) for 10 min and heated at 90^{o}C for 10 min. The mixtures were then centrifuged at 10,000×g, 25^{o}C for 20 min. The supernatants were collected and then 2-folds concentrated loading buffer containing β-mercaptoethanol was added. The samples were heat at 90^{o}C for 3 min and SDS-PAGE was performed according to Laemmli (1970). Samples (25 µg) were loaded onto 7.5% acrylamide gel. Gels were run at a constant voltage at 120 V, stained with 0.125% Comassie Brilliant blue R-250 and destained in a solution containing 25% ethanol and 10% acetic acid.

3.5 Production of Thai Panga skin hydrolysates

To determine the optimal substrate and protease, 9 mL of DI water were added into 0.1 g protein of each sample (defatted skin, alkaline-acid pretreated skin and gelatin powder). The suspension was adjusted pH and pre-incubated in a shaking

water bath for 5 min under the optimal condition of protease applied: pH 8, 55°C for Alcalase; pH 2, 37°C for pepsin; pH 8, 37°C for trypsin; pH 7, 55°C for papain; pH 7.5, 55°C for Protamex; and pH 8, 60°C for protease from *Virgibacillus halodenitrificans* SK 1-3-7. Protein hydrolysis was carried out at enzyme to substrate ratio of 1 : 100 (w/w) for all commercial proteases. *V. halodenitrificans* SK 1-3-7 protease was added to the reaction mixture at a final unit activity of 2.5 U. Samples were hydrolyzed for 12 h and then heated at 95°C for 10 min to terminate the reaction. The samples were centrifuged at 10,000×g, 4°C for 20 min. Samples were adjusted to pH 7 using 10 N NaOH or 6 N HCl. The supernatants were referred to as crude hydrolysates and used for ACE inhibitory activity assay.

As alkaline-acid pretreated skin and pepsin showed the highest ACE inhibitory activity, the effect of hydrolysis time on ACE inhibitory activity was studied. Alkaline-acid pretreated skin powder was added DI water (10% w/v) and adjusted to pH 2. The mixtures were pre-incubated at 37°C for 5 min. The samples were then added pepsin with the enzyme/substrate ratio of 1:100 (w/w). The samples were taken for the measurement of degree of hydrolysis (DH) and ACE inhibitory activity at various time intervals of 0, 2, 4, 8, and 12 h. Samples at each time interval were heated at 95°C for 10 min to terminate the reaction and centrifuged 10,000 × g at 4°C for 20 min.

3.6 Degree of hydrolysis (DH)

DH was determined according to Adler-Nissen (1979). Fifty microliters of hydrolysates was mixed with 0.5 mL of 0.2125 M phosphate buffer, pH 8.2 and 0.5 mL of 0.05% TNBS reagent. The mixture was incubated at 50°C for 1 h. The reactions

were terminated by adding 0.1 N HCl and then left at room temperature for 30 min. Absorbance was monitored at 420 nm using glycine which is a major amino acid in skins of Thai Panga as a standard. To determine total amino acid content, dried powder of all samples was hydrolyzed with 6 N HCl at a ratio of 1 : 100 at 120^{o}C for 24 h using an autoclave. The degree of hydrolysis was calculated as follow:

$$DH\ (\%) = \frac{h_s - h_0}{h_t} \times 100\%$$

where h_s is α-amino group content of sample at time t, h_0 is α-amino content of sample at 0 h, and h_t is the total α-amino content obtained after acid hydrolysis.

3.7 ACE inhibitory activity assay

The *in vitro* assay was determined according to Cushman and Cheng (1971) with slight modifications. Protein hydrolysates (50 µL) and 150 µL of ACE substrate (8.3 mM HHL in 50 mM sodium borate buffer containing 0.5 M NaCl, pH 8.3) were pre-incubated at 37^{o}C for 10 min. Then, 50 µL of 25 mU/mL of ACE was added and reaction was incubated at 37^{o}C for 60 min in a shaking water bath with a set speed of 180 rpm. The reaction was terminated by adding 250 µL of 1 N HCl. Hippuric acid (HA) was extracted with 1.5 mL ethyl acetate. After centrifugation at 800×g, 4^{o}C for 15 min, 1 mL of the supernatant was transferred into a test tube and solvent was evaporated at 80^{o}C on a sand bath. The dried samples were re-dissolved in 1 mL of DI water and filtered through a 0.45-µm membrane filter before subjected to HPLC (1260 Infinity, Multiple Wavelength Detector, Agilent Technologies, Santa Clara, CA, USA). Twenty µL of samples were injected into a Zorbax Eclipse XDB-C18 column

(4.6 × 150 mm, Agilent Technologies, Santa Clara, CA, USA). The column was eluted with a mobile phase A of 0.05% TFA in DI water and mobile phase B of 0.05% TFA in acetonitrile at a flow rate of 0.5 mL/min. The separation condition was performed with 0-60% B for the first 10 min, maintained at 60% B for 2 min, subsequently returned to 5% B for 1 min and maintained at 5% B for 5 min. The chromatogram was monitored at 228 nm and HA was used as an external standard. Blanks were performed by adding 0.1 N HCl prior to ACE. The ACE inhibitory activity (%) was calculated following the equation:

$$\text{ACE inhibitory activity (\%)} = \frac{(C_0 - C_B) - (I_0 - I_B)}{(C_0 - C_B)} \times 100$$

where C_0 is HA content in the control (without protein hydrolysates), C_B is HA content in the control blank (without protein hydrolysates), I_0 is HA content in the sample in the presence of protein hydrolysates, and I_B is HA content of blank in the presence of protein hydrolysates.

3.8 Purification of ACE inhibitory peptides

3.8.1 Ultrafiltration

Pepsin-hydrolyzed alkaline-acid pretreated skin which showed the highest ACE inhibitory activity was selected for further purification. The sample was firstly subjected to the sequential ultrafiltration fractionation using 5-L Ultralab™ System (PALL Corporation, Ann Arbor, MI, USA). Crude hydrolysate (1L) was passed through a 30-kDa molecular weight cut-off (MWCO) membrane (PALL Corporation, Ann Arbor, MI, USA), the retentate was collected for ACE inhibitory activity and TNBS determination, while permeate was fractionated sequentially through a 5-kDa

and 1-kDa MWCO membrane (PALL Corporation, Ann Arbor, MI, USA). The permeate and retentate fractions obtained from each membrane were also collected for ACE inhibitory activity and TNBS detemination. The fraction exhibiting the highest ACE inhibitory activity was subjected to ion exchange chromatography**.**

3.8.2 Ion exchange chromatography

The purification was performed using AKTA Purifier FPLC with UNICORN software version 5.01 (GE Healthcare, Piscataway, NJ, USA). DEAE-Sephacel (GE Healthcare, Piscataway, NJ, USA) was packed in a column (2.6 × 6.5 cm) and equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The lyophilized fraction of hydrolysate with MW < 1-kDa (100 mg) was dissolved in 1 mL of DI water, then filtered through a 0.45-μm membrane and subsequently loaded into the column. The column was washed with 0.05 M Tris-HCl for 35 mL and eluted with a linear gradient of NaCl (0-1.0 M). The elution was performed at a flow rate of 1 mL/min and 5-mL fractions were collected. The absorbance was monitored at 215 nm. ACE inhibitory activity and peptide content were determined. The fraction showing the highest ACE inhibitory activity was lyophillized and collected for size exclusion chromatography.

3.8.3 Size exclusion chromatography

The lyophilized powder obtained from 3.8.2 was dissolved in DI water (100 mg/mL). A hundred μL of peptide solution was loaded into a Superdex Peptide 10/300 GL column (10 mm × 300 mm; GE Healthcare, Piscataway, NJ, USA) pre-equilibrated with DI water. The elution was performed using AKTA purifier (GE Healthcare, Piscataway, NJ, USA) at a flow rate of 0.2 mL/min and absorbance was monitored at 215 nm. Fractions (1 mL) were collected and determined for ACE inhibition and peptide content. The fraction with the highest ACE inhibitory activity

was used for IC_{50} determination. *In vitro* pepsin-pancreatin simulated gastrointestinal (GI) digestion was also evaluated.

For IC_{50} determination, peptides at various concentrations of 1.25, 2.5, 5.0, 10.0, 25.0, 50.0 µg Gly eq./mL were prepared and determined for ACE inhibitory activity. IC_{50} value defined as the concentration of peptide at which 50% inhibition is attained was calculated using GraphPad® Prism 6.0.

3.8.4 Peptide identification

The potent fraction obtained from a Superdex Peptide 10/300 GL column was analyzed for *de novo* peptide sequencing using a Waters SYNAPT™ HDMS™ system. The 1D-nanoLC was carried out with a Waters nanoACQUITY UPLC system. Four µL of digests was injected onto the RP analytical column (20 cm × 75 µm) packed with a 1.7 µm Bridged Ethyl Hybrid (BEH) C18 (Waters Corporation, MA, USA). Peptides were eluted with a linear gradient from 2% to 40% acetonitrile developed over 60 min at a flow rate of 350 nL/min. This was followed by a 15 min period of 80% acetonitrile to clean the column before returning to 2% acetonitrile for the next sample. The effluent samples were electrosprayed into a mass spectrometer (Synapt HDMS) for MS/MS analysis of peptides. Argon gas was used in the collision cell to obtain MS/MS data. MS/MS spectra thus obtained were processed using Max Ent 3, a deconvolution software for peptides (Ensemble 1, Iterations 50, auto peak width determination) within MassLynx 4.0. The deconvoluted spectra were then imported into Mass Seq (MassLynx's denovo sequencing tool) to obtain a sequence tag.

3.9 *In vitro* pepsin-pancreatin simulated GI digestion

Simulated GI digestion using an *in vitro* pepsin-pancreatin hydrolysis was performed according to Rao et al. (2012) with some modifications. The partially purified peptides from Superdex Peptide 10/300 GL column (60 µg. Gly eq./mL) were lyophilized. Peptide powder was re-dissolved with 2.2 mL DI water, pH 2 and pre-incubated at 37^{o}C for 5 min. Pepsin (concentration 1.2 µg/mL, 0.5 mL) was added to provide enzyme to substrate ratio of 1:100 (w/w). The mixture were incubated at 37^{o}C for 3 h and then adjusted to pH 7.5 with 10 N NaOH. The mixture volume was adjusted to 2.9 mL. Neutralized mixture (0.3 mL) was collected and centrifuged at 10,000 × g for 10 min. The supernatant was collected for determination of α-amino acid group content and ACE inhibitory activity. The suspension was further digested by 2% (w/w) porcine pancreatin at 37^{o}C for 4 h. The digestion was terminated at 90^{o}C for 10 min and centrifuged at 10,000 × g for 10 min. The supernatant was collected to determine α-amino acid group content and ACE inhibitory activity.

3.10 Statistical analyses

All experiments were repeated twice. Measurements were carried out in a duplication. Data (n = 4) were expressed as mean ± standard deviation. The statistical analysis was performed using SPSS Statistics Program (Version 17.0, SPSS Inc, Chicago, IL, USA). Data were analyzed using analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used in a mean comparison at a statistical level as *P*-value < 0.05.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Chemical characteristics of skin and gelatin

4.1.1 Proximate composition

The proximate composition of various forms of skin and gelatin was summarized in Table 4.1. Protein is a major component in Thai Panga skin. Protein content is higher than skins of fish without scale including bamboo shark skin (65.1%) (Kittiphattanabawon, Benjakul, Visessanguan, Kishimura, and Shahidi, 2010) and shortfin scad skin (60.9%) (Cheow, Norizah, Kyaw, and Howell, 2007) but lower than the skin of walking catfish (82.6%) (Jamilah, Tan, Hartina, and Azizah, 2011), striped catfish (85.4%) (Jamilah, Tan, Hartina, and Azizah, 2011), and channel catfish (76.8%) (Liu, Li, and Guo, 2007). High amount of protein indicated that Thai Panga can be a good source for the bioactive peptide production. The skin also contained relatively higher fat than those previously reported which were in range of 0.5-24.3% (Cheow, Norizah, Kyaw, and Howell, 2007; Kittiphattanabawon, Benjakul, Visessanguan, Kishimura, and Shahidi, 2010; Liu, Li, and Guo, 2007). Skin of Thai Panga contain trace amount of ash. Ash content of striped catfish was 11.7% and walking catfish was 1.4% (Jamilah, Tan, Hartina, and Azizah, 2011).

Butyl alcohol extraction and alkaline-acid pretreatment only removed about 36.2% fat from the skin. Fat was removed up to 70% after thermal hydrolysis into gelatin. However, it is relatively high when compared with that of other fish skin

gelatins (< 1.0%) (Binsi, Shamasundar, Dileep, Badii, and Howell, 2009; Cheow, Norizah, Kyaw, and Howell, 2007; Jellouli et al., 2011; Jongjareonrak et al., 2010; Lassoued et al., 2014). These results reflect that pretreatment condition was not efficient to remove fat from Thai Panga skin. High polarity of buthanol may cause insufficient removal of fat. In general, commercial gelatin contain 84-90% protein with free of fat and containing ash lower than 2% (Gelatine Manufacturers of Europe, 2015).

Table 4.1 Chemical compositions (% dry basis) of dried skin, defatted skin, alkaline-acid pretreated skin and gelatin of Thai Panga.

Compositions	Skin	Defatted skin	Alkaline-acid pretreated skin	Gelatin
Crude protein	71.4^{c}	81.9^{b}	85.2^{b}	91.8^{a}
Crude fat	28.4^{a}	18.1^{b}	14.8^{b}	8.1^{c}
Ash	0.3^{a}	0.1^{b}	0.0^{c}	0.0^{c}

Note: Values are expressed as mean ± SD (n = 4). Superscript indicates a significant difference of skin pre-treatment at $P<0.05$.

