PHYSICO-CHEMICAL PROPERTIES AND ANGIOTENSIN I CONVERTING ENZYME INHIBITORY PEPTIDES OF COLLAGEN PREPARED FROM TILAPIA (*Oreochromis niloticus*) AND HYBRID CATFISH (*Clarias macrocephalus*×*C. gariepinus*) SKIN

Pornpimol Sungperm

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คุณสมบัติทางเคมีกายภาพและเพปไทด์ยับยั้งเอนไซม์แอนจิโอเทนซิน ของคอลลาเจนจากหนังปลานิลและหนังปลาดุกบิ๊กอุย

นางสาวพรพิมล สังข์เพิ่ม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2555

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

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พรพิมล สังข์เพิ่ม : คุณสมบัติทางเคมีกายภาพและเพปไทด์ยับยั้งเอนไซม์แอนจิโอเทนซิน ของคอลลาเจนจากหนังปลานิลและหนังปลาดุกบิ๊กอุย (PHYSICO-CHEMICAL PROPERTIES AND ANGIOTENSIN I CONVERTING ENZYME INHIBITORY PEPTIDES OF COLLAGEN PREPARED FROM TILAPIA (*Oreochromis niloticus*) AND HYBRID CATFISH (*Clarias macrocephalus*$\times$*C. gariepinus*) SKIN) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. จิรวัฒน์ ยงสวัสดิกุล, 97 หน้า.

หนังปลานิลและหนังปลาดุกบิ๊กอุยคือผลพลอยได้จากอุตสาหกรรมแปรรูปเนื้อปลาซึ่งปกติแล้วนำไปผลิตเป็นอาหารสัตว์ที่มีมูลค่าต่ำ วัตถุประสงค์ของงานวิจัยนี้คือ ศึกษาคุณสมบัติเคมีกายภาพของคอลลาเจนที่สกัดได้จากหนังปลานิล และหนังปลาดุกบิ๊กอุย รวมถึงผลิตเพปไทด์ที่มีความสามารถยับยั้งเอนไซม์แอนจิโอเทนซินโดยโปรติเนส ผลิตผล (Yield) ของคอลลาเจนจากหนังปลานิลและปลาดุกบิ๊กอุยที่สกัดด้วยกรดมีค่าเท่ากับ 58.9% และ 48.0 % ต่อน้ำหนักหนังปลาแห้งตามลำดับ กรดอะมิโนหลักของคอลลาเจนจากหนังปลาทั้งสองชนิดคือ ไกลซีน โพรลีน และ ไลซีน ความสามารถในการละลายของคอลลาเจนจากหนังปลานิลและปลาดุกบิ๊กอุยมีค่าสูงสุดที่พีเอช 3 และความสามารถในการละลายลดลงอย่างรวดเร็วเมื่อมีเกลืออยู่ในสารละลายมากกว่าหรือเท่ากับ 3% การสูญเสียสภาพธรรมชาติเมื่อวิเคราะห์ด้วย Differential scanning calorimetry (DSC) พบว่าอุณหภูมิในการเสียสภาพ (T_d) ของคอลลาเจนหนังปลานิลและหนังปลาดุกบิ๊กอุยในสารละลายกรดอะซิติกเข้มข้น 0.05 โมลาร์เท่ากับ 36.42 และ 35.15 องศาเซลเซียส ในขณะที่อุณหภูมิในการสูญเสียสภาพของคอลลาเจนในน้ำปราศจากไอออนคือ 47.12 และ 38.27 องศาเซลเซียสตามลำดับ ผลจากวิเคราะห์รูปแบบเพปไทด์ (peptide mapping) แสดงให้เห็นว่าการจัดเรียงตัวของลำดับกรดอะมิโนและโครงสร้างของคอลลาเจนหนังปลานิลและปลาดุกบิ๊กอุยนั้นแตกต่างกัน รูปแบบของสเปคตรา (spectra) จากฟูเรียร์ทรานสฟอร์มอินฟาเรด (Fourier transform- infrared spectroscopy) ของคอลลาเจนหนังปลานิลและปลาดุกบิ๊กอุยคล้ายคลึงกับคอลลาเจนจากหนังลูกวัว แสดงว่าโครงสร้างของคอลลาเจนจากหนังปลาทั้งสองชนิดเป็น คอลลาเจนชนิดที่ 1 (Type I) และคอลลาเจนจากปลาทั้งสองชนิดยังคงสภาพของโครงสร้างทุติภูมิ

จากการเปรียบเทียบไฮโดรไลเสทของคอลลาเจนหนังปลานิลและหนังปลาดุกบิ๊กอุยจากการย่อยด้วยเพปซิน อัลคาเลส ทริปซินและโปรตีเนสจาก *Vigibacillus* sp. SK 39 พบว่าไฮโดรไลเสทจากเพปซินมีความสามารถยับยั้งกิจกรรมของเอนไซม์แอนจิโอเทนซินได้สูงสุดที่ระดับการย่อย (Degree of hydrolysate) 30% ผลจากการแยกไฮโดรไลเสทด้วยการกรองผ่านเยื่อกรอง สามารถจำแนกได้สามส่วนคือส่วนที่มีขนาดมากกว่า 30 กิโลดาลตัน ขนาดระหว่าง 5-30 กิโลดาลตัน และขนาดน้อยกว่า 5 กิโลดาลตัน ไฮโดรไลเสทที่มีขนาดน้ำหนักโมเลกุลต่ำกว่า 5 กิโลดาลตัน แสดง