4.1.2 Protein patterns

Main protein component of all samples is collagen as seen on SDS-PAGE (Figure 4.1). Defatted and alkaline-acid pretreated skin contained β-component (MW ~200 kDa), α1 and α2 chain (MW ~100 kDa) as major constituents which were similar to the pattern of bovine tendon type I collagen (TC). High molecular mass component (MW > 200 kDa), which are intra- and intermolecular crosslinks of collagen were also observed. However, low molecular mass components (MW ~100

Da) did not appear in all skins. Intensity of β-component of gelatin is lighter than others. The band of α2-chain of gelatin disappeared with a concomitant formation of the low molecular mass peptides (MW ~31 kDa). These were possibly due to the transition of the collagen triple helix to coil structure and some peptide bonds cleavage induced by thermal extraction. This phenomenon has been reported in fish gelatin extracted at temperature higher than 45^{o}C (Kittiphattanabawon, Benjakul, Visessanguan, and Shahidi, 2010; Muyonga, Cole, and Duodu, 2004; Nagarajan, Benjakul, Prodpran, Songtipya, and Kishimura, 2012; Sinthusamran, Benjakul, and Kishimura, 2014)

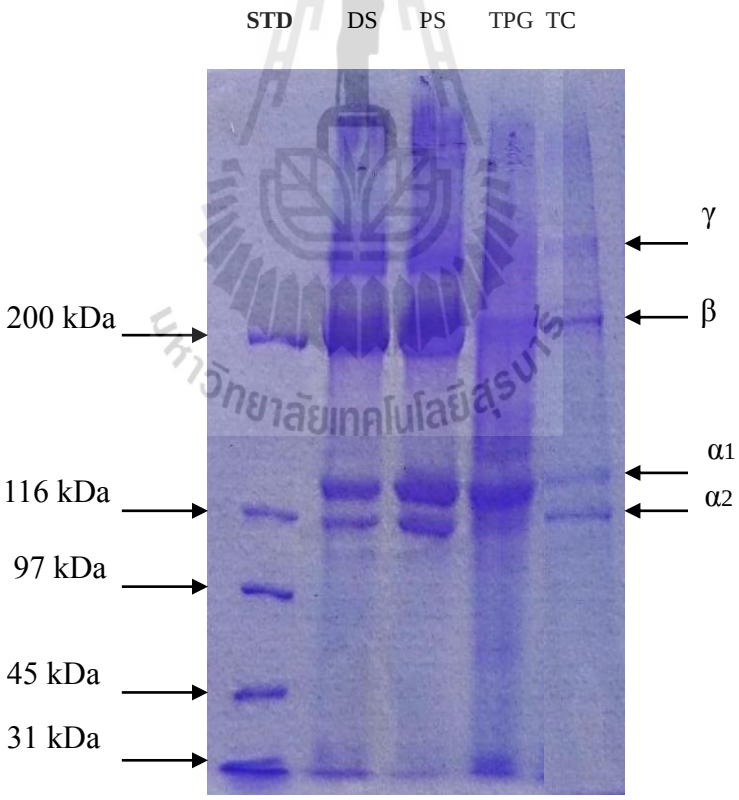

Figure 4.1 SDS-PAGE pattern (7.5% polyacrylamide) of a standard marker (STD), defatted skin (DS), alkalie-acid pretreated skin (PS), gelatin (TPG) of Thai Panga, and type I tendon collagen (TC).

4.1.3 Amino acid composition

Amino acid compositions are a critical factor providing bioactivity of peptides. The amino acid compositions of all samples were shown in Table 4.2. The most abundant amino acids in all samples are Gly, Pro, Hyp, Arg, Ala, Glu/Gln. In general, Gly exists uniformly in the α-chain as a repeating formula Gly-X-Y (X and Y is mainly Pro and Hyp, respectively) throughout the collagen molecules (Asghar and Henrickson, 1982). Likewise, Ala, Pro and Hyp are usually found in collagen molecules, while Cys and Tyr are not common (Muyonga, Cole, and Duodu, 2004). In general, amino acid composition is not different among samples (P>0.05). High proportion of Gly, Ala, Pro, Glu/Gln and Arg were also observed in chum salmon, channel catfish, farmed Amur sturgeon and striped catfish skin (Lee, Jeon, and Byun, 2014; Liu, Li, and Guo, 2007; Nikoo et al., 2014; Singh, Benjakul, Maqsood, and Kishimura, 2011). Amino acid contents of gelatin were in the range found in other fish species (19.6-37.6 g/100 g protein for Gly, 8.3-11.9 g. /100 g. protein for Ala, 1.9-13.6 g/100 g protein for Pro, 3.3-10.5 g./100 g. protein for Glu/Gln, and 3.5-8.7 g/100 g protein for Arg) (Binsi, Shamasundar, Dileep, Badii, and Howell, 2009; Cheow, Norizah, Kyaw, and Howell, 2007; Duan, Zhang, Xing, Konno, and Xu, 2011; Jamilah, Tan, Hartin, and Azizah, 2011; Lee, Jeon, and Byun, 2014; Liu, Li, and Guo, 2007; Nagai, Araki, and Suzukia, 2002; Nikoo et al., 2014; Sila et al., 2015; Singh, Benjakul, Maqsood, and Kishimura, 2011; Zeng et al., 2009; Zhang et al., 2007). The contents of Asp, Glu/Gln, and Ser of defatted and alkaline-acid pretreated skin are higher than those of gelatin (P<0.05). These amino acids possibly lost during the thermal extraction of gelatin. Alkaline-acid pretreated skin and gelatin had higher amount Pro than defatted skin (P<0.05). Removal of non-collagen substance by alkaline-acid pretreatment as well as thermal extraction leads to an increase in

collagen proportion, and higher amount of Pro in these two samples. Potent ACE inhibitory peptides found in previous studies usually contain Pro, Ala, Asp, Gly, Glu in their sequences (Table 2.1) such as protein hydrolysates from *Rhopilema esculentum* which contained high level of Gly, Glu, Pro, Asp and Ala (Liu, Zhang, Zhang, and Liu, 2012). Strong ACE inhibitory activity evidently associated with Pro residues in the peptide, particularly at the C-terminal position (Korhonen and Pihlanto, 2006). Furthermore, peptides containing Pro is known to be resistant to the intestinal enzyme digestion and pass easily into the blood circulation (Walter, Kissel, and Amidon, 1996). Cushman and Ondetti (1984) proposed that Pro at C-terminal position of the peptides showed effective binding to ACE. In addition, most commercial ACE inhibitor drugs (Captopril and Enalapril) also contained Pro in their structure (Otte, Shalaby, Zakola, Pripp, and El-Shabrawy, 2007). Amino acid composition appeared to suggest that Thai Panga skin would contain the potent ACE inhibitory peptides.

Table 4.2 Amino acids composition of defatted skins, pretreated skins and gelatin powder of Thai Panga (g/100 g protein).

Amino acid	Defatted skin	Pretreated skin	Gelatin
Alanine	8.6	8.1	7.6
Arginine	8.7 [b]	9.3 [a]	8.3 [b]
Aspartic acid/Asparagine	3.9 [a]	3.6 [a]	2.2 [b]
Cysteine	3.1	1.2	3.7
Glutamic acid/Glutamine	9.9 [a]	9.8 [a]	6.6 [b]
Glycine	21.3	23.3	25.4
Histidine	0.9	0.7	0.6
Hydroxylysine	ND	ND	ND

Table 4.2 Amino acids composition of defatted skins, pretreated skins and gelatin powder of Thai Panga (g/100 g protein) (Continued).

Amino acid	Defatted skin	Pretreated skin	Gelatin
Hydroxyproline	6.3	8.9	6.4
Isoleucine	1.9	1.7	1.6
Leucine	3.7	3.3	3.1
Lysine	6.9	4.4	9.5
Methionine	2.6 [a]	1.6 [b]	2.7 [a]
Phenylalanine	1.9	1.8	2.1
Proline	10.5 [b]	13.2[a]	12.6 [a]
Serine	3.1 [a]	3.2 [a]	2.0 [b]
Threonine	2.8	2.7	2.5
Trptophan	ND	ND	ND
Tyrosine	0.9	0.5	0.5
Valine	3.0	2.8	2.6
Total	100.00	100.00	100.00

Data are expressed as mean ± SD (n = 2).

4.2 Screening of substrate and protease for ACE inhibitory peptides production

Overall, there was a significant effect of enzyme on degree of hydrolysis (DH) (P<0.05). The highest DH was found in hydrolysates prepared by Alcalase (P<0.05, Table 4.3). Alcalase is an endoprotease with broad specificity and high affinity toward aromatic amino acids, Glu, Met, Leu, Ala and Ser in the peptide chains (Barrett, Rawlings, and Woessner, 2004). Although Protamex, papain and *V. halodenitrificans* SK 1-3-7 protease have a broad specificity, they showed lower DH (P<0.05, Table 4.4). Alcalase was recommended as the most effective enzyme in hydrolyzing

collagen (He, Liu, and Ma, 2013). It has been reported that skate skin hydrolysate by Alcalase showed higher DH (65.9%) than that of chymotrypsin (43.1%) and papain (45.4%) (Lee, Jeon, and Byun, 2011). It also showed relatively higher DH than that obtained from collagenase, Proteinase K, trypsin, thermolysin and pepsin in the hydrolysis of bovine collagen at the same hydrolysis time (Zhang, Olsen, Grossi, and Otte, 2013). Trypsin predominantly cleaves peptide bonds at carboxyl side of Lys and Arg (Barrett, Rawlings, and Woessner, 2004). Rich Pro in the repeating sequence of Gly-Pro-Hyp in collagen probably obstructs the hydrolysis of trypsin. Pepsin preferably cleaves peptide bonds between hydrophobic and aromatic amino acids (Phe, Trp, and Tyr) (Barrett, Rawlings, and Woessner, 2004; Whitaker, 1994). Less content of these amino acids in collagen may limit pepsin hydrolysis.

It was observed that hydrolysates prepared at high temperature (50-60^{o}C) showed higher DH than those produced at a low temperature (37^{o}C for trypsin and pepsin) (P<0.05, Table 4.4). Thai Panga skin has a collagen as a major structural protein (Figure 4.1). This rigid structure is difficult to breakdown by a protease except for collagenase (Zhang, Olsen, Grossi, and Otte, 2013). However, it could be transformed to random coil of gelatin at 40^{o}C (Karim and Bhat, 2009). When all substrates were subjected into the hydrolysis by Alcalase, *V. halodenitrificans* SK 1-3-7 protease, Protamex, papain at temperatures above 50^{o}C for 12 h, inter- and intra-hydrogen bonds were likely to be disrupted. Particularly, heating collagen molecules in alkaline condition as in hydrolysis by Alcalase, papain, and *V. halodenitrificans* SK 1-3-7 would weaken covalent crosslinks. The triple helical structure of collagen was uncoiled, converting to gelatin. This would, in turn, increase enzyme accessibility. Under pepsin and trypsin hydrolysis, which was performed in acidic and alkaline

condition at 37^{o}C, respectively, the triple helix structure of collagen may partially uncoil, but covalent crosslinks among tropocollagen in the skin matrix remained, leading to the limited enzymatic hydrolysis, especially in defatted skin.

DH of gelatin was comparable to that of alkaline-acid pretreated skin (P>0.05, Table 4.4). Alkaline and acid pretreatment could open up the matrix structure of skin, allowing enzyme accessibility to the similar level as uncoiled structure of gelatin. On the other hand, gelatin showed DH value higher than defatted skin (P<0.05, Table 4.4). This was because proteases could directly hydrolyze peptide bonds in individual random coil of gelatin easier than tightly packed matrix of collagen in defatted skin. Collagen in defatted skin is stabilized by numerous hydrogen bonds and covalent cross-links, leading to more protease resistance. Based on DH, Alcalase and defatted skin would be recommended to use to produce Thai Panga skin protein hydrolysate since a high yield would be obtained.

Type of protease and form of substrate have an overall effect on specific ACE inhibitory activity (P<0.05, Table 4.4). Pepsin hydrolysate showed the highest ACE inhibitory activity. Potency of ACE inhibitory peptides is strongly influenced by hydrophobicity of amino acid residues at the ultimate position of peptides since it has tightly bound at the sub-active site of ACE (Hong et al., 2008; Murray and FitzGerald, 2007; Ondetti and Cushman, 1982). Pepsin may produce peptides with some hydrophobic amino acids at the C-terminal position, resulting in high ACE inhibitory activity. Protamex, papain, and Alcalase also produced hydrolysates with ACE inhibitory activity (Table 4.3). According to their broad specificity, the high amounts of small peptides were produced. The smaller peptides are well known to exhibit higher potent ACE inhibitory activity than the larger peptides since the latter is

hindered by the small cavity of ACE active site (Natesh, Schwager, Sturrock, and Acharya, 2003). For trypsin hydrolysate, ACE inhibitory potency was related to positively charged amino acid at C-terminal of peptides, which could interact with anionic binding site of ACE (FitzGerald and Meisel, 2000). The hydrolysate produced by *V. halodenitrificans* SK 1-3-7 protease showed lowest ACE inhibitory activity in this study. The ultimate position and length of peptides may not be favored for ACE active site.

ACE inhibitory activity also appears to vary with form of substrate. Alkaline-acid pretreated skin hydrolysates showed ACE inhibitory activity higher than defatted skin and gelatin ($P<0.05$, Table 4.4). This might be due to the difference in amino acid composition among three substrates. Higher proportion of Pro and Arg (Table 4.2) in alkaline-acid pretreated skin may associate with its high specific ACE inhibitory activity. Interaction between substrate and enzyme used was significant ($P<0.05$).

Specific ACE inhibitory activity did not vary with form of substrate for Alcalase hydrolysis ($P>0.05$). Alcalase has been reported to degrade the triple helix of bovine collagen within a short time (Zhang, Olsen, Grossi, and Otte, 2013). Both defatted skin and alkaline-acid pretreated skin of Thai Panga were equally accessed by Alcalase. The collagen networks that were destabilized at 50^{o}C could promote the accessibility of Alcalase to the skins, resulting in a similar specific ACE inhibitory activity.

Alkaline-acid pretreated skin and gelatin hydrolysate showed higher specific ACE inhibitory activity than defatted skin in pepsin hydrolysis ($P<0.05$, Table 4.3). At 37^{o}C, covalent cross-links of collagen fibrils in the defatted skin were still stabilized. Accessibility of pepsin to collagen in the defatted skin would be more difficult than in

alkaline-acid pretreated skin and gelatin. Collagen is a hydrophilic protein which can solubilize in polar solution (Shoulders and Raines, 2009). At pH 2, random coil gelatin and partially uncoiled collagen structure of alkaline-acid pretreated skin may ease them to be more solubilized in hydrochloric solution than a close packed structure of defatted skin. This would promote pepsin hydrolysis, leading to more release of ACE inhibitory peptides. In addition, alkaline-acid pretreated skin and gelatin comprised of higher proportion Pro in their structure than defatted skin (P<0.05, Table 4.2). Strong ACE inhibitory activity evidently associated with Pro residues in the peptide, particularly at the C-terminal (Korhonen and Pihlanto, 2006). The most potent ACE inhibitory peptides with Pro at C-terminal produced by pepsin included Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Pro-Pro-Lys-Tyr-Leu-Asp-Thr-Pro derived from tuna frame hydrolysate (Lee, Qian, and Kim, 2010).

For trypsin hydrolysis, only the hydrolysate produced by alkaline-acid pretreated skin show specific ACE inhibitory activity (P<0.05, Table 4.3). Trypsin typically produces peptides with C-terminal Arg or Lys, whose guanidine or ε-amino can interact with an anionic binding site of ACE (Murray and FitzGerald, 2007). Peptides in trypsin-hydrolyzed gelatin and defatted skin hydrolysate may be devoid of these residues in their ultimate position, resulting in limited ACE inhibitory activity. Amino acid profile clearly showed that alkaline-acid pretreated skin has relatively higher proportion of Arg than others (P<0.05, Table 4.2). It could possibly be that alkaline-acid pretreated skin would release peptide containing Arg after trypsin hydrolysis, resulting in high specific ACE inhibitory activity. Several previous studies showed that the most potent ACE inhibitory peptides derived from trypsin hydrolysates of wheat gluten, β-lactoglobulin, buffalo colostrum, ostrich egg white,

chum salmon skin, and Alaska pollack frame contained peptide containing Arg at their C-terminal position (Asoodeh et al., 2014; Ferreiraa, et al., 2007; Hou et al., 2012; Lee, Jeon, and Byun, 2014; Rohit, Sathisha, and Aparna., 2012; Tanzadehpanah, Asoodeh, Saberi, and Chamani., 2013).

Specific ACE inhibitory activity of papain hydrolysate was also varied with form of substrate (P<0.05, Table 4.3). The highest specific ACE inhibitory activity was observed in papain-hydrolyzed alkaline-acid pretreated skin. It has been reported that the most potent ACE inhibitory peptides derived from cod skin and Atlantic salmon collagen hydrolysate was produced by papain hydrolysis, which contained Pro in their ultimate position (Gu, Li, Liu, Yi, and Cai., 2011; Ngo, Ryu, Voa, Himaya, and Kim, 2011). The cod skin peptide, Thr-Cys-Ser-Pro showed ACE inhibitory activity about 81% at 0.5 mg/mL, while a dipeptide, Ala-Pro, from Atlantic salmon collagen exhibited IC_{50} = 60 µM (Gu, Li, Liu, Yi, and Cai, 2011; Ngo, Ryu, Voa, Himaya, and Kim, 2011). High content of Pro in alkaline-acid pretreated skin (Table 4.2) may provide the opportunity to release peptides with Pro at their C-terminal for papain hydrolysate, resulting in the highest ACE inhibitory activity.

Protamex hydrolysis of defatted and alkaline-acid pretreated skin showed higher specific ACE inhibitory activity than gelatin (P<0.05, Table 4.3). It has been found that a dipeptide, Tyr-Asn, derived from hard calm nuscle hydrolysate was the potent candidate in ACE inhibition with IC_{50} = 51 µM (Tsai, Chen, and Pan, 2008). High proportion of Asp/Asn in both defatted and alkaline-acid pretreated skin might generate some peptides rendering relatively high specific ACE inhibitory activity.

Specific ACE inhibitory activity of *V. halodenitrificans* SK 1-3-7 protease hydrolysis was only detected in alkaline-acid pretreated skin hydrolysate, while DH

and peptide content of all hydrolysates produced by this enzyme were comparable (P>0.05, Table 4.3). This suggested that peptide sequence was a critical factor for ACE inhibition. As reported by Toopcham, Roytrakul, and Yongsawatdigul (2015), the most potent ACE inhibitory peptide derived from tilapia hydrolysates composed of Arg at the C-terminal position (Met-Met-Leu-Leu-Leu-Phe-Arg, IC_{50} = 0.1 μM). High proportion of Arg in alkaline-acid pretreated skin may provide hydrolasate containing some peptides with C-terminal Arg, resulting in high specific ACE inhibitory activity.

These results imply that protease and form of substrate affected ACE inhibitory potency of Thai Panga skin hydrolysate. Protease governs the length and amino acid at the C-terminal end of peptide, while skin pretreatment contributed to improving the accessibility of protease to the substrate. The highest ACE inhibitory activity was detected in pepsin-hydrolyzed pretreated skins. Thus, pepsin and pretreated skin were selected for further studies.

Specific ACE inhibitory activity of hydrolysate did not follow the same trend as DH (P<0.05, Table 4.4). DH indicates the extent of protein degradation (Adler-Nissen, 1979). Thus, single polypeptide chain of gelatin or opened collagen structure of alkaline-acid pretreated skin which was easily to cleave by the enzyme could generate higher amount of peptide fragments than complex structure of defatted skin. ACE inhibitory potency of peptides strongly depends on amino acid at C-terminal of peptides. Therefore, the proportion of some specific amino acids (Arg, Pro) is a critical factor providing high ACE inhibitory activity in alkaline-acid pretreated skin.

Table 4.3 Degree of hydrolysis, ACE inhibitory activity, peptide content and specific ACE inhibitory activity of hydrolysate produced by various enzyme and substrates.

Enzyme	Substrate	ACE inhibition (%)	DH (%)	Peptide content (µg Gly eq.)	Specific ACE inhibitory activity (%/µg Gly eq.)
Alcalase	Gelatin	32.3	35.1	68.5	0.5e[b]
	Defatted skin	44.1	38.4	77.3	0.6e[ab]
	Alkaline-acid pretreated skin	45.8	33.2	66.5	0.7e[a]
Pepsin	Gelatin	48.5	7.7	15.5	3.1b[a]
	Defatted skin	21.5	6.9	15.5	1.4cd[b]
	Alkaline-acid pretreated skin	71.0	7.7	14.5	4.9a[a]
Trypsin	Gelatin	ND	12.3	35.8	ND
	Defatted skin	ND	10.6	27.0	ND
	Alkaline-acid pretreated skin	62.6	11.7	34.3	1.9[c]
Papain	Gelatin	ND	18.9	37.0	ND
	Defatted skin	25.4	13.9	30.9	0.8de[a]
	Alkaline-acid pretreated skin	63.4	17.0	36.0	1.8c[b]
Protamex	Gelatin	21.5	17.6	35.9	0.6c[b]
	Defatted skin	45.9	12.3	26.8	1.7c[a]
	Alkaline-acid pretreated skin	59.4	15.8	34.5	1.7e[a]

Table 4.3 Degree of hydrolysis, ACE inhibitory activity, peptide content and specific ACE inhibitory activity of hydrolysate produced by various enzyme and substrates (Continued).

Enzyme	Substrate	ACE inhibition (%)	DH (%)	Peptide content (µg Gly eq.)	Specific ACE inhibitory activity (%/µg Gly eq.)
SK 1-3-7	Gelatin	ND	18.9	46.3	ND
	Defatted skin	ND	20.3	47.0	ND
	Alkaline-acid pretreated skin	29.1	22.4	48.5	0.6^{e}

Data are express as mean ± SD (n = 4). SK 1-3-7 is *V. halodenitrificans* SK 1-3-7 protease. Letter is denoted as a significant difference among sample at $P<0.05$.

Superscripts indicate significant difference on the individual protease at $P<0.05$.

Table 4.4 Effect of substrates and enzymes on DH and specific ACE inhibitory activity.

Factor	**Significance level (*P*)****	**Effect on DH***	**Effect on specific ACE inhibitory activity***
Substrate	<0.0001	Gelatin ≥ Alkaline-acid pretrated skin ≥ Defatted skin	Alkaline-acid pretreated skin > Defatted skin ≥ Gelatin
Enzyme	<0.0001	Alcalase > SK 1-3-7 > papain ≥ Protamex > trypsin > pepsin	Pepsin > Protamex > Papain ≥ trypsin ≥ Alcalase ≥ SK 1-3-7
Enzyme * Substrate	<0.0001		

** Analysis based on sum of square. * Data were compared by DMRT. > indicates significantly higher than (P<0.05).

≥ is higher than but not significant (P>0.05). SK 1-3-7 is *V. halodenitrificans* SK 1-3-7 protease.

4.3 Purification of ACE inhibitory peptides

Both DH and ACE inhibitory activity increased with hydrolysis time (Figure 4.2), indicating that ACE inhibitory activity of pepsin-hydrolyzed alkaline-acid pretreated Thai Panga skin hydrolysate relied on DH. During the progression of proteolysis, numbers of smaller peptides were released from the skin structure. Some of them might possess the chain length or C-terminal amino acid residues which could exert inhibitory activity towards ACE (Nasria et al., 2013; Salampessy, Reddy, Kailasapathy, and Phillips, 2015). ACE inhibitory activity rapidly increased within 2 h and became steady afterward. Therefore, two hour hydrolysis is suitable for the production of potent ACE inhibitory hydrolysate.

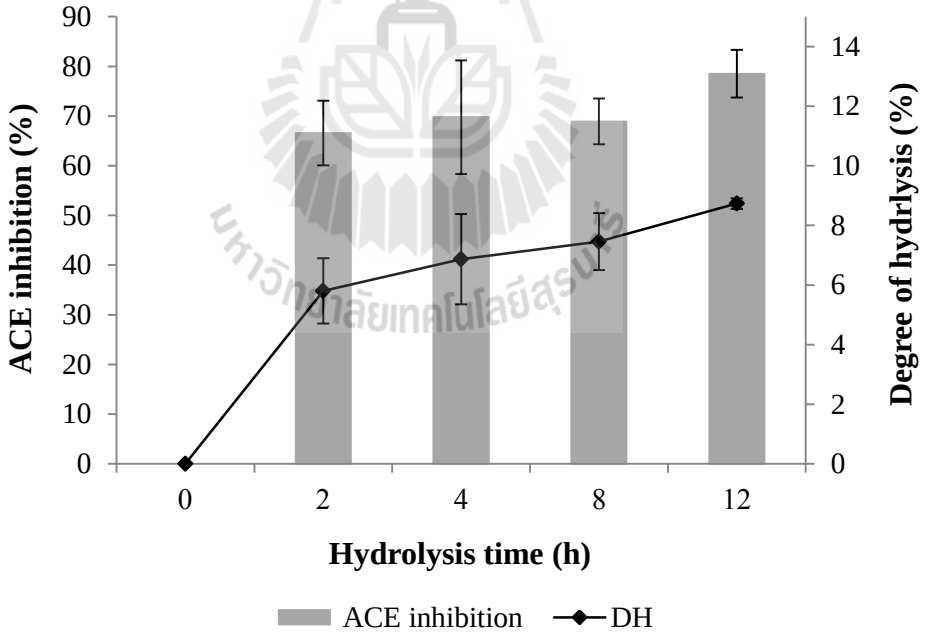

Figure 4.2 ACE inhibition (%) and degree of hydrolysis (%) of alkaline-acid pretreated skin produced by pepsin at different hydrolysis time. Values are expressed as mean ± SD (n=4).

Results of ultrafiltration showed that peptides with MW < 1 kDa exhibited the highest ACE inhibitory activity and yield (P<0.05, Table 4.5). This result corresponds to the fact that small peptides contribute to the ACE inhibitory activity of protein hydrolysates. ACE has a small groove which restricts the access of large polypeptide to its active site (Hernández-Ledesma, Contreras, and Recio, 2011; Natesh, Schwager, Sturrock, and Acharya, 2003). The hydrolysate have MW less than that of the most potent ACE inhibitory peptide from tuna frame hydrolysate produced by pepsin which has MW 2,482 Da and IC_{50} = 11.3 µM (Lee, Qian, and Kim, 2010). The sequential ultrafiltration could effectively increase purity approximately 5 folds as compared to crude hydrolysates. Thus, the active fraction was further purified.

Table 4.5 Ultrafiltration of pepsin-hydrolysed alkaline-acid pretreated skin.

Fraction	**Peptide content (mg.Gly eq.)**	**Yield (%)**	**ACE inhibition (%)**
Crude	290.5 ± 1.4	100.0	9.3[c]
> 30 kDa	34.6 ± 0.4	11.9	0.0[d]
5-30 kDa	6.5 ± 0.1	2.2	45.5[b]
1-5 kDa	3.0 ± 0.2	1.0	46.5[b]
< 1 kDa	146.8 ± 2.3	50.5	49.8[a]

Values are expressed as mean ± SD (n = 4). Yields were calculated based on peptide content (mg Gly eq.). Different letters indicate significant difference of ACE inhibition at the level of P<0.05. ACE inhibitions of fractions were determined at the same concentration of peptide (25 µg.Gly eq./mL).

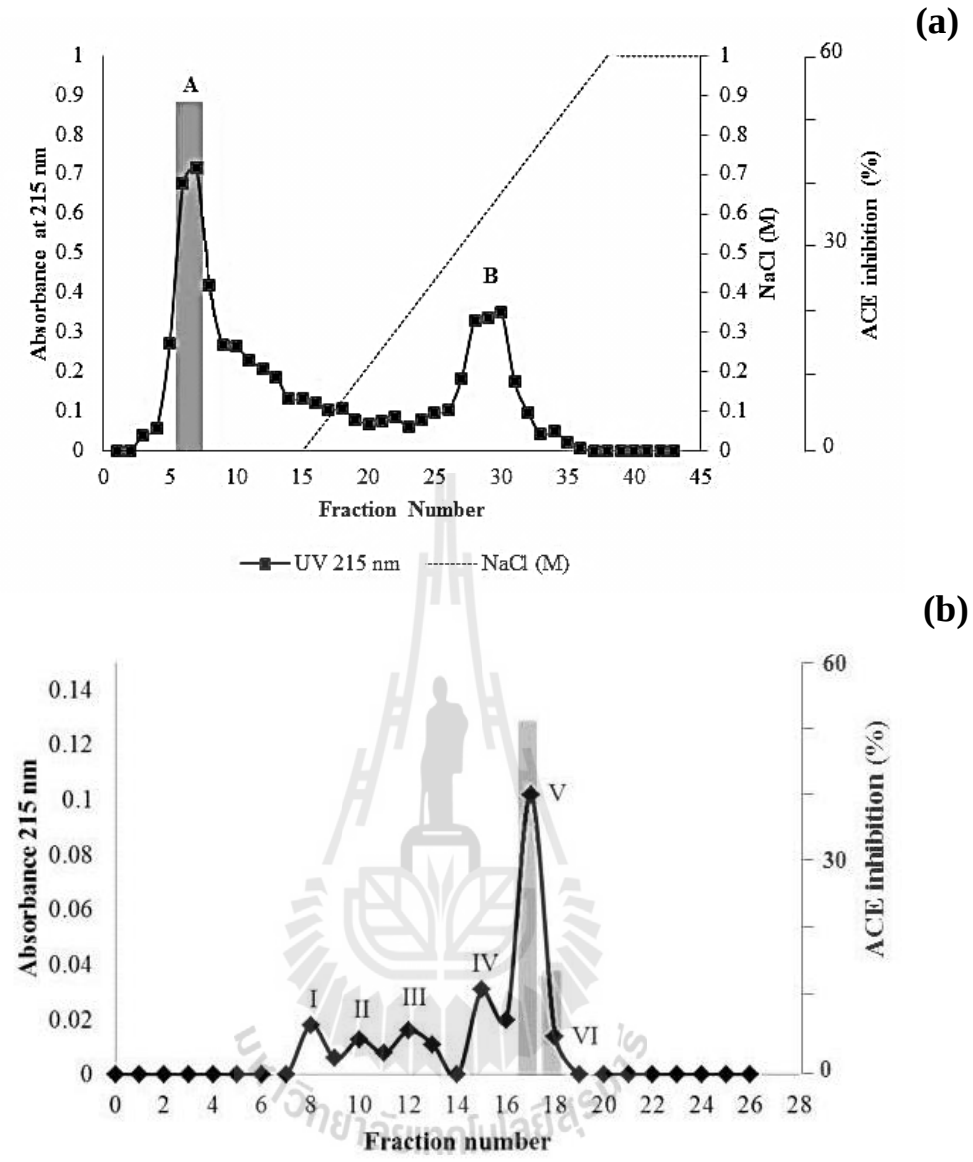

Figure 4.3 (a) DEAE-Sephacel anion exchange chromatogram of pepsin-hydrolysed alkaline-acid pretreated skins hydrolysate (MW <1-kDa). Elution was performed at a flow rate of 1 mL/min with linear gradient of NaCl (0-1 M) in 50 mM Tris-HCl buffer at pH 8.0. Bar graph indicates ACE inhibition (%). (b) Gel filtration chromatogram of active fraction A by DEAE-Sephacel column. Elution was performed at 0.2 mL/min with DI water. The fractions designated as I-VI were assayed for ACE inhibition (%).