กิจกรรมยับยั้งเอนไซม์แอนจิโอเทนซินสูงที่สุด และความเข้มข้นที่ยับยั้งกิจกรรมของเอนไซม์แอนจิโอเทนซิน 50% (IC_{50}) มีค่าเท่ากับ 9.01±0.04 ไมโครกรัม (สมมูลไกลซีน) ต่อมิลลิลิตร เมื่อนำส่วนที่ผ่านเยื่อกรองมาทำบริสุทธิ์ด้วยวิธีการแลกเปลี่ยนประจุ (Ion exchange chromatography) และการแยกตามขนาดโมเลกุล (Gel filtration) เพปไทด์หลังผ่านการแยกตามขนาดโมเลกุลสามารถยับยั้งเอนไซม์แอนจิโอเทนซินได้ 72.06% ที่ระดับความเข้มข้น 0.2 ไมโครกรัม สมมูลไกลซีน ผลการวิเคราะห์ด้วย LC-Tandem mass spectrometry ของเพปไทด์ที่มีกิจกรรมยับยั้งเอนไซม์แอนจิโอเทนซินสูงสุดประกอบด้วยกรดอะมิโนอาร์จินีนและไกลซีนที่ปลายสายคาร์บอกซิลของเพปไทด์ (C-terminus) และกรดอะมิโนด้านปลายสายอะมิโน (N-terminus) ส่วนมากประกอบด้วยกรดอะมิโนที่มีโซ่ข้างเป็นคาร์บอนสายตรง (Aliphatic amino acid)

การศึกษานี้แสดงให้เห็นว่าคุณสมบัติทางเคมีกายภาพของหนังปลาจากปลานิลและปลาดุกบิ๊กอุยไม่แตกต่างกัน และมีศักยภาพในการเป็นแหล่งคอลลาเจนทางเลือกแทนหนังโคและสุกร นอกจากนี้ไฮโดรไลเสทจากหนังปลาดุกบิ๊กอุยที่ได้จากเอนไซม์เพปซินแสดงกิจกรรมยับยั้งเอนไซม์แอนจิโอเทนซิน ซึ่งสามารถนำไปพัฒนาเป็นผลิตภัณฑ์อาหารเสริมที่มีคุณสมบัติควบคุมความดันโลหิตสูง

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา_____

PORNPIMOL SUNGPERM : PHYSICO-CHEMICAL PROPERTIES AND ANGIOTENSIN I CONVERTING ENZYME INHIBITORY PEPTIDES OF COLLAGEN PREPARED FROM TILAPIA (*Oreochromis niloticus*) AND HYBRID CATFISH (*Clarias macrocephalus* × *C. gariepinus*) SKIN

THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 97 PP.

PHYSICO-CHEMICAL PROPERTIES/FISH COLLAGEN / ANGIOTENSIN I CONVERTING ENZYME INHIBITORY PEPTIDE

Skins of tilapia (*Oreochromis niloticus*) and hybrid catfish (*C. macrocephalus* ×*C. gariepinus*) are byproducts of fish processing and are normally turned into low value fish meal. The objectives of this study were to investigate and compare the physico-chemical properties of collagen extracted from tilapia skin (TS) and hybrid catfish skin (HS). In addition, the study aimed to produce angiotensin converting enzyme (ACE) inhibitory peptides using suitable proteinase. The yield of collagen from TS and HS by acid extraction was 58.9 and 48.0% of dried skin, respectively. Major amino acids of both collagens were glycine, proline and lysine. The maximum solubility of the collagens from TS and HS was at pH 3, whereas the solubility of both collagens rapidly decreased at ≥ 3% NaCl. Differential scanning calorimetry (DSC) revealed that denaturation temperature (T_d) of TS and HS collagens in the presence of 0.05 M acetic acid was at 36.42 and 35.15°C, while those dissolved in deionized water (DI) were at 47.12 and 38.27°C, respectively. Based on peptide mapping, TS and HS collagen showed differences in amino acid sequences and collagen

conformation as compared to calf collagen. The Fourier transform- infrared (FT-IR) spectra of TS and HS collagen were similar to that of calf collagen, suggesting that a native conformation of TS and HS was classified as type I collagen. Their secondary structure remained.