The fraction sample (MW < 1 kDa) was subjected to DEAE-Sephacel anion exchange chromatography. Two fractions were obtained (Figure 4.3). The first elution (fraction A) was the active fraction with 51.2% ACE inhibition, while fraction B did not show ACE inhibitory activity at the same peptide concentration (25 µg. Gly eq./mL). The potent fraction A, was further separated by size-exclusion chromatography on Superdex Peptide 10/300 GL column. Five major peptide peaks were obtained. Only fraction V and its shoulder (fraction VI) exhibited ACE inhibitory activity. IC_{50} value of fraction V was determined to be 19 µg. Gly eq./mL.

Peptide sequences of fraction V from Superdex peptide 10/300 were analyzed by LC-MS/MS (Table 4.5). The potent fraction contained nine peptides consisting of 7-13 amino acids with molecular mass between 0.7 and 1.5 kDa. The identified peptides contained a high proportion of hydrophobic amino acids (Phe, Pro, Leu, Val, Met, Tyr, and Trp). Amongst them, five peptides contained either hydrophobic (Leu or Val) or aromatic amino acids (Trp or Tyr) at the C-terminal while other four sequences possess Arg. Hydrophobic amino acids (Pro, Ala, Leu, Phe, and Val) were also located at the N-terminus of those peptides except for KEKLNPQPFVPR.

The molecular size (between 500-1500 Da and 3-13 amino acids length) and specific amino acids at the C-terminal of the identified peptides seem to be crucial factors regulating ACE inhibitory activity (Byun and Kim, 2002; Cheung, Wang, Ondetti, Sabo, and Cushman, 1980; Li, Leemail, Shi, and Shrestha, 2004; Jiangping Wu, Aluko, and Nakai, 2006). The sequences LNRVVERL, FLMKMRALFY, VFLVQRWWWV, VFLVQRWVWW and VFLRVQWREW showed some characteristics of a potent ACE inhibitory peptide according to Cheung, Wang, Ondetti, Sabo, and Cushman, (1980) who reported that Pro, hydrophobic, and aromatic amino acids at the C-terminal and branched-chain aliphatic amino acids (Val,

Ile, Leu) at the N-terminal can bind to ACE as a competitive inhibitor. Those preferred amino acids at the C-terminal tripeptide could tightly bind with the subsites (S', S_1' and S_2') of the ACE catalytic site (Ondetti and Cushman, 1982).

Table 4.6 ACE inhibitory peptide sequences identified by *de novo* peptide sequencing.

Sequence no.	Charge[a] (H^+)	m/z[b]	MW (Da)	Score	Sequence
1	2	499.80	997.60	82.22	LNRVVERL
2	2	363.20	724.42	58.97	PSLPVGR
3	2	363.20	724.45	53.84	ALLPVGR
4	3	440.56	1318.69	45.61	FLMKMRALFY
5	3	473.59	1417.76	45.61	VFLVQRWVWW
6	3	473.59	1417.76	36.84	VFLVQRWWWV
7	3	473.59	1417.76	33.33	VFLRVQWREW
8	4	363.96	1451.82	31.88	KEKLNPQPFVPR
9	4	363.96	1451.79	25.33	FPQPPLGTPFVPR

[a] Charge state of the observed peptides.

[b] Relation of mass/charge (m/z) observed in the LC-MS/MS system

The ACE inhibitory potency of Arg residue at the C-terminal was described by Meisel (1993). The positive charge on the guanidine or ε-amino group of the C-terminal arginine and lysine side chain interacted with an anionic binding site of ACE other than the catalytic site (FitzGerald and Meisel, 2000). Peptides with Arg at C-terminal have also been reported as the most potent ACE inhibitory peptide including: ALPMHIR (IC_{50} = 42.6 μM) derived from β-lactoglobulin of whey protein concentrate (Ferreiraa et al., 2007), IPALLKR (IC_{50} = 43 ± 1.3 μM) and AQQLAAQLPAMCR (IC_{50} = 68 ± 2.8 μM) from gluten (Asoodeh et al., 2014),

FQSNFNTQATNR (IC_{50} = 30 µM) from lysozyme (Asoodeh, Yazdi, and Chamani, 2012), and QLGPLGPR (IC_{50} = 148 µM) from skate skin collagen (Lee, Jeon, and Byun, 2011). Those peptides behaved as a non-competitive, competitive, uncompetitive inhibitor and non-competitive inhibitor, respectively (Asoodeh et al., 2014; Asoodeh, Yazdi, and Chamani, 2012; Lee, Jeon, and Byun, 2011).

Peptide sequences found in this study have not yet been reported in the literatures. Nevertheless, amino acid residues at the ultimate and penultimate position of Thai Panga skin peptides, FPQPPLGTPFVPR and KEKLNPQPFVPR, had a homology with the first two residues from C-terminal of ACE inhibitory peptide from soya milk (WNPR, IC_{50} = 880 µM) (Tomatsu, Shimakage, Shinbo, Yamada, and Takahashi, 2013), skate skin (QLGFLGPR, IC_{50} = 148 µM) (Lee, Jeon, and Byun, 2011), and skipjack roe (YVNDAATLLPR, IC_{50} = 105.9 µM) (Intarasirisawat, Benjakul, Wu, and Visessanguan, 2013). In addition, the first two residues from C-terminal of VFLVQRWVWW of Thai Panga alkaline-acid pretreated skin was similar to the potent ACE inhibitory peptide from bullfrog muscle (GAAGLPCSADWW, IC_{50} = 1.0 µM) (Qian, Jung, Lee, Byun, and Kim, 2007).

4.4 Effect of gastrointestinal proteases on ACE inhibitory activity

The effective ACE inhibitory peptides need to preserve their active form until reach the target site *in vivo*. Gastrointestinal digestion might increase or decrease ACE inhibitory activity of the peptide (Jao, Huang, and Hsu, 2012). A simulated peptic-pancreatic digestion can be used to predict the fate of ACE inhibitory potency after orally ingested (Hur, Lim, Decker, and McClements, 2011). After pepsin hydrolysis, ACE inhibitory activity remained the same, but decreased after pancreatic digestion. The α-amino acid group content increased after peptic-pancreatic incubation (Figure

4.5), indicating that those peptides were mostly degraded by pancreatin. Long chain peptides (more than 5 amino acid residues) appeared to be prone to pancreatin digestion via several proteases (trypsin, chymotrypsin, elastase, carboxypeptidases A and B) (Barrett, Rawlings, and Woessner, 2004; Coffee, 1998). The potent amino acids at C-terminal (Arg, Trp, Phe, Tyr, and Leu) of some ACE inhibitory peptides could be liberated by carboxypeptidase A and B, resulting in inactive forms. The peptides may be classified as a substrate of ACE (Fujita, Yokoyama, and Yoshikawa, 2000), which they lost their ACE inhibitory activity after gastrointestinal digestion.

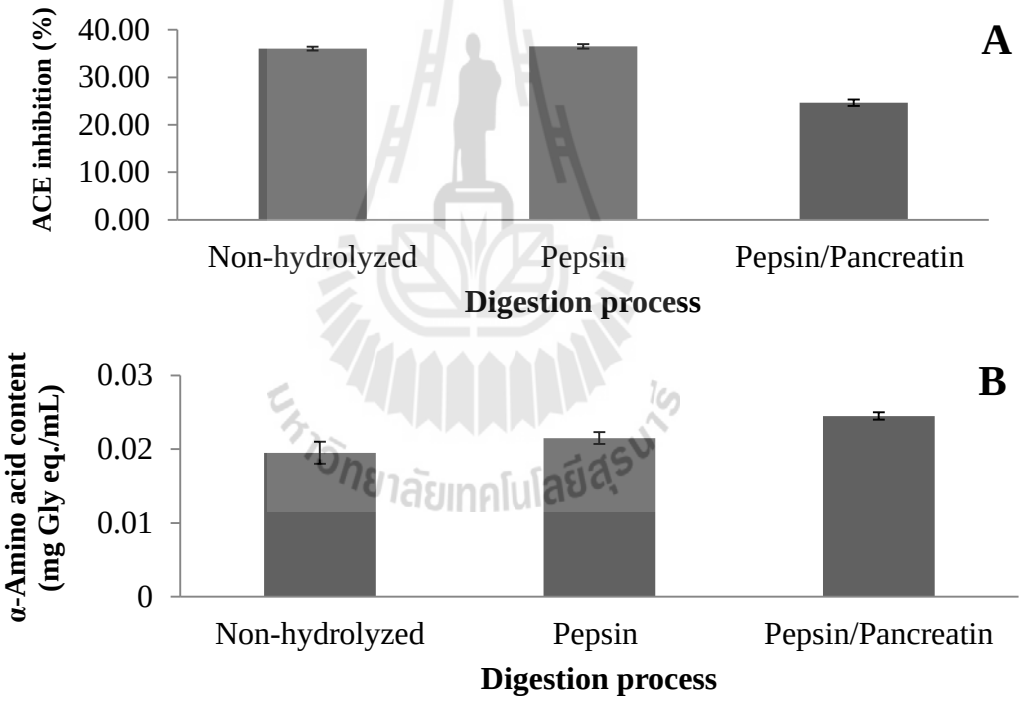

Figure 4.5 (A) α-Amino acid content (mg. Gly eq./mL) and (B) ACE inhibitory activity of the partially-purified peptides at the same peptide concentration (20 µg. Gly eq./mL) after incubation by pepsin for 3 h followed by pancreatin for 4 h.

CHAPTER V

SUMMARY

Thai Panga skin is a rich source of protein. Pretreatment of Thai Panga skin with butyl alcohol or alkaline-acid extraction increased protein content. Defatted and alkaline-acid pretreatred skin composed of type I collagen. Thermal extraction process of gelatin at 50^{o}C induced degradation of α1-chain into smaller peptide fragments. Defatted and alkaline-acid pretreated skin contained Asp, Glu/Gln and Ser higher than gelatin, while alkaline-acid pretreated skin and gelatin contained Pro higher than defatted skin. Enzymatic protein hydrolysates from Thai Panga skin showed potential to inhibit ACE when prepared using alkaline-acid pretreated skin and pepsin at 2 h of hydrolysis time. The consecutive fractionation of ultrafiltration, anion exchanger and size-exclusion chromatography could improve the purity approximately 5 folds as compared to crude hydrolysates. The partially-purified peptides showed IC_{50} of 19 µg Gly eq./mL with molecular mass between 0.7-1.5 kDa and composed of hydrophobic, aromatic amino acids or Arg at their C-terminal position. ACE inhibitory peptides from Thai Panga skin were classified as a substrate type ACE inhibitor as their ACE inhibitory activity decreased after a simulated *in vitro* peptic-pancreatic incubation. Production of ACE inhibitory peptides could be an efficient utilization of by-product from Thai Panga industry. To develop a functional food, the *in vivo* efficacy is essential to validate their role in lowering blood pressure.

REFERENCES

REFERENCES

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. **Journal of Agricultural and Food Chemistry.** 27: 1256-1262.

Agyei, D., and Danquah, M. K. (2011). Industrial-scale manufactering of pharmaceutical-grade bioactive peptides. **Biotechnology Advances.** 29: 272-277.

Ahhmed, A. M., and Muguruma, M. (2010). A review of meat protein hydrolysates and hypertension. **Meat Science.** 8: 110-118.

Ahmad, M., Benjakul, S., and Nalinanon, S. (2010). Compositional and physicochemical characteristics of acid solubilized collagen extracted from the skin of unicorn leatherjacket (*Aluterus monoceros*). **Food Hydrocolloids.** 24: 588-594.

Ahn, C. B., Jeon, Y. J., Kim, Y. T., and Je, J. Y. (2012). Angiotensin I converting enzyme (ACE) inhibitory peptides from salmon byproduct protein hydrolysate by Alcalase hydrolysis. **Process Biochemistry.** 47: 2240-2245.

Albert-Weißenbergera, C., Sirén, A. L., and Kleinschnitz, C. (2013). Ischemic stroke and traumatic brain injury: The role of the kallikrein-kinin system. **Progress in Neurobiology.** 101-102: 65-82.

Alemán, A., Giménez, B., Pérez-Santin, E., Gómez-Guillén, M. C., and Montero, P. (2011). Contribution of Leu and Hyp residues to antioxidant and ACE-

inhibitory activities of peptide sequences isolated from squid gelatin hydrolysate. **Food Chemistry.** 125: 334-341.

Alemán, A., Gómez-Guillén, M. C., and Montero, P. (2013). Identification of ace-inhibitory peptides from squid skin collagen after *in vitro* gastrointestinal digestion. **Food Research International.** 54: 790-795.

Alemána, A., Pérez-Santína, E., Bordenave-Juchereaub, S., Arnaudinb, I., Gómez-Guilléna, M. C., and Monteroa, P. (2011). Squid gelatin hydrolysates with antihypertensive, anticancer and antioxidant activity. **Food Research International.** 44: 1044-1051.

AOAC. (2000). **Official methods of analysis** (17th ed.). Gaithersberg, Maryland: Association of Official Chemists.

AOAC. (2006). **Official methods of analysis** (18th ed.). Gaithersberg, Maryland: Association of Official Chemists.

Arihara, K., Nakashima, Y., Mukai, T., Ishikawa, S., and Itoh, M. (2001). Peptide inhibitors for angiotensin I-converting enzyme from enzymatic hydrolysates of porcine skeletal muscle proteins. **Meat Science.** 57: 319-324.

Arunachalam, K., Amirtham, R., and Appadorai, J. (2013). Antioxidant potential and biochemical evaluation of metabolites from the marine bacteria *Virgibacillus sp*. associated with the sponge Callyspongia diffusa. **Free Radicals and Antioxidants.** 3: 47-51.

Asghar, A., and Henrickson, R. L. (1982). Chemical, Biochemical, Functional, and Nutritional Characteristics of Collagen in Food Systems. **Advances in Food Research.** 28: 231-372.

Asoodeha, A., Haghighib, L., Chamanib, J., Ansari-Ogholbeykb, M. A., Mojallal-Tabatabaeia, Z., and Lagzianc, M. (2014). Potential angiotensin I converting enzyme inhibitory peptides from gluten hydrolysate: Biochemical characterization and molecular docking study. **Journal of Cereal Science.** 60: 92-98.

Asoodeh, A., Yazdi, M. M., and Chamani, J. (2012). Purification and characterisation of angiotensin I converting enzyme inhibitory peptides from lysozyme hydrolysates. **Food Chemistry.** 131: 291-295.

Atlas, S. A. (2007). The Renin-Angiotensin Aldosterone System: Pathophysiological Role and Pharmacologic Inhibition. **Journal of Managed Care Pharmacy.** 13: 9-20.

Balti, R., Bougatef, A., Sila, A., Guillochon, D., Dhulster, P., and Nedjar-Arroume, N. (2015). Nine novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish *(Sepia officinalis)* muscle protein hydrolysates and antihypertensive effect of the potent active peptide in spontaneously hypertensive rats. **Food Chemistry.** 170: 519-525.