TS and HS collagen hydrolysates were obtained using pepsin, Alcalase, trypsin and proteinase from *Vigibacillus* sp. SK 39. Pepsin produced peptides exhibiting the highest ACE inhibitory activity with a degree of hydrolysis (DH) of 30%. The hydrolysate was fractionated into 3 fractions: MW >30 kDa, 5–30 kDa and <5 kDa using cross-flow ultrafiltration. The fraction with MW <5 kDa showed the highest ACE inhibitory activity with IC_{50} of 9.01±0.04 μg (glycine equivalent)/ml. The ultrafiltrated fraction was further purified using ion exchange chromatography and gel filtration. The pooled fraction after gel filtration showed ACE inhibitory activity at 72.06% at 0.2 μg glycine equivalent. LC-Tandem mass spectrometry of the pooled fraction revealed that peptides exhibiting ACE inhibitory activity were composed of arginine and lysine at C-termini, while N-termini contained aliphatic amino acids. This study revealed that collagen from tilapia and hybrid catfish skin showed comparable physico-chemical characteristics and could provide potential alternative resources for collagen. In addition, ACE inhibitory peptides derived from HS collagen could be developed as a functional food with antihypertensive property.

School of Food Technology Student's Signature _____

Academic Year 2012 Advisor's Signature _____

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Finally, I would like to dedicate this thesis to my beloved parents for their moral support. There have been a constant source of love, always believe in me and give me the great opportunity even my family is not rich but they never say No when I need supporting.

Pornpimol Sungperm

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LIST OF ABBREVIATIONS

ACE	=	Angiotensin I-Converting enzyme
Ala	=	Alanine
Arg	=	Arginine
Asn	=	Asparagine
Asp	=	Aspartic acid
^{o}C	=	Degree celsius
CFU/mL	=	Colonies forming unit per milliliter
CM	=	Carboxymethyl
cm^{-1}	=	Reciprocal centimeter
DI	=	Deionized water
DSC	=	Differential scanning calorimetry
FT-IR	=	ourier transform- infrared spectroscopy
G	=	gram
×g	=	Relative centrifugal fields
Glu	=	Glutamic acid
Gln	=	Glutamine
Gly	=	Glycine
ΔH	=	Enthalpy
Ile	=	Isoleucine
J/g	=	Joules/gram

LIST OF ABBREVIATIONS (Continued)

kDa	=	Kilodalton
Leu	=	Leucine
Lys	=	Lysine
Met	=	Methionine
M	=	Molar
Mg	=	Milligram
Min	=	Minute
mL	=	Milliliter
mm	=	Millimeter
MW	=	Molecular weight
MWC	=	Molecular weight cut-off
Phe	=	Phenylalanine
pI	=	Isoelectric point
Pro	=	Proline
S	=	Second
Ser	=	Serine
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T_d	=	Denaturation temperature
T_m	=	Melting temperature
TCA	=	Trichloroacetic acid
Trp	=	Tryptophan

LIST OF ABBREVIATIONS (Continued)

Tyr	=	Tyrosine
µL	=	Microliter
µm	=	Micrometer
U	=	Unit activity
V	=	Volt
V	=	Valine

CHAPTER I

INTRODUCTION

1.1 Introduction

Collagen is the predominant structural protein of animal, comprising approximately 30% of total animal protein (Lee et al., 2001). Twenty eight different types of collagen composed of at least 27 distinct polypeptide chains (types I-XXVIII) have been identified (Brik and Brucker, 2005). Primary structure of collagen is a repeating sequence of glycine-proline-hydroxyproline which is the most common triplet in collagen polypeptide. Secondary structure of collagen is arranged as a left-handed helix and three of these combine to form a right hand triple helix. In animals, individual collagens triple helices are known as tropocollagen. The tropocollagen molecules assemble to form fibril which aggregates to form fiber. Collagen fibrous contributes to connective tissue of skins, tendons, bones and cartilages (Matthew, Shoulder and Raines, 2009).

Collagen has very broad industrial applications, such as photographic, gelatin and leather industry and sausage casings. Furthermore, soluble collagen serves in biomedical and pharmaceutical industry including the treatment of hypertension, osteoarthritis, tissue engineering for implants in humans, treatment of hypertension and used in cosmetics industry (Lee et al., 2001). The denatured form is widely used in the food industries as food supplements and food additives. (Karim and Bhat, 2009)

The increasing attention of alternative sources for replacement mammalian

collagen has been paid due to the outbreaks of bovine spongiform encephalopathy (BSE) and the food-and-mount disease (FMD). There is a strong need to develop new collagen sources. Safe sources of collagen have been focused and isolated from many animals, such as poultry, chicken feet, bird feet (Cheng et al., 2009; Lin and Liu, 2006), marine fish, such as Japanese sea bass, brownstrip red snapper, common mackerel, bullhead shark, Baltic cod (Nagai et al., 2002; Jongjareanra et al., 2005; Kimura et al., 1988; Nagai and Suzuki, 2000; Sadowska et al., 2008), freshwater fish, such as carp, channel catfish, young and adult nile perch (Duan et al., 2009; Liu et al., 2007; Muyonga et al., 2004) and other sources including frog skin, jellyfish and cuttlefish (Li et al., 2004; Nagai et al., 2000; Nagai et al., 2001). Collagen derived from fish and poultry can be potential source. Schrieber, et al (2007) reported that poultry skin and bone are limited by low yield for the commercial production. Fish skin would be an alternative source for collagen extraction.

Fish provide the new source of material because of availability without religious barriers and no risk of disease transmission. Fish fillet industry generated 75% of fish waste based on weight of catches. This has led to the generation of a large biomass of byproduct which is generally discarded as the mixture of head, fin, scale, bone, viscera as well as skins. Fish skin can be the promising source for collagen extraction (Kelleher, et al., 2005).

However, physical and chemical properties of fish collagen are different from those of mammalian collagen (Bailey and Light 1989). Fish skin collagens are known to have low thermal stability when compared to mammalian collagen, limiting the use in some applications. Recent studies have demonstrated that thermal stability of collagen from cold water fish is lower than that extracted from warm water fish. In

addition, collagen from marine fish tends to have significantly poorer thermal stability than mammalian and warm freshwater fish collagen. Collagens derived from fish species living in cold water have lower content of imino acid and they exhibited lower thermal stability than those of warm water fish (Muyonga et al., 2004). Tropical freshwater fish collagens could have more potential as they might have higher thermal stability.

Recently, most studies have focused on marine fish (Jongjareonrak et al., 2005; Kittiphattabawon et al., 2005 Duan et al., 2009; Bae et al., 2008). Freshwater fish collagens reported so far include nile perch, grass carp, channel catfish and silver carp (Muyonga et al., 2004; Zhang et al, 2007; Liu, Li and Guo, 2007; Rodziewicz-Motowidło et al., 2008). Young and adult Nile perch are subtropical fish with the denaturation temperature of collagen at 36ºC comparable to that of mammalian collagen.

Freshwater aquaculture plays an important role in food security and economy of Thailand. Two main cultured freshwater fish species were Nile tilapia (*Oreochromis niloticus*) and hybrid catfish (*Clarias macrocephalus* × *C. gariepinus*). Tilapia bas become the most important fish in aquaculture because of their large size and rapid growth. Originally, the majority of tilapia fishery was in Africa and it was introduced to Asia since 1962. Thailand is currently the third top tilapia producer from aquaculture in Asia after China and Philippines (FAO, 2012). Hybrid catfish is another cultured species with approximately 86,000 tons per year or 30% of total freshwater fish production in Thailand (FAO, 2011). It was become the most preferred species because it grows faster than the native one. Collagen extraction would increase the full utilization of tilapia and hybrid catfish skins, which are byproducts from fillet processing.

Protein hydrolysate from byproducts of several fish species have been analyzed for their bioactive properties (Kim et al., 2006). Bioactive peptides isolated from various fish hydrolasate have shown bioactivities (Kim et al., 2000). Some peptides showed antihypertensive activity by inhibiting angiotensin I converting enzyme (ACE) activity (Je et al., 2005). Hypertension is a worldwide major problem of epidermic proportions. It is the most common serious chronic health problem and carries a high risk factor for arteriosclerosis stroke (Jung et al., 2006). Angiotensin converting enzyme (ACE) is a Zn metallopeptidase and plays an important role in regulating blood pressure (Wang, Cui, Bai, Du, Miyaguchi and Lin, 2008). Since the discovery of ACE inhibitor in snake venom, many studies have been carried out in synthesizing ACE inhibitors, such as captopril, enalapril, alacepril and lisnopril. However, the patients encounter with side effects, such as hypotension, headache, insomnia and fever. Recently, many inhibitory peptides against ACE are reported as natural alternative functional peptides that are safer than the artificial synthesised ACE inhibitory drugs (Lee, Jeon and Byun, 2011). The improved nutritional and potent functional peptides have been produced by enzymatic digestion (Fahmi et al., 2004). Commercial enzymes including pepsin, trypsin, alcalase, collagenase and pornase E have been reported to librates strong angiotensin I inhibitory peptide from various fish species (Jeon, Byun and Kim, 1999; Byun and Kim, 2001; Zhao et al., 2007; Lee, Kyun and Byun, 2011).

Peptides present protein hydrolysates have exhibited different biological activities depending on their molecular weights and amino acid sequences. It has been demonstrated that short peptide with C-terminal proline and hydroxyproline residues are generally strong ACE inhibitors (Erdmann et al., 2008). Therefore, peptide

purification and characterization is needed. Ultrafiltration and chromatographic method are typically useful process for peptide purification.

The objective of this research was to investigate and compare physico-chemical properties of collagen from freshwater fish, namely Tilapia (fish with scale) and hybrid catfish skin (fish without scale). In addition, collagen hydrolysates from skin of both species were produced using enzyme technology to attain peptide with ACE inhibitory activity.

1.2 Research objectives

The objectives of this research were:

1) To investigate and compare physico-chemical properties of collagen from tilapia (*Oreochromis niloticus*) and hybrid catfish (*Clarias macrocephalus* × *C. gariepinus*) skin.
2) To study the potential of hydrolysate produced from tilapia and hybrid catfish skin with ACE inhibitory activity,
3) To fractionate peptides with most potent ACE inhibitory activity.

1.3 Research hypotheses

Physico-chemical properties of tilapia (fish with scale) and hybrid catfish skin (fish without scale) collagen will be different. ACE inhibitory peptide could be produced from collagen hydrolysate. The degree of hydrolysis may influence ACE inhibitory activity.