Balti, R., Nedjar-Arroume, N., Bougatef, A., Guillochon, D., and Nasri, M. (2010). Three novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (*Sepia officinalis*) using digestive proteases. **Food Research International.** 43: 1136-1143.

Banerjee, P., and Shanthi, C. (2012). Isolation of novel bioactive regions from bovine *Achilles* tendon collagen having angiotensin I-converting enzyme-inhibitory properties. **Process Biochemistry.** 47: 2335-2346.

Barbana, C., and Boye, J. I. (2010). Angiotensin I-converting enzyme inhibitory activity of chickpea and pea protein hydrolysates. **Food Research International.** 43: 1642-1649.

Barbana, C., and Boye, J. I. (2011). Angiotensin I-converting enzyme inhibitory properties of lentil protein hydrolysates: Determination of the kinetics of inhibition. **Food Chemistry.** 127: 94-101.

Barrett, A. J., Rawlings, N. D., and Woessner, J. F. (2004). **Handbook of proteolytic enzymes** (Vol. 2). London: Elsevier Academic Press.

Barrett, A. J., Rawlings, N. D., and Woessner, J. F. (2013). **Handbook of proteolytic enzymes** (Vol. 1). UK: Academic Press.

Barrett, K. E., Ghishan, F. K., Merchant, J. L., Said, H. M., and Wood, J. D. (2006). **Physiology of the gastrointestinal tract**. London: Academic Press.

Bernardini R. D., Mullen, A. M., Bolton, D., Kerry, J., O'Neill, E., and Hayes, M. (2012). Assessment of the angiotensin-I-converting enzyme (ACE-I) inhibitory and antioxidant activities of hydrolysates of bovine brisket sarcoplasmic proteins produced by papain and characterisation of associated bioactive peptidic fractions. **Meat Science.** 90: 226-235.

Bernstein, K. E., Ong, F. S., Blackwell, W. L., Shah, K. H., Giani, J. F., Gonzalez-Villalobos, R. A., Shen, X. Z., Fuchs, S., and Touyz, R. M. (2013). A Modern understanding of the traditional and nontraditional biological functions of angiotensin-converting enzyme. **Pharmacological Reviews.** 65: 1-46.

Bhuyan, B. J., and Mugesh, G. (2011). Angiotensin converting enzyme inhibitors inthe treatment of hypertension. **Current Science.** 101: 881-887.

Bidasolo, I. B., Ramos, M., and Gomez-Ruiz, J. A. (2012). *In vitro* simulated gastrointestinal digestion of donkeys' milk. Peptide characterization by high performance liquid chromatographyetandem mass spectrometry. **International Dairy Journal.** 24: 146-152.

Binsi, P. K., Shamasundar, B. A., Dileep, A. O., Badii, F., and Howell, N. K. (2009). Rheological and functional properties of gelatin from the skin of Bigeye snapper (*Priacanthus hamrur*) fish: Influence of gelatin on the gel-forming ability of fish mince. **Food Hydrocolloids.** 23: 132-145.

Boschin, G., Scigliuolo, G. M., Resta, D., and Arnoldi, A. (2014). ACE-inhibitory activity of enzymatic protein hydrolysates from lupin and other legumes. **Food Chemistry.** 145: 34-40.

Bougatef, A., Nedjar-Arroume, N., Ravallec-Plé, R., Leroy, Y., Guillochon, D., Barkia, A., and Nasri, M. (2008). Angiotensin I-converting enzyme (ACE) inhibitory activities of sardinelle (*Sardinella aurita*) by-products protein hydrolysates obtained by treatment with microbial and visceral fish serine proteases. **Food Chemistry.** 111: 350-356.

Boye, J. I., Roufik, S., Pesta, N., and Barbana, C. (2010). Angiotensin I-converting enzyme inhibitory properties and SDS-PAGE of red lentil protein hydrolysates. **LWT - Food Science and Technology.** 43: 987-991.

Brandelli, A., Daroit, D. J., and Corrêa, A. P. F. (2015). Whey as a source of peptides with remarkable biological activities. **Food Research International.** 73:149-161.

Butylina, S., Luque, S., and Nystrom, M. (2006). Fractionation of whey-derived peptides using a combination of ultrafiltration and nanofiltration. **Journal of Membrane Science.** 280: 418-426.

Byun, H. G., and Kim, S. K. (2001). Purification and characterization of angiotensin I converting enzyme (ACE) inhibitory peptides from Alaska pollack (*Theragra chalcogramma*) skin. **Process Biochemistry.** 36: 1155-1162.

Byun, H. G., and Kim, S. K. (2002). Structure and activity of angiotensin I converting enzyme inhibitory peptides derived from Alaskan pollack skin. **Journal of Biochemistry and Molecular Biology.** 35: 239-243.

Castellano, P., Aristoy, M. C., Sentandreu, M. Á., Vignolo, G., and Toldráb, F. (2013). Peptides with angiotensin I converting enzyme (ACE) inhibitory activity generated from porcine skeletal muscle proteins by the action of meat-borne Lactobacillus. **Journal of Proteomics.** 89: 183-190.

Cheison, S. C., Schmitt, M., Lee, E., Letzel, T., and Kulozikd, U. (2010). Influence of temperature and degree of hydrolysis on the peptide composition of trypsin hydrolysates of β-lactoglobulin: Analysis by LC–ESI-TOF/MS. **Food Chemistry.** 121: 457-467.

Chena, J., Liua, S., Yeb, R., Caia, G., Jic, B., and Wua, Y. (2013). Angiotensin-I converting enzyme (ACE) inhibitory tripeptides from rice protein hydrolysate: Purification and characterization. **Journal of Functional Food.** 5: 1684-1692.

Chen, J., Wang, Y., Zhong, Q., Wu, Y., and Xia, W. (2012). Purification and characterization of a novel angiotensin-I converting enzyme (ACE) inhibitory peptide derived from enzymatic hydrolysate of grass carp protein. **Peptides.** 33: 52-58.

Cheow, C. S., Norizah, M. S., Kyaw, Z. Y., and Howell, N. K. (2007). Preparation and characterisation of gelatins from the skins of sin croaker (*Johnius*

dussumieri) and shortfin scad *(Decapterus macrosoma)*. **Food Chemistry.** 101: 386-391.

Cheung, H. S., Wang, F. L., Ondetti, M. A., Sabo, E. F., and Cushman, D. W. (1980). Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the COOH-terminal dipeptide sequence. **The Journal of Biochemistry.** 255: 401-407.

Cho, J. K., Jin, Y. G., Rha, S. J., Kim, S. J., and Hwang, J. H. (2014). Biochemical characteristics of four marine fish skins in Korea. **Food Chemistry.** 159: 200-207.

Clemente, A. (2000). Enzymatic protein hydrolysates in human nutrition. **Trends in Food Science and Technology.** 11: 254-262.

Coffee, C. J. (1998). **Metabolism**. USA: Wiley-Blackwell.

Connolly, A., O'Keeffe, M. B., Piggott, C. O., Nongonierma, A. B., and FitzGerald, R. J. (2015). Generation and identification of angiotensin converting enzyme (ACE) inhibitory peptides from a brewers' spent grain protein isolate. **Food Chemistry.** 176: 64-71.

Contrerasa, F., de la Partea, A., Cabreraa, J., Ospinoa, N., Hernándezb, R., Hernández, J., Lezamaa, E., and Velascoa, M. (2002). Functional aspects of the renin-angiotensin-aldosterone system and angiotensin II receptor AT1 blockers in arterial hypertension. **International Congress Series.** 1237: 87-98.

Contreras, M. d. M., Carrón, R., Montero, M. J., Ramos, M., and Recio, I. (2009). Novel casein-derived peptides with antihypertensive activity. **International Dairy Journal.** 19: 566-573.

Contreras, M. d. M., Sanchez, D., Sevilla, M. Á., Recio, I., and Amigo, L. (2013). Resistance of casein-derived bioactive peptides to simulated gastrointestinal digestion**. International Dairy Journal.** 32: 71-78.

Cushman, D. W., and Cheng, H. S. (1971). Concentrations of angiotensin-converting enzyme in tissue rat. **Biochimica et Biophysica.** 250: 201-265.

Cushman, D. W., Cheung, H. S., Sabo, E. F., and Ondetti, M. A. (1977). Design of potent competitive inhibitors of angiotensin-converting enzyme. Carboxyalkanoyl and mercaptoalkanoyl amino acids. **Biochemistry.** 16: 5484-5491.

Cushman, D. W., and Ondetti, M. A. (1984). Angiotensin-converting enzyme inhibitors: biochemical properties and biological actions. **Critical Reviews in Biochemistry and Molecular Biology.** 16: 381-411.

Duan, R., Zhang, J., Xing, F., Konno, K., and Xu, B. (2011). Study on the properties of gelatins from skin of carp (*Cyprinus carpio*) caught in winter and summer season. **Food Hydrocolloids.** 25: 368-373.

Dub, L., Fangb, M., Wuc, H., Xiea, J., Wub, Y., Lib, P., Zhangb, D., Huangc, Z., Xiaa, Y., Zhoua, L., and Weia, D. (2013). A novel angiotensin I-converting enzyme inhibitory peptide from *Phascolosoma esculenta* water-soluble protein hydrolysate. **Journal of Functional Foods.** 5: 475-483.

Durak, A., Baraniak, B., Jakubczyk, A., and Wieca, M. S. (2013). Biologically active peptides obtained by enzymatic hydrolysis of Adzuki bean seeds. **Food Chemistry.** 141: 2177-2183.

Eckert, E., Zambrowicz, A., Pokora, M., Setner, B., Dąbrowska, A., Szołtysik, M., Szewczuk, Z., Polanowski, A., Trziszka, T., and Chrzanowska, J. (2014). Egg-

yolk protein by-product as a source of ACE-inhibitory peptides obtained with using unconventional proteinase from Asian pumpkin (*Cucurbita ficifolia*). **Journal of Proteomics.** 110: 107-116.

Ehlers, M. R. W., Fox, E. A., Strydom, D. J., and Riordan, J. F. (1989). Molecular cloning of human testicular angiotensin-converting enzyme: The testis isozyme is identical to the C-terminal half of endothelial angiotensin-converting enzyme. **Proceedings of the National Academy of Sciences.** 86: 7741-7745.

Erdmann, K., Cheung, B. W. Y., and Schröder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. **The Journal of Nutritional Biochemistry.** 19: 643-654.

Escudero, E., Mora, L., and Toldrá, F. (2014). Stability of ACE inhibitory ham peptides against heat treatment and *in vitro* digestion. **Food Chemistry**: 161 305-311.

Espejo-Carpio, F. J., Gobba, C. D., Guadix, A., Guadix, E. M., and Otte, J. (2013). Angiotensin I-converting enzyme inhibitory activity of enzymatic hydrolysates of goat milk protein fractions. **International Dairy Journal.** 32: 175-183.

Fahmia, A., Morimuraa, S., Guob, H. C., Shigematsua, T., Kidaa, K., and Uemurac, Y. (2004). Production of angiotensin I converting enzyme inhibitory peptides from sea bream scales. **Process Biochemistry.** 39: 1195-1200.

Food and Agriculture Organization of the United Nation. (2014). **Pangasius-November 2014** [On-line]. Available: http://www.globefish.org/pangasius-november-2014.html

Ferreira, S. H., Bartelt, D. C., and Greene, L. J. (1970). Isolation of bradykinin-potentiating peptides from *Bothrops jararaca* venom. **Biochemistry.** 13: 2583-2593.

Ferreiraa, I. M. P. L. V. O., Pinhoa, O., Motaa, M. V., Tavaresc, P., Pereirac, A., Gonçalvesd, M. P., Torresd, D., Rochae, C., and Teixeirae, J. A. (2007). Preparation of ingredients containing an ACE-inhibitory peptide by tryptic hydrolysis of whey protein concentrates. **International Dairy Journal.** 17: 481-487.

FitzGerald, R. J., and Meisel, H. (2000). Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. **British Journal of Nutrition.** 84: 33-37.

Fuchs, S., Xiao, H. D., Hubert, C., Michaud, A., Campbell, D. J., Adams, J. W., Capecchi, M. R., Corvol, P., and Bernstein, K. E. (2008). Angiotensin-converting enzyme C-terminal catalytic domain is the main site of angiotensin I cleavage *in vivo*. **Hypertension.** 51: 267-274.

Fujita, H., Eiichiyokoyama, K., and Yoshikawa, M. (2000). Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. **Journal of Food Science.** 65:564-569.

Fujita, H., Yamagami, T., and Ohshima, K. (2001). Effects of an ace-inhibitory agent, katsuobushi oligopeptide, in the spontaneously hypertensive rat and in borderline and mildly hypertensive subjects. **Nutrition Research.** 21: 1149-1158.

Ghassem, M., Arihara, K., Babji, A. S., Said, M., and Ibrahim, S. (2011). Purification and identification of ACE inhibitory peptides from Haruan (*Channa striatus*)

myofibrillar protein hydrolysate using HPLC-ESI-TOF MS/MS**. Food Chemistry.** 129: 1770-1777.

Girgiha, A. T., Hea, R., Malomoa, S., Offengenden M., Wub, J., and Alukoa, R. E. (2014). Structural and functional characterization of hemp seed (*Cannabis sativa* L.) protein-derived antioxidant and antihypertensive peptides. **Journal of Functional Foods.** 6: 384-394.

Gelatine Manufacturers of Europe. (2015). **Properties** [On-line]. Available: http://www.gelatine.org/about-gelatine/properties.html

Golias, C., Charalabopoulos, A., Stagikas, D., Charalabopoulos, K., and Batistatou, A. (2007). The kinin system-bradykinin: biological effects and clinical implications. Multiple role of the kinin system-bradykinin. **Hippokratia.** 11: 124-128.

Gómez-Guillén, M. C., Giménez, B., López-Caballero, M. E., and Montero, M. P. (2011). Functional and bioactive properties of collagen and gelatin from alternative sources: A review. **Food Hydrocolloids.** 25: 1813-1827.

Gorgieva, S., and Kokol, V. (2011). Collagen- vs. Gleatine-Based Biomaterials and Their Biocompatibility. In P. Rosario (ed.). **Biomaterials applications for nanomedicine** (pp 17-37). Rijeka: Intech.

Gu, R. Z., Li, C. Y., Liu, W. Y., Yi, W. X., and Cai, M. Y. (2011). Angiotensin I-converting enzyme inhibitory activity of low-molecular-weight peptides from Atlantic salmon (*Salmo salar* L.) skin. **Food Research International.** 44: 1536-1540.

Gu, Y., Majumder, K., and Wu, J. (2011). QSAR-aided *in silico* approach in evaluation of food proteins as precursors of ACE inhibitory peptides. **Food Research International.** 44: 2465-2474.

Gu, Y., and Wu, J. (2013). LC-MS/MS coupled with QSAR modeling in characterising of angiotensin I-converting enzyme inhibitory peptides from soybean proteins. **Food Chemistry.** 141: 2682-2690.

Guang, C., Phillips, R. D., Jiang, B., and Milani, F. (2012). Three key proteases angiotensin-I-converting enzyme (ACE), ACE2 and renin within and beyond the renin-angiotensin system. **Archives of Cardiovascular Disease.** 105: 373-385.