1.4 Scope of this study

Collagen from two species of freshwater fish namely, tilapia and hybrid catfish were extracted. Physico-chemical properties of both species were studied, including thermal stability, solubility, peptide mapping and molecular weight distribution. Collagen hydrolysates were produced using commercial enzymes (Alcalase, pepsin and trypsin) and enzyme from *Vigibacillus* sp.SK 39. ACE inhibitory activity of hydrolysates were studied. The most potent ACE inhibitory peptides were fractionated using ultrafiltration, ion exchange and gel filtration technique. Amino sequences of the fractionated peptides were determined using LC-MS/MS.

CHAPTER II

LITERATURE REVIEWS

2.1 Collagen

Collagen is the fibrous protein synthesized by fibroblasts (Figure 2.1).The fibrous collagen constitutes 25-30% of total animal protein content (Lee et al., 2001). Collagen is the major component of several connective tissues, such as skin, bone, tendon, blood vessel, basement membrane, cartilage, and teeth (Morimura et al., 2002; Nagai and Suzuki, 2000).

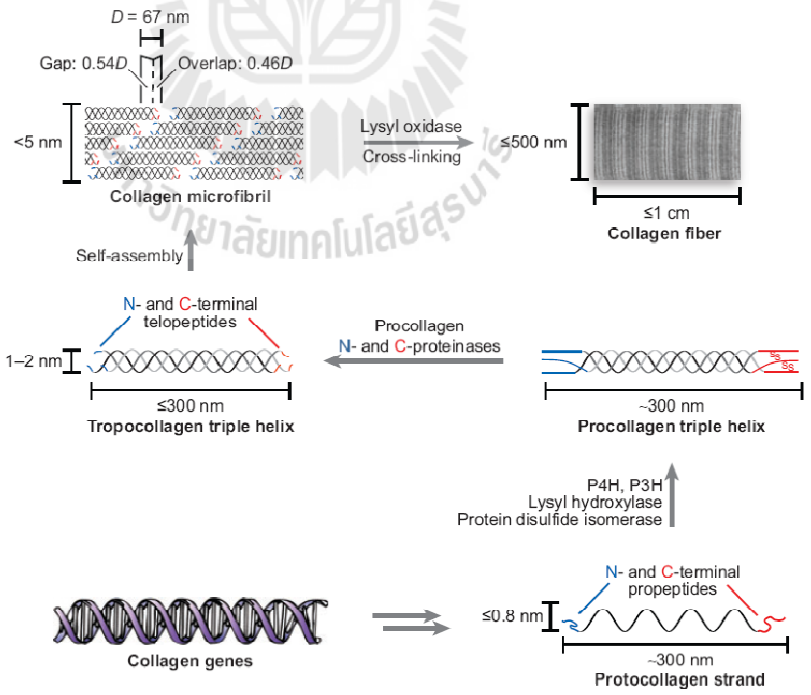


Figure 2.1 Biosynthesis of collagen structure.

From: Shoulders and Raines (2009).

Collagen fibrous comprises a right handed bundle of three α-chains which can be formed by three homotrimers chains as collagen type II, III, VII or formed by heterotrimers chains as collagen types I, IV, V and VI. At present, 28 different types of collagen have been identified as shown in Table 2.1. Different types of collagen are characterized by considerable complexity and diversity in their structure, splice variants, presence of additional, non-helical domains, assembly and function (Gelse, Pöschl and Aigner, 2003). Based on structure and supramolecular organization, collagen can be classified into 4 groups, fibril-forming collagens, fibril-associated collagen (FACIT), network-forming collagens and anchoring fibrils respectively. (Xiong, 1997; Shoulders and Raines, 2009).

Table 2.1 Type of collagen and their distribution.

Type	Class	Composition	Distribution
I	Fibrillar	$\alpha1(I)_2$ α2(I)	Abunadant and widespread: dermis, bone, tendon and ligament
II	Fibrillar	$\alpha1(II)_3$	Cartilage, vitreous
III	Fibrillar	$\alpha1(III)_3$	Skin, blood vessel, intestine
IV	Network	α1(IV)α2(IV)α3(IV)α4(IV)α5(IV)α6(IV)	Basement membranes
V	Fibrillar	$\alpha1(V)_3\alpha1(V)_2$ α2(V)α1(V)α2(V)α3(V)	Widespread:bone, dermis, cornea, placenta
VI	Network	α1(VI)α2(VI)α3(VI)α1(VI)α2(VI)α4(VI)	Widespread:bone, cartilage, cornea, dermis
VII	Anchoring fibril	$\alpha1(VII)_2\alpha2(VII)$	Dermis, bladder
VIII	Network	$\alpha1(VIII)_3$ $\alpha2(VIII)_3$ $\alpha1(VIII)_2$ α2(VIII)	Widespread: dermis, brain, heart, kidney
IX	FACIT[a]	α1(IX)α2(IX)α3(IX)	Cartilage, cornea, vitreous
X	Network	$\alpha1(X)_3$	Cartilage
XI	Fibrillar	α1(XI)α2(XI)α3(XI)	Cartilage, intervertebral disc
XII	FACIT	$\alpha1(XII)_3$	Dermis, tendon
XIII	MACIT[a]	-	Endothelial cells, dermis, eye, heart
XIV	FACIT	$\alpha1(XIV)_3$	Widespread: bone, dermis, cartilage
XV	MULTIPLEXIN[a]	-	Capillaries, testis, kidney, heart
XVI	FACIT	-	Dermis, kidney

Table 2.1 (Continued)

Type	Class	Composition	Distribution
XVII	MACIT	$\alpha1(XVII)_3$	Hemidesmosomes in epithelia
XVIII	MULTIPLEXIN	-	Basement membranes, liver
XIX	FACIT	-	Basement membranes
XX	FACIT	-	Cornea(chick)
XXI	FACIT	-	Stomach, kidney
XXII	FACIT	-	Tissue junctions
XXIII	MACIT	-	Heart, retina
XXIV	Fibrillar	-	Bone, cornea
XXV	MACIT	-	Brain, heart, testis
XXVI	FACIT	-	Testis, ovary
XXVII	Fibrillar	-	Cartilage
XXVIII	-	-	Dermis, sciatic nerve

From: Shoulders and Raines (2009)

Abbreviations: FACIT, fibril-associated collagen with interrupted triple helices; MACIT, membrane-associated collagen with interrupted triple helices; MULTRIPLEXIN, multriple triple-helix domains and interrupted.

Type I and V collagen are fibrils type and contribute to structural backbone of bone and dermis, whereas type II and XI are similar fibrils collagen but predominantly contribute to the matrix of articular cartilage (Angele et al., 2004). The microfibrillar type such as, VI collagen is highly disulfide cross-linked and contributes to a network of breaded filaments interwoven with other collagen fibrils (von der mark, Aumailley, Wick, Fleischmajer and Timpl, 1984). Furthermore, different type of collagen considerably varies with the triple helix length. The length of helix collagen type I, II and III approximately 300 nm which corresponds to about 1000 amino acids. Collagen type VI and X contain triple helices with approximately 200 and 460 amino acids, respectively (Bateman, Lamende and Ramsha, 1996).

Type I collagen is the most abundant contributing to organic mass of bone and is the major collagen of tendon, skin, ligament, cornea. Cartilage mainly contains type II collagen, and some type I collagen (Wardale and Duance, 1993). Type I collagen is

also widespread in the animal kingdom, from invertebrates to vertebrates (Exposito et al., 1992). Type I collagen is used in the gelatin industry and in many biomaterials and leather. The structure of type I procollagen is similar to other fibrillar collagens. Molecules are comprised of three heterotrimer polypeptide chains (α-chains), two α_1 (I) and one α_2 (I) chains. The α_1 (I) chain is more conserved than the α_2 (I) chain. Type I collagen molecule contains an uninterrupted triple helix of approximately 300 nm in length and 1.5 nm in diameter flanked by short nonhelical telopeptides. (Kimura, 1983).

2.1.1 Collagen composition and structure

Collagen structure has been proposed since 1940. Rich and Crick proposed that collagen structure has a single interstrand ($N\text{-}H_{(Gly)}\ldots O{=}C_{(Xaa)}$) hydrogen bond per triplet of amino acid and a tenfold helical symmetry with a 28.6-Å axial repeat (10/3 helical pitch) and this structure model is accepted today (Shoulders and Raines, 2009). The collagen monomer is a long cylindrical protein about 2800 Å long and 14-15 Å in diameter (Foegeding et al., 1996). Collagen is consisted of three polypeptides unit (called α-chains) intertwined to form a triple-helical structure. Each α-chains coils in a left-handed helix with three residues per turn and three chains are twisted right-handed to form the triple helix. Triple helix of collagen is stabilized by the formation of two inter-chain hydrogen bonds between the amino acid group of Gly N-H and the carbonyl of X residue ($N\text{-}H_{(Gly)}\ldots O{=}C_{(Xaa)}$) show in Figure 2.2. Different types of collagen are contained with different α-chains or other composition as illustrated in Table 2.1 Different α-chains, designated α1, α2 or α3, have different amino acid sequences and composition. Distribution of α1, α2 and α3-chains of collagen molecule varies depending on the specific genetic variants (Xiong, 1997).

Amino acid composition of collagen is characterized by a repeating sequence of Gly-X-Y triplets, where X is proline and Y is hydroxyproline. Rigby (1968) reported that the most important amino acids in collagen are glycine, proline, and hydroxyproline, approximately 33%, 12% and 11%, respectively. Additionally, Xu et al. (2002) reported that hydroxyproline and hydroxylysine are located at Y-position, while proline can be found in either X- or Y-position. Previous studies showed that collagen is the only protein that is rich in hydroxyproline (up to 10% in mammalian collagen), thus it is used as a measure of collagen content (Foegeding et al., 1996).

Glycine represents nearly one-third of total residues at every third position throughout polypeptide chains (Foegeding et al., 1996). The presence of glycine at every three residues is a critical requirement for collagen superhelix structure because of glycine contains no side chain (Regenstein and Zhou, 2007). This allows a close packing structure along the central axis of the triple helix molecule (Gelse, Pöschl and Aigner, 2003).