Guy, J. L., Lambert, D. W., Warner, F. J., Hooper, N. M., and Turner, A. J. (2005). Membrane-associated zinc peptidase families: Comparing ACE and ACE2. **Biochimica et Biophysica Acta.** 1751: 2-8.

Hammond, B. G., and Jez, J. M. (2011). Impact of food processing on the safety assessment for proteins introduced into biotechnology-derived soybean and corn crops. **Food and Chemical Toxicology.** 49: 711-721.

Harnedy, P. A., and FitzGerald, R. J. (2012). Bioactive peptides from marine processing waste and shellfish: A review. **Journal of Functional Foods.** 4: 6-24.

Hartmann, R., and Meisel, H. (2007). Food-derived peptides with biological activity: from research to food applications. **Current Opinion in Biotechnology.** 18: 163-169.

Hatachote, S, Sriphairoj, K., and Na-Nakorn, U., (2009). Performance of Striped catfish (*Pangasianodon hypophthalmus*), Pla mong (*Pangasius bocourti*) and their hybrid (*P. hypophthalmus* × *P. bocourti*). In **Proceedings of 48th Kasetsart University Annual Conference: Fisheries**. (pp 210-217). Bangkok: Kasetsart University.

Hatti-Kaul, R., and Mattiasson, B. (2003). **Isolation and purification of proteins.** New York: Marcel Dekker.

He, H. L., Chen, X. L., Wu, H., Sun, C. Y., Zhang, Y. Z., and Zhou, B. C. (2007). High throughput and rapid screening of marine protein hydrolysates enriched in peptides with angiotensin-I-converting enzyme inhibitory activity by capillary electrophoresis. **Bioresource Technology.** 98(18): 3499-3505.

He, H. L., Liu, D., and Ma, C. B. (2013). Review on the angiotensin-I-converting enzyme (ACE) inhibitor peptides from marine proteins. **Applied Biochemistry and Biotechnology.** 169:738-749.

He, H. L., Wu, H., Chen, X. L., Shi, M., Zhang, X. Y., Sun, C. Y., Zhang, Y. Z., and Zhou, B. C. (2008). Pilot and plant scaled production of ACE inhibitory hydrolysates from *Acetes chinensis* and its *in vivo* antihypertensive effect. **Bioresource Technology.** 99: 5956-5959.

He, R., Malomo, S. A., Alashi, A., Girgih, A. T., Ju, X., and Aluko, R. E., (2013). Purification and hypotensive activity of rapeseed protein-derived renin and angiotensin converting enzyme inhibitory peptides. **Journal of Functional Foods.** 5: 781-789.

He, S., Franco, C., and Zhang, W. (2013). Functions, applications and production of protein hydrolysates from fish processing co-products (FPCP). **Food Research International.** 50: 280-297.

Hernández-Ledesma, B., Contreras, M. d. M., and Recio, I. (2011). Antihypertensive peptides: Production, bioavailability and incorporation into foods. **Advances in Colloid and Interface Science.** 165: 23-35.

Himay, S. W. A., Ngo, D. H., Ryu, B., and Kim, S. K. (2012). An active peptide purified from gastrointestinal enzyme hydrolysate of Pacific cod skin gelatin attenuates angiotensin-1 converting enzyme (ACE) activity and cellular oxidative stress. **Food Chemistry.** 132: 1872-1882.

Hong, F., Ming, L., Yi, S., Zhanxia, L., Yongquan, W., and Chi, L. (2008). The antihypertensive effect of peptides: a novel alternative to drugs?. **Peptides.** 29: 1062-1071.

Hou, H., Fan, Y., Li, B., Xue, C., Yu, G., Zhang, Z., and Zhao, X. (2012). Purification and identification of immunomodulating peptides from enzymatic hydrolysates of Alaska pollock frame. **Food Chemistry.** 134: 821-828.

Hur, S. J., Lim, B. O., Decker, E. A., and McClements, J. (2011). *In vitro* human digestion models for food applications. **Food Chemistry.** 125: 1-12.

Hwang, J. S. (2010). Impact of processing on stability of angiotensin I-converting enzyme (ACE) inhibitory peptides obtained from tuna cooking juice. **Food Research International.** 43: 902-906.

Intarasirisawat, R., Benjakul, S., Wu, J., and Visessanguan, W. (2013). Isolation of antioxidative and ACE inhibitory peptides from protein hydrolysate of skipjack (*Katsuwana pelamis*) roe. **Journal of Functional Food.** 5: 1854-1862.

Jamilah, B., Tan, K. W., Hartin, M. R. U., and Azizah, A. (2011). Gelatins from three cultured freshwater fish skins obtained by liming process. **Food Hydrocolloids.** 25: 1256-1260.

Jang, A., and Lee, M. (2005). Purification and identification of angiotensin converting enzyme inhibitory peptides from beef hydrolysates. **Meat Science.** 69: 653-661.

Janson, J. C. (2011). **Protein purification** (Vol. 54). (3 ed.). New York: John Wiley and Sons.

Jao, C. L., Huang, S. L., and Hsu, K. C. (2012). Angiotensin I-converting enzyme inhibitory peptides: Inhibition mode, bioavailability, and antihypertensive effects. **Biomedicine.** 2:130-136.

Jellouli, K., Balti, R., Bougatef, A., Hmidet, N., Barkia, A., and Nasri, M. (2011). Chemical composition and characteristics of skin gelatin from grey triggerfish (*Balistes capriscus*). **LWT- Food Science and Technology.** 44: 1965-1970.

Jensen, I. J., Eysturskarð, J., Madetoja, M., and Eilertsen, K. E. (2014). The potential of cod hydrolyzate to inhibit blood pressure in spontaneously hypertensive rats. **Nutrition Research.** 34: 168-173.

Jia, J., Ma, H., Zhao, W., Wang, Z., Tian, W., Luo, L., and He, R. (2010). The use of ultrasound for enzymatic preparation of ACE-inhibitory peptides from wheat germ protein. **Food Chemistry.** 119: 336-342.

Jia, J., Wu, Q., Yan, H., and Gui, Z. (2015). Purification and molecular docking study of a novel angiotensin-I converting enzyme (ACE) inhibitory peptide from alcalase hydrolysate of ultrasonic-pretreated silkworm pupa (*Bombyx mori*) protein. **Process Biochemistry.** 50: 876-883.

Jiang, Z., Tian, B., Brodkor, A., and Huo, G. (2010). Production, analysis and *in vivo* evaluation of novel angiotensin-I-converting enzyme inhibitory peptides from bovine casein. **Food Chemistry.** 123: 779-786.

Jimsheena, V. K., and Gowda, L. R. (2010). Arachin derived peptides as selective angiotensin I-converting enzyme (ACE) inhibitors: Structure-activity relationship. **Peptides.** 31: 1165-1176.

Jimsheena, V. K., and Gowda, L. R. (2011). Angiotensin I-converting enzyme (ACE) inhibitory peptides derived from arachin by simulated gastric digestion. **Food Chemistry.** 125: 561-569.

Jongjareonrak, A., Rawdkuen, S., Chaijan, M., Benjakul, S., Osako, K., and Tanaka, M. (2010). Chemical compositions and characterisation of skin gelatin from farmed giant catfish (*Pangasianodon gigas*). **LWT-Food Science and Technology.** 43: 161-165.

Jung, W. Y., Mendis, E., Je, J. Y., Park, P. J., Wha Son, B. W., Chin Kim, H. C., Choi, Y. K., and Kim, S. K. Angiotensin I-converting enzyme inhibitory peptide from yellowfin sole (*Limanda aspera*) frame protein and its antihypertensive effect in spontaneously hypertensive rats. **Food Chemistry.** 94: 26-32.

Kaewla, S., Arpakulanu, U., Chumnongsittathum, B., and Nilsri, A. (2011). **Production of Thai Panga (*Pangasius hypophthamus* × *Pangasius bocourti*) fry with different densities in water recirculation system** [On-line]. Available: http://www.inlandfisheries.go.th/research/files/full/F42554.pdf

Karim, A., and Bhat, R. (2009). Fish gelatin: Properties, challenges, and prospects as an alternative to mammalian gelatins. **Food Hydrocolloids.** 23: 563-576.

Kasankal, L. M., Xue, Y., Weilong, Y., Hong, S. D., and He, Q. (2007). Optimization of gelatine extraction from grass carp (*Catenopharyngodon idella*) fish skin by response surface methodology. **Bioresource Technology.** 98: 3338-3343.

Kearney, P. M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P. K., and He, J. (2005). Global burden of hypertension: analysis of worldwide data. **The Lancet.** 365: 217-223.

Kim, S. K., and Wijesekara, I. (2010). Development and biological activities of marine-derived bioactive peptides: A review. **Journal of Functional Foods.** 2: 1-9.

Kinenen, S. M., Johannson, T., Gerd, V., Pihlava, J. M., and Pihlanto, A. (2012). Angiotensin I-converting enzyme inhibitory and antioxidant properties of rapeseed hydrolysates. **Journal of Functional Foods.** 4: 575-583.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Kishimura, H., and Shahidi, F. (2010). Isolation and Characterisation of collagen from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*). **Food Chemistry.** 119: 1519-1526.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., and Shahidi, F. (2010). Comparative study on characteristics of gelatin from the skins of brownbanded bamboo shark and blacktip shark as affected by extraction conditions. **Food Hydrocolloids.** 24: 164-171.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., and Shahidi, F. (2013). Inhibition of angiotensin converting enzyme, human LDL cholesterol and DNA oxidation by hydrolysates from blacktip shark gelatin. **LWT - Food Science and Technology.** 51: 177-182.

Kloet, A. D., Krause, E. G., and Woods, S. C. (2010). The renin angiotensin system and the metabolic syndrome. **Physiology and Behavior.** 100: 525-534.

Koa, S. C., Kang, N., Kim, E. A., Kang, M. C., Lee, S. H., Kang, S. M., Lee, J. B., Jeon, B. T., Kim, S. K., Park, S. J., Park, P. J., Jung, W. K., Kim, D., and Jeon, Y. J. (2012). A novel angiotensin I-converting enzyme (ACE) inhibitory peptide from a marine Chlorella ellipsoidea and its antihypertensive effect in spontaneously hypertensive rats. **Process Biochemistry.** 47: 2005-2011.

Kongo-Dia-Moukala, J. U., Zhang, H., and Irakoze, P. C. (2011). *In vitro* Binding Capacity of Bile Acids by Defatted Corn Protein Hydrolysate. **International Journal of Molecular Sciences.** 12: 1066-1080.

Korhonen, H., and Pihlanto, A. (2006). Bioactive peptides: Production and functionality. **International Dairy Journal.** 16: 945-960.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the hHead of bacteriophage T4. **Nature.** 227: 680-685.

Lafarga, T., and Hayes, M. (2014). Bioactive peptides from meat muscle and by-products: generation, functionality and application as functional ingredients. **Meat Science.** 98: 227-239.

Lapsongphon, N., and Yongsawatdigul, J. (2013). Production and purification of antioxidant peptides from a mungbean meal hydrolysate by *Virgibacillus sp.* SK37 proteinase. **Food Chemistry.** 141: 992-999.

Lassoued, I., Jridi, M., Nasri, R., Dammak, A., Hajji, M., Nasri, M., and Barkia, A. (2014). Characteristics and functional properties of gelatin from thornback ray skin obtained by pepsin-aided process in comparison with commercial halal bovine gelatin. **Food Hydrocolloids.** 41: 309-318.

Lau, C. C., Abdullah, N., Shui, A. S., and Aminudin, N. (2014). Novel angiotensin I-converting enzyme inhibitory peptides derived from edible mushroom

Agaricus bisporus (J. E. Lange) Imbach identified by LC-MS/MS. **Food Chemistry.** 148: 396-401.

Lee, J. K., Hong, S., Jeon, J. K., Kim, S. K., and Byun, H. G. (2009). Purification and characterization of angiotensin I converting enzyme inhibitory peptides from the rotifer, Brachionus rotundiformis. **Bioresource Technology.** 100: 5255-5259.

Lee, J. K., Jeon, J. K., and Byun, H. G. (2011). Effect of angiotensin I converting enzyme inhibitory peptide purified from skate skin hydrolysate. **Food Chemistry.** 125: 495-499.

Lee, J. K., Jeon, J. K., and Byun, H. G. (2014). Antihypertensive effect of novel angiotensin I converting enzyme inhibitory peptide from chum salmon (*Oncorhynchus keta*) skin in spontaneously hypertensive rats. **Journal of Functional Food.** 7: 381-389.

Lee, S. H., Qian, Z. J., and Kim, S. K. (2010). A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. **Food Chemistry.** 118: 96-102.

Lee, S. J., Kim, K. H., Kim, Y. S., Kim, E. K., Hwang, J. W., Lim, B. O., Moon, S. H., Jeon, B. T., Jeon, Y. J., Ahn, C. B., and Park, P. J. (2012). Biological activity from the gelatin hydrolysates of duck skin by-products. **Process Biochemistry.** 47:1150-1154.

Li-Chan, E. C. (2015). Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients. **Current Opinion in Food Science.** 1: 28-37.

Li, G. H., Leemail, G. W., Shi, Y. H., and Shrestha, S. (2004). Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. **Nutrition Research.** 24: 469-486.

Lia, P., Jiab, J., Fanga, M., Zhanga, L., Guoa, M., Xiea, J., Xiaa, Y., Zhoua, L., and Weia, D. (2014). *In vitro* and *in vivo* ACE inhibitory of pistachio hydrolysates and in silico mechanism of identified peptide binding with ACE. **Process Biochemistry.** 49: 898-904.

Lin, H. C., Chou, S. T., Chuang, M. Y., Liao, T.Y., Tsai, W. S., and Chiu, T. H. (2012). The effects of *Caulerpa microphysa* enzyme-digested extracts on ACE-inhibitory activity and *in vitro* anti-tumour properties. **Food Chemistry.** 134: 2235-2241.

Lin, L., Lv, S., and Li, B. (2012). Angiotensin-I-converting enzyme (ACE)-inhibitory and antihypertensive properties of squid skin gelatin hydrolysates. **Food Chemistry.** 131: 225-230.

Liu, H., Li, D., and Guo, S. (2007). Studies on collagen from the skin of channel catfish (*Ictalurus punctaus*). **Food Chemistry.** 101: 621-625.

Liu, J. B., Yu, Z. P., Zhao, W. Z., Lin, S. Y., Wang, E. L., Zhang, Y., Hao, H., Wang, Z. Z., and Chen, F. (2010). Isolation and identification of angiotensin-converting enzyme inhibitory peptides from egg white protein hydrolysates. **Food Chemistry.** 122:1159-1163.

Liu, X., Zhang, M., Jia, A., Zhang, Y., Zhu, H., Zhang, C., Sun, Z., and Liu, C. (2013). Purification and characterization of angiotensin I converting enzyme inhibitory peptides from jellyfish *Rhopilema esculentum*. **Food Research International.** 50: 339-343.