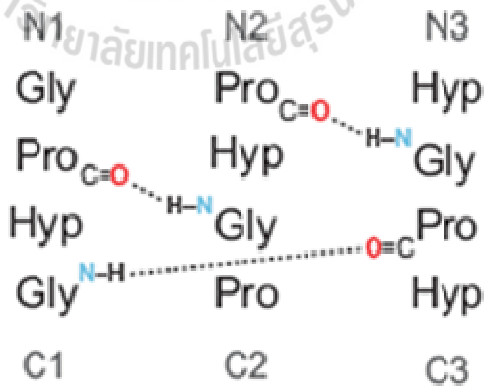


Figure 2.2 Inter-strand hydrogen bonds along the collagen structure

From: Shoulders and Raines (2009).

Amino acid composition of collagen is unique. Collagens are rich in glycine, proline and alanine, and contain low or no cysteine and tryptophan and relatively low content of tyrosine and histidine. Collagen is a hydrophilic protein because it contains of acidic and hydroxylated amino acid residues than lipophilic residues (Table 2.2) Thus, it swells in polar solution. Alanine is a major amino acid after glycine and proline or hydroxyproline, but it does not provide any additional stability or any important role (Bhattacharjee et al., 2005). Glutamic acid, leucine and phenylalanine are preferred at the X position, whereas arginine and lysine are seen in the Y position. The residues at Y position can help stabilize triple helices as well as assemblies of triple helices through additional interactions (Bhattacharjee et al., 2005).

Table 2.2 Amino acid composition of collagen from fish and mammalian skin (residue/1000 residues)

Amino acid	Calf [a]	Porcine [b]	Cod [c]	Ocellate puffer fish [c]	Tiger puffer [d]	Carp [e]	Tilapia[f]	Young nile perch [g]	Adult nile perch [g]	Grass carp [h]	Chanel catfish [i]	Largefin longbarbel catfish [j]
Alanine	119	115	107	106	118	118	119	131	134	135	127	116
Arginine	50	48	54	54	53	55	58	55	55	57	52	49
Aspartic Acid	45	44	53	50	45	48	42	55	53	42	48	50
Cysteine	0	0	0	2	0	0	0	0	0	2	0	4
Glutamic Acid	75	72	80	87	68	76	69	0	0	61	80	68
Glycine	330	341	342	351	350	332	356	335	349	334	349	317
Histidine	5	7	8	8	4	5	6	9	8	5	5	6
Hydroxylysine	-	7	7	0	5	7	0	11	10	8	0	11
Hydroxyproline	94	97	51	67		76	82	72	73	65	61	74
Isoleucine	11	10	12	12	7	10	8	13	11	10	12	13
Leucine	23	22	22	23	15	22	20	35	26	22	22	22
Lysine	26	27	29	19	29	28	20	33	31	23	32	27
Methionine	6	6	15	14	14	14	5	14	13	6	10	10
Phenylalanine	3	12	12	10	12	13	13	18	17	17	14	13
Proline	121	123	123	103	113	114	128	118	123	121	93	139
Serine	33	33	59	48	43	35	32	39	38	39	44	36
Threonine	18	16	23	25	29	24	22	32	30	24	26	23
Tryptophan	0	0	0	0	0	0	0	0	0	0	0	0
Tyrosine	3	1	4	4	2	3	3	6	6	2	3	4
Valine	21	22	19	17	22	19	17	25	24	31	23	24

Source: (a) Giraud-Guille et al. (2000); (b) Ikoma et al. (2003); (c) Nagai et al. (2002); (d) Bae et al. (2008);(e) Duan et al. (2009); (f) Zheng et al. (2009); (g) Muyonga et al.(2004); (h) Zhang et al. (2007);(i) Liu, Li and Gue (2007); (j) Zhang, Liu and Li (2009)

The telopeptide is the end of collagen terminal. Glycine is absent in the first fourteen amino acid residues from N-terminus and the first ten residues from the C-terminus. Structure of telopeptide is non-helical accounting for 2% of the molecule (Kadler et al. 1996). Telopeptide are essential for fibril formation of collagen because collagen intermolecular cross links are confined to the end overlap region involving a lysine aldehyde in the telopeptide of one chain and a hydroxylysine of an adjacent chain (Figure 2.3) (Foegeding et al. 1996). In addition, the strength of the collagen fibers and collagen solubility, depending on the formation of covalent cross-links between the telopeptide and adjacent helical domains of collagen molecules (Orgel et al. 2000). Thus, the typical strong rigid nature of skins, tendons and bone are due to the basic structure formed by many of these cross-link collagen fibril.