Liu, X., Zhang, M., Zhang, C., and Liu, C. (2012). Angiotensin converting enzyme (ACE) inhibitory, antihypertensive and antihyperlipidaemic activities of protein hydrolysates from *Rhopilema esculentum*. **Food Chemistry.** 134: 2134-2121.

Liu, X., Zhang, M., Zhang, C., and Liu, C. (2012). Angiotensin converting enzyme (ACE) inhibitory, antihypertensive and antihyperlipidaemic activities of protein hydrolysates from *Rhopilema esculentum*. **Food Chemistry.** 134: 2134-2140.

Ma, H., Huang, L., Peng, L., Wang, Z., and Yang, Q. (2015). Pretreatment of garlic powder using sweep frequency ultrasound and single frequency countercurrent ultrasound: Optimization and comparison for ACE inhibitory activities. **Ultrasonics Sonochemistry.** 23: 109-115.

Majumder, K., and Wu, J. (2011). Purification and characterisation of angiotensin I converting enzyme (ACE) inhibitory peptides derived from enzymatic hydrolysate of ovotransferrin. **Food Chemistry.** 126: 1614-1619.

Manee, N., and Tangprakhon, T. (2008). **Nursing of Thai Panga (*Pangasius hypophthalmus* × *Pangasius bocourti*) fry in cage at different stocking densities** [On-line]. Available: http://www.inlandfisheries.go.th/research/files /full/F392551.pdf

Mao, X. Y., Ni, J. R., Sun, W. L., Hao, P. P., and Fan, L. (2007). Value-added utilization of yak milk casein for the production of angiotensin-I-converting enzyme inhibitory peptides. **Food Chemistry.** 103: 1282-1287.

Marczak, E. D., Usui, H., Fujita, H., Yang, Y., Yokoo, M., Lipkowski, A. W., and Yoshikawa, M. (2003). New antihypertensive peptides isolated from rapeseed. **Peptides.** 24: 791-798.

Megías, C., Pedroche, J., del Mar Yust, M., Alaiz, M., Girón-Calle, J., Millán, F., and Vioque, J. (2009). Purification of angiotensin converting enzyme inhibitory peptides from sunflower protein hydrolysates by reverse-phase chromatography following affinity purification. **LWT - Food Science and Technology.** 42: 228-232.

Mentz, R. J., Bakris, G. L., Waeber, B., McMurray, J. J., Gheorghiade, M., Ruilope, L. M., Maggioni, A. P., Swedberg, K., Piña, I. L., Fiuzat, M., O'Connor, C. M., Zannad, F., and Pitt, B. (2013). The past, present and future of renin-angiotensin aldosterone system inhibition. **International Journal of Cardiology.** 167: 1677-1687.

Miguel, M., Aleixandre, M. A., Ramos, M., and López-Fandiño, R. (2006). Effect of Simulated Gastrointestinal Digestion on the Antihypertensive Properties of ACE-Inhibitory Peptides Derived from Ovalbumin. **Journal of Agricultural and Food Chemistry.** 54: 726-731.

Miguel, M., Alonso, M. a. J., Salaices, M., Aleixandre, A., and Lo´pez-Fandin˜o, R. (2007). Antihypertensive, ACE-inhibitory and vasodilator properties of an egg white hydrolysate: Effect of a simulated intestinal digestion. **Food Chemistry.** 104: 163-168.

Millsa, S., Ross, R. P., Hill, C., Fitzgerald, G. F., and Stanton, C. (2011). Milk intelligence: Mining milk for bioactive substances associated with human health. **International Dairy Journal.** 21: 377-401.

Montriwong, A., Kaewphuak, S., Rodtong, S., Roytrakul, S., and Yongsawatdigul, J. (2012). Novel fibrinolytic enzymes from *Virgibacillus halodenitrificans* SK1-3-7 isolated from fish sauce fermentation. **Process Biochemistry.** 47: 2379-2387.

Morimur, S., Nagata, H., Uemura, Y., Fahmi, A., Shigematsu, T., and Kida, K. (2002). Development of an effective process for utilization of collagen from livestock and fish waste. **Process Biochemistry.** 37: 1403-1412.

Muguruma, M., Ahhmed, A.M., Katayam, K., Kawahara, S., Maruyama, M., and Nakamura, T. (2009). Identification of pro-drug type ACE inhibitory peptide sourced from porcine myosin B: Evaluation of its antihypertensive effects *in vivo*. **Food Chemistry.** 114: 516-522.

Murray, B. A., and FitzGerald, R. J. (2007). Angiotensin Converting Enzyme Inhibitory Peptides Derived from Food Proteins: Biochemistry, Bioactivity and Production. **Current Pharmaceutical Design.** 19: 773-791.

Muyonga, J. H., Cole, C. G. B., and Duodu, K. G. (2004). Extraction and physico-chemical characterisation of Nile perch (*Lates niloticus*) skin and bone gelatin. **Food Hydrocolloids.** 18: 581-592.

Nagai, T., Araki, Y., and Suzukia, N. (2002). Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*). **Food Chemistry.** 78: 173-177.

Nagai, T., and Suzuki, N. (2002). Preparation and partial characterization of collagen from paper nautilus (*Argonauta argo, Linnaeus*) outer skin. **Food Chemistry.** 76: 149-153.

Nagai, T., Yamashita, E., Taniguchi, K., Kanamori, N., and Suzuki, N. (2001). Isolation and characterisation of collagen from the outer skin waste material of cuttlefish (*Sepia lycidas*). **Food Chemistry.** 72: 425-429.

Nagarajan, M., Benjakul, S., Prodpran, T., Songtipya, P., and Kishimura, H. (2012). Characteristics and functional properties of gelatin from splendid squid (*Loligo formosana*) skin as affected by extraction temperatures. **Food Hydrocolloids.** 29: 389-397.

Nakajima, K., Yoshie-Starka, Y., and Ogushi, M. (2009). Comparison of ACE inhibitory and DPPH radical scavenging activities of fish muscle hydrolysates. **Food Chemistry.** 114: 844-851.

Nasri, R., Chataigné, G., Bougatef, A., Chaâbouni, M. K., Dhulster, P., Nasri, M., and Nedjar-Arroume, N. (2013). Novel angiotensin I-converting enzyme inhibitory peptides from enzymatic hydrolysates of goby (*Zosterisessor ophiocephalus*) muscle proteins. **Journal of Proteomics.** 91: 444-452.

Natesh, R., Schwager, S. L. U., Sturrock, E. D., and Acharya, K. R. (2003). Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. **Nature.** 421: 551-554.

Ng, K. K. F., and Vane, J. R. (1970). Some Properties of Angiotensin Converting Enzyme in the Lung *in vivo*. **Nature.** 225:1142 - 1144.

Ngo, D. H., Ryu, B., and Kim, S. K. (2014). Active peptides from skate (*Okamejei kenojei*) skin gelatin diminish angiotensin-I converting enzyme activity and intracellular free radical-mediated oxidation. **Food Chemistry.** 143: 246-255.

Ngo, D. H., Ryu, B., Vo, T. S., Himaya, S. W. A., and Kim, I. W. K. (2011). Free radical scavenging and angiotensin-I converting enzyme inhibitory peptides from Pacific cod (*Gadus macrocephalus*) skin gelatin. **International Journal of Biological Macromolecules.** 49: 1110-1116.

Nikoo, M., Benjakul, S., Bashari, M., Alekhorshied, M., Cissouma, A. I., Yang, N., and Xu, X. (2014). Physicochemical properties of skin gelatin from farmed

Amur sturgeon (*Acipenser schrenckii*) as influenced byacidpretreatment. **Food Bioscience.** 5: 19-26.

Olsen, R. L., Toppe, J., and Karunasagar, I. (2014). Challenges and realistic opportunities in the use of by-products from processing of fish and shellfish. **Trends in Food Science and Technology.** 36: 144-151.

Ondetti, M. A., and Cushman, D. W. (1982). Enzymes of the Renin-Angiotensin System and their Inhibitors. **Annual Review of Biochemistry.** 51: 283-308.

Onuh, J. O., Girgih, A. T., Aluko, R. E., and Aliani, M. (2013). Inhibitions of renin and angiotensin converting enzyme activities by enzymatic chicken skin protein hydrolysates. **Food Research International.** 53: 260-267.

Ortiz-Chao, P., Gómez-Ruiz, J. A., Rastall, R. A., Mills, D., Cramer, R., Pihlanto, A., Korhonen, H., and Jauregi, P. (2009) Production of novel ACE inhibitory peptides from β-lactoglobulin using Protease N Amano. **International Dairy Journal**. 19(2): 69-76.

Otte, J., Shalaby, S. M., Zakola, M., Pripp, A. H., and El-Shabrawy, S. A. (2007). Angiotensin-converting enzyme inhibitory activity of milk protein hydrolysates: Effect of substrate, enzyme and time of hydrolysis. **International Dairy Journal.** 17: 488-503.

Pan, D., Cao, J., Guo, H., and Zhao, B. (2012). Studies on purification and the molecular mechanism of a novel ACE inhibitory peptide from whey protein hydrolysate. **Food Chemistry.** 130: 121-126.

Pokora, M., Zambrowicz, A., Dąbrowska, A., Eckert, E., Setner, B., Szołtysik, M., Szewczuk, Z., Zabłocka, A., Polanowski, A., Trziszka, T., and Chrzanowska, J. (2014). An attractive way of egg white protein by-product use for producing of novel anti-hypertensive peptides. **Food Chemistry.** 151: 500- 505.

Power, O., Fernández, A., Norris, R., Riera, F. A., and FitzGerald, R. J. (2014). Selective enrichment of bioactive properties during ultrafiltration of a tryptic digest of β-lactoglobulin. **Journal of Functional Food.** 9: 38-47.

Qian, Z. J., Je, J. Y., and Kim, S. K. (2007). Antihypertensive Effect of Angiotensin I Converting Enzyme-Inhibitory Peptide from Hydrolysates of Bigeye Tuna Dark Muscle, Thunnus obesus. **Journal of Agricultural Food and Food Chemistry.** 21: 8398-8403.

Qian, Z. J., Jung, W. K., Lee, S. H., Byun, H. G., and Kim, S. K. (2007). Antihypertensive effect of an angiotensin I-converting enzyme inhibitory peptide from bullfrog (*Rana catesbeiana Shaw*) muscle protein in spontaneously hypertensive rats. **Process Biochemistry.** 42: 1443-1448.

Qu, W., Ma, H., Pan, Z., Luo, L., Wang, Z., and He, R. (2010). Preparation and antihypertensive activity of peptides from *Porphyra yezoensis*. **Food Chemistry.** 123: 14-20.

Qu, W., Ma, H., Zhao, W., and Pan, Z. (2013). ACE-inhibitory peptides production from defatted wheat germ protein by continuous coupling of enzymatic hydrolysis and membrane separation: Modeling and experimental studies. **Chemical Engineering Journal.** 226: 139-145.

Quiros, A., Contreras, M., Ramos, M., Amigo, L., and Recio, I. (2009). Stability to gastrointestinal enzymes and structure–activity relationship of β-casein-peptides with antihypertensive properties. **Peptides.** 30: 1848-1853.

Quist, E. E., Phillips, R. D., and Saalia, F. K. (2009). Angiotensin converting enzyme inhibitory activity of proteolytic digests of peanut (*Arachis hypogaea* L.) flour. **LWT - Food Science and Technology.** 42: 694-699.

Raghavan, S., and Kristinsson, H. G. (2009). ACE-inhibitory activity of tilapia protein hydrolysates. **Food Chemistry.** 117: 582-588

Rao, S. Q., Ju, T., Sun, J., Su, Y. J., Xu, R. R., and Yang, Y. J. (2012). Purification and characterization of angiotensin I-converting enzyme inhibitory peptides from enzymatic hydrolysate of hen egg white lysozyme. **Food Research International.** 46: 127-134.

Rao, S., Liu, S., Ju, T., Xu, W., Mei, G., and Xu, Y. (2012). Design of substrate-type ACE inhibitory pentapeptides with an antepenultimate C-terminal proline for efficient release of inhibitory activity. **Biochemical Engineering Journal.** 60: 50-55.

Rawendra, R. D., Aisha, Chang, C. I., Aulanni'am, Chen, H. H., Huang, T. C., and Hsu, J. L. (2013). A novel angiotensin converting enzyme inhibitory peptide derived from proteolytic digest of Chinese soft-shelled turtle egg white proteins. **Journal of Proteomics.** 94: 359-369.

Regoli, D., and Jr., F. G. (2015). Critical insights into the beneficial and protective actions of the kallikrein–kinin system. **Vascular Pharmacology.** 64: 1-10.

Regulska, K., Stanisz, B., Regulski, M., and Murias, M. (2014). How to design a potent, specific, and stable angiotensin-converting enzyme inhibitor. **Drug Discovery Today.** 11: 1731-1743.

Rioli, V., Gozzo, F. C., Heimann, A. S., Linardi, A., Krieger, J. E., Shida, C. S., Almeida, P. C., Hyslop, S., Eberlin, M. N., and Ferro, E. S. (2003). Novel natural peptide substrates for endopeptidase 24.15, neurolysin, and angiotensin-converting enzyme. **Journal of Biological Chemistry.** 7: 8547-8555.

Riordan, J. F. (2003). Angiotensin-I-converting enzyme and its relatives. **Genome Biology.** 4: 225.221-225.225.

Rohit, A. C., Sathisha, K., and Aparna, H. S. (2012). A variant peptide of buffalo colostrum β-lactoglobulin inhibits angiotensin I-converting enzyme activity. **European Journal of Medicinal Chemistry.** 53: 211-219.

Roufik, S., Gauthier, S. F., and Turgeon, S. L. (2006). *In vitro* digestibility of bioactive peptides derived from bovine β-lactoglobulin. **International Dairy Journal.** 16: 294-302.

Rui, X., Boye, J. I., Simpson, B. K., and Prasher, S. O. (2012). Angiotensin I-converting enzyme inhibitory properties of *Phaseolus vulgaris* bean hydrolysates: Effects of different thermal and enzymatic digestion treatments. **Food Research International.** 49: 739-746.

Rui, X., Boye, J. I., Simpson, B. K., and Prasher, S. O. (2013). Purification and characterization of angiotensin I-converting enzyme inhibitory peptides of small red bean (*Phaseolus vulgaris*) hydrolysates. **Journal of Functional Foods.** 5:1116-1124.

Ruiz-Giménez, P., Salom, J. B., Marcos, J. F., Vallés, S., Martínez-Maqueda, D., Recio I., Torregrosab, G., Alborchb, E., and Manzanaresa, P. (2012). Antihypertensive effect of a bovine lactoferrin pepsin hydrolysate: Identification of novel active peptides. **Food Chemistry.** 131: 266-273.

Salampessy, J., Reddy, N., Kailasapathy, K., and Phillips, M. (2015). Functional and potential therapeutic ACE-inhibitory peptides derived from bromelain hydrolysis of trevally proteins. **Journal of Functional Food.** 14: 716-725.

Saxena, P., Thompson, P., d'Udekem, Y., and Konstantinov, I. E. (2011). Kallikrein-kinin system: A surgical perspective in post-aprotinin era. **Journal of Surginal Research.** 167: 70-77.

Sayer, G., and Bhat, G. (2014). The renin-angiotensin-aldosterone system and heart failure. **Cardiology Clinics.** 32: 21-32.

Segura-Campos, M. R., Chel-Guerrero, L. A., and Betancur-Ancona, D. A. (2011). Purification of angiotensin I-converting enzyme inhibitory peptides from a cowpea (*Vigna unguiculata*) enzymatic hydrolysate. **Process Biochemistry.** 46: 864-872.

Segura-Campos, M. R., Salazar-Vega, I. M., Chel-Guerrero L. A., and Betancur-Ancona, D. A. (2013). Biological potential of chia (*Salvia hispanica* L.) protein hydrolysates and their incorporation into functional foods. **LWT - Food Science and Technology.** 50: 723-731.

Sheih, I. C., Fang, T. J., and Wu, T. K. (2009). Isolation and characterisation of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from the algae protein waste. **Food Chemistry.** 115: 279-284.

Shoulders, M. D., and Raines, R. T. (2009). Collagen structure and stability. **Annual Review of Biochemistry.** 78: 929-958.

Sica, D. A. (2010). The evolution of renin-angiotensin blockade: angiotensin-converting enzyme inhibitors as the starting point. **Current Hypertension Reports.** 12: 67-73.

Sila, A., Martinez-Alvarez, O., Haddar, A., Gómez-Guillén, M. C., Nasri, M., Montero, M. P., and Bougatef, A. (2015). Recovery, viscoelastic and functional properties of Barbel skin gelatine: investigation of anti-DPP-IV and

anti-prolyl endopeptidase activities of generated gelatine polypeptides. **Food Chemistry.** 168: 478-486.

Silva, R. S. G., Bandeira, S. F., and Pinto, L. A. A. (2014). Characteristics and chemical composition of skins gelatin from cobia (*Rachycentron canadum*). **LWT - Food Science and Technology.** 57: 580-585.

Silverman, R. B., and Holladay, M. W. (2015). **The organic chemistry of drug design and drug action**. USA: Academic Press.

Singh, P., Benjakul, S., Maqsood, S., and Kishimura, H. (2011). Isolation and characterisation of collagen extracted from the skin of striped catfish (*Pangasianodon hypophthalmus*). **Food Chemistry.** 124: 97-105.

Sinsuwan, S., Rodtong, S., and Yongsawatdigul, J. (2010). Purification and characterization of a salt-activated and organic solvent-stable heterotrimer proteinase from *virgibacillus sp. SK33* isolated from Thai fish sauce. **Journal of Agricultural Food and Food Chemistry.** 58: 248-256.

Sinthusamran, S., Benjakul, S., and Kishimura, H. (2014). Characteristics and gel properties of gelatin from skin of seabass (*Lates calcarifer*) as influenced by extraction conditions. **Food Chemistry.** 152: 276-284.

Siow, H. L., and Gan, C. Y. (2013). Extraction of antioxidative and antihypertensive bioactive peptides from Parkia speciosa seeds. **Food Chemistry.** 141: 3435-3442.

Skierka, E. Z., and Sadowska, M. (2007). The influence of different acids and pepsin on the extractability of collagen from the skin of Baltic cod (*Gadus morhua*). **Food Chemistry.** 105: 1302-1306.

Spellman, D., McEvoy, E., O'Cuinn, G., and FitzGerald, R. J. (2003). Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. **International Dairy Journal.** 13: 447-453.

Stressler, T., Eisele, T., and Fischer, L. (2013). Simultaneous monitoring of twelve angiotensin I converting enzyme inhibitory peptides during enzymatic β-casein hydrolysis using lactobacillus peptidases. **International Dairy Journal.** 30: 96-102.

Sturrock, E. D., Natesh, R., Rooyen, J. M. v., and Acharya, K. R. (2004). Structure of angiotensin I-converting enzyme. **Cellular and Molecular Life Sciences.** 61: 2677-2686.

Tanzadehpanah, H., Asoodeh, A., Saberi, M. R., and Chamani, J. (2013). Identification of a novel angiotensin-I converting enzyme inhibitory peptide from ostrich egg white and studying its interactions with the enzyme. **Innovative Food Science and Emerging Technologies.** 18: 212-219.

Thi, H., Sylla, K. S. B., Randriamahatody, Z., Donnay-Moreno, C., Moreau, J., Tran, L. T., and Bergé, J. P. (2011). Enzymatic hydrolysis of yellowfin tuna (*Thunnus albacares*) by-products using protamex protease. **Food Technology Biotechnology.** 49: 48-55.

Thipbharos, P. (2014). Reduction of earthy-flavour and Geosmin content in frozen Thai Panga fish (*Pangasius sp*.) fillets. **KKU Science Journal.** 42: 380-392.

Tikellis, C., Cooper, M. E., and Thomas, M. C. (2006). Role of the renin–angiotensin system in the endocrine pancreas: Implications for the development of

diabetes. **The International Journal of Biochemistry and Cell Biology.** 38: 737-751.

Tomatsu, M., Shimakage, A., Shinbo, M., Yamada, S., and Takahashi, S. (2013). Novel angiotensin I-converting enzyme inhibitory peptides derived from soya milk. **Food Chemistry.** 136: 612-616.

Toopcham, T., Roytrakul, S., and Yongsawatdigul, J. (2015). Characterization and identification of angiotensin I-converting enzyme (ACE) inhibitory peptides derived from tilapia using *Virgibacillus halodenitrificans* SK1-3-7 proteinases. **Journal of Functional Foods.** 14: 435-444.

Torruco-Uco, J., Chel-Guerrero, L., Martı´nez-Ayala, A., Da´vila-Ortı´z, G., and Betancur-Ancona, D. (2009). Angiotensin-I converting enzyme inhibitory and antioxidant activities of protein hydrolysates from Phaseolus lunatus and Phaseolus vulgaris seeds. **LWT - Food Science and Technology.** 42: 1597-1604.

Tovar-Pérez, E. G., Guerrero-Legarreta, I., Farrés-González, A., and Soriano-Santos, J. (2009). Angiotensin I-converting enzyme-inhibitory peptide fractions from albumin 1 and globulin as obtained of amaranth grain. **Food Chemistry.** 116: 437-444.

Tsai, J. S., Chen, J. L., and Pan, B. S. (2008). ACE-inhibitory peptides identified from the muscle protein hydrolysate of hard clam (*Meretrix lusoria*). **Process Biochemistry.** 43: 743-747.

Tsai, J. S., Lin, T. C., Chen, J. L., and Pan, B. S. (2006). The inhibitory effects of freshwater clam (*Corbicula fluminea*, *Muller*) muscle protein hydrolysates on angiotensin I converting enzyme. **Process Biochemistry.** 41: 2276-2281.

Tulyapongrak, S., Ratana-Arporn, P., and Runglerdkriangkrai, J. (2008). Physical, chemical and microbiological changes of Thai Panga fish during ice storage. In **The 45th Kasetsart University Annual Conference**. Bangkok: Kasetsart.

Turner, A. J., and Hooper, N. M. (2002). The angiotensin-converting enzyme gene family: Genomics and pharmacology. **Trend in Pharmacological Sciences.** 23: 177-183.

Vaštag, Z. U. A., Popovic´, L., Popovic´, S., Krimer, V., and Pericˇin, D. (2011). Production of enzymatic hydrolysates with antioxidant and angiotensin-I converting enzyme inhibitory activity from pumpkin oil cake protein isolate**.** **Food Chemistry.** 124:1316-1321.

Vercruysse, L., Van Camp, J., Morel, N., Rougé, P., Herregods, G., and Smagghe, G. (2010). Ala-Val-Phe and Val-Phe: ACE inhibitory peptides derived from insect protein with antihypertensive activity in spontaneously hypertensive rats. **Peptides.** 31: 482-488.

Vercruysse, L., Smagghe, G., Matsui, T., and Camp, J. V. (2008). Purification and identification of an angiotensin I converting enzyme (ACE) inhibitory peptide from the gastrointestinal hydrolysate of the cotton leafworm, *Spodoptera littoralis*. **Process Biochemistry.** 43: 900-904.

Verhoeckx, K. C., Vissers, Y. M., Baumert, J. L., Faludi, R., Feys, M., Flanagan, S, Herouet-Guicheney, C., Holzhauser, T., Shimojo, R., van der Bolt, N., Wichers, H., and Kimber, I. (2015). Food processing and allergenicity. **Food and Chemical Toxicology.** 80: 223-240.

Walter, E., Kissel, T., and Amidon, G. L. (1996). The intestinal peptide carrier: A potential transport system for small peptide derived drugs. **Advanced Drug Delivery Reviews.** 20: 33-58.

Wang, J., Hu, J., Cui, J., Bai, X., Du, Y., Miyaguchi, Y., and Lin, B. (2008). Purification and identification of a ACE inhibitory peptide from oyster proteins hydrolysate and the antihypertensive effect of hydrolysate in spontaneously hypertensive rats. **Food Chemistry.** 111: 302-308.

Wang, L., Mao, X., Cheng, X., Xiong, X., and Ren, F. (2010). Effect of enzyme type and hydrolysis conditions on the *in vitro* angiotensin I-converting enzyme inhibitory activity and ash content of hydrolysed whey protein isolate. **International Journal of Food Science and Technology.** 45: 807-812.

Whitaker, J. R. (1994). **Principles of enzymology for the food sciences**. New York: Marcel Decker.

World Health Organization. (2013). **A global brief on hypertension. Silent killer, global public health crisis**. Geneva: WHO Press.

Wijesekara, I., Qian, Z. J., Ryu, B., Ngo, D. H., and Kim, S. K. (2011). Purification and identification of antihypertensive peptides from seaweed pipefish (*Syngnathus schlegeli*) muscle protein hydrolysate. **Food Research International.** 44: 703-707.

Williams, T. A., Corvol, P., and Soubr, F. (1994). Identification of two active site residues in human angiotensin I-converting enzyme. **Journal of Biological Chemistry.** 269: 29430-29434.

Williams, T. A., Michaud, A., Houard, X., Chauvet, M. T., Soubrier, F. and P Corvol, P. (1996). Drosophila melanogaster angiotensin I-converting enzyme

expressed in Pichia pastoris resembles the C domain of the mammalian homologue and does not require glycosylation for secretion and enzymic activity. **Biochemical Journal.** 318:121-131.

Wiriyaphan, C., Xiao, H., Decker, E. A., and Yongsawatdigul, J. (2015). Chemical and cellular antioxidative properties of threadfin bream (*Nemipterus spp*.) surimi byproduct hydrolysates fractionated by ultrafiltration. **Food Chemistry.** 167: 7-15.

Wong, D. W. S. (1995). **Food enzymes: Structure and mechanism**. New York: Chapman and Hall.

Wu, H., He, H. L., Chen, X. L., Sun, C. Y., Zhang, Y. Z., and Zhou, B. C. (2008). Purification and identification of novel angiotensin-I-converting enzyme inhibitory peptides from shark meat hydrolysate. **Process Biochemistry.** 43: 457-461.

Wu, J., Aluko, R. E., and Muir, A. D. (2008). Purification of angiotensin I-converting enzyme-inhibitory peptides from the enzymatic hydrolysate of defatted canola meal. **Food Chemistry.** 111: 942-950.

Wu, J., Aluko, R. E., and Muir, A. D. (2009). Production of angiotensin I-converting enzyme inhibitory peptides from defatted canola meal. **Bioresource Technology.** 100: 5283-5287.

Wu, J., Aluko, R. E., and Nakai, S. (2006a). Structural requirements of angiotensin I-converting enzyme inhibitory peptides: Quantitative structure-activity relationship modeling of peptides containing 4-10 amino acid residues. **QSAR and Combinatorial Science.** 25: 873-880.

Wu, J., Aluko, R. E., and Nakai, S. (2006b). Structural requirements of angiotensin I-converting enzyme inhibitory peptides: Quantitative structure-activity

relationship study of di- and tripeptides. **Journal of Agricultural Food and Food Chemistry.** 54: 732-738.

Wu, Q., Jia, J., Yan, H., Du, J., and Gui, Z. (2014). A novel angiotensin-I converting enzyme (ACE) inhibitory peptidefrom gastrointestinal protease hydrolysate of silkworm pupa (*Bombyx mori*) protein: Biochemical characterization and molecular dockingstudy. **Peptides.** 68: 17-24

Wu, S., Feng, X., Lan, X., Xu, Y., and Liao, D. (2015). Purification and identification of Angiotensin-I Converting Enzyme (ACE) inhibitory peptide from lizard fish (*Saurida elongata*) hydrolysate. **Journal of Functional Foods.** 13: 295-299.

Yamada, A., Sakurai, T., Ochi, D., Mitsuyama, E., Yamauchi, K., and Abe, F. (2013). Novel angiotensin I-converting enzyme inhibitory peptide derived from bovine casein. **Food Chemistry.** 141: 3781-3789.

Yu, W., Gao, J., Xue, Z., Kou, X., Wang, Y., and Zhai, L. (2013). Radical-scavenging activity, ACE-inhibiting capability and identification of rapeseed albumin hydrolysate. **Food Science and Human Wellness.** 2: 93-98.

Yu, Y., Hu, J., Miyaguchi, Y., Bai, X., Du, Y., and Lin, B. (2006). Isolation and characterization of angiotensin I-converting enzyme inhibitory peptides derived from porcine hemoglobin. **Peptides.** 27: 2950-2956.

Yu, Z., Liu, B., Zhao, W., Yin, Y., Liu, J., and Chen, F. (2012). Primary and secondary structure of novel ACE-inhibitory peptides from egg white protein. **Food Chemistry.** 133: 315-322.

Zeng, S. K., Zhang, C. H., Lin, H., Yang, P., Hong, P. Z., and Jiang, Z. (2009). Isolation and characterisation of acid-solubilised collagen from the skin of Nile tilapia (*Oreochromis niloticus*). **Food Chemistry.** 116: 879-883.

Zhang, Y., Liu, W., Li, G., Shi, B., Miao, Y., and Wu, X. (2007). Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*). **Food Chemistry.** 103: 906-912.

Zhang, Y., Olsen, K., Grossi, A., and Otte, J. (2013). Effect of pretreatment on enzymatic hydrolysis of bovine collagen and formation of ACE-inhibitory peptides. **Food Chemistry.** 141: 2343-2354.

Zhao, Y., Li, B., Dong, S., Liu, Z., Zhao, X., Wang, J., and Zeng, M. (2009). A novel ACE inhibitory peptide isolated from Acaudina molpadioidea hydrolysate. **Peptides.** 30:1028-1033.

Zhao, Y., Li, B., Liu, Z., Dong, S., Zhao, X., and Zeng, M. (2007). Antihypertensive effect and purification of an ACE inhibitory peptide from sea cucumber gelatin hydrolysate. **Process Biochemistry.** 42: 1586-1591.

Zhou, C., Ma, H., Yu, X., Liu, B., Yagoub, Ael-G., and Pan, Z. (2013). Pretreatment of defatted wheat germ proteins (by-products of flour mill industry) using ultrasonic horn and bath reactors: Effect on structure and preparation of ACE-inhibitory peptides. **Ultrasonics Sonochemistry.** 20: 1390-1400.

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