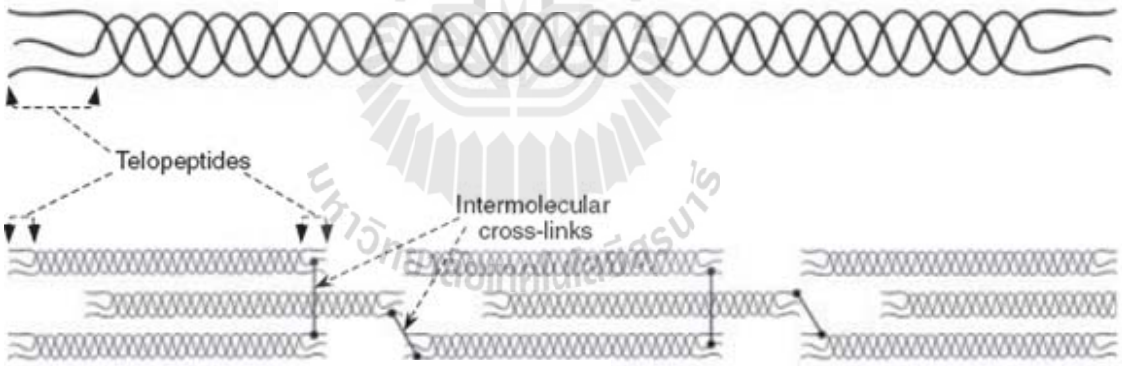


Figure 2.3 Collagen telopeptides and intermolecular cross-links

From: Gelse, Pöschl and Aigner (2003).

2.2 Stability of collagen

2.2.1. Amino acid sequence

The relationship between amino acid composition of collagen and its stability

have been reported. Generally, GlyProHyp sequence is the most common triplet approximately 10.5% in collagen (Ramshaw, Zhah and Brodsky, 1998). Burjandze (2000) reported that total Gly-Pro-Hyp sequence content is one of the main factors affecting collagen thermal stability. Francesco (1998) reported that denaturation temperature (T_d) of $(GlyProPro)_{10}$, $(GlyHypHyp)_{10}$ and $(GlyProHyp)_{10}$ was 41, 65 and 69 °C, respectively. Pro+Hyp rich zones of the molecule are the most likely to be involved in the formation of nucleation zones (Ledward, 1968), leading to higher stability than other sequences. Furthermore, the length of GlyProHyp also affected thermal stability. T_d of $(GlyProHyp)_7$ and $(GlyProHyp)_{10}$ was 36 and 69°C, respectively (Bretscher, Jenkins, Taylor, DeRider and Raines, 2001). Replacement of amino acid residues in X and Y position of Gly-X-Y sequence also affected thermal stability. The most stable tripeptide unit was Gly-Pro-Hyp with T_d 47.3°C. Replacing Pro in the X position led to a decrease in stability like Gly-Glu-Hyp (T_d = 42.9°C) and 15°C for Gly-Trp-Hyp (T_d = 31.9°C) (Persikov, Ramshaw and Brosky, 2005).

2.2.2 Amino acid residue

Proline and hydroxyproline are called imino acid with pyrrolidine ring. Josse and Harrington (1963) demonstrated that pyrrolidine ring residue in the polypeptide chain contributed to structural stability. The content of pyrrolidine ring in *Ascaris* and earthworm collagen was 29% Pro+2%Hyp and 0.8% Pro+17%Hyp, respectively. *Ascaris* and earthworm collagen showed different thermal transition of 22 °C and 52 °C, respectively, indicating that high content of hydroxyproline contributed to high thermal stability. Table 2.3 shows relationship between imino acid content and thermal stability of collagens from fish and mammalian skin. Hydroxyproline has an essential role in stabilizing the triple helix of collagen by inter-chain hydrogen bonding via a

bridging water molecules as well as direct hydrogen bonding to a carbonyl (Bae et al. 2008).Thus, collagen polypeptides that lack hydroxyproline can fold into a triple-helical conformation at low temperatures (Prockop et al. 1979). Proline and hydroxyproline contents vary with species and their living habitat (Jongjareonrak et al. 2005). Fish collagens contain less imino acid than mammalian collagen approximately 5-10%. In addition, lower content of imino acid is generally found in cold-water fish as compared with warm water fish (Easton and Leach, 1977; Muyonga et al., 2004).

Table 2.3 The maximum transition temperature (T_m) or denaturation (T_d) temperature of collagens from various sources.

Sources of skin collagen	Imino acid content[a] (residues/1000 residues)		Tm or Td (°C)		Reference
	ASC	PSC	ASC	PSC	
Calf[b]	215	-	36.3	-	Ogawa et al. (2003)
Young Nile perch[b]	189	-	36.0	-	Muyonga et al. (2004)
Adult Nile perch[b]	196	-	36.0	-	Muyonga et al. (2004)
Unicorn leatherjack[b]	190	-	27.7	-	Ahmad et al. (2010)
Black drum[b]	199	197	34.2	35.8	Ogawa et al. (2003)
Sheepshead sea bream[b]	204	194	34.0	34.3	Ogawa et al. (2003)
Brownstrip red snapper[b]	212	221	31.5	31.0	Jongjareonrak et al. (2005)
Bigeye snapper[c]	193	-	32.5	31.5	Nalinanon et al. (2007)
Carp[c]	190	-	28.0	-	Duan et al. (2009)
Channel catfish[b]	154	-	32.5	-	Liu et al. (2007)
Pacific whiting[b]	160	-	21.7	-	Kim and Park (2004)
Eagle ray[b]	193	-	34.1	-	Nalinanon et al. (2010)
Largefin longbarbel catfish[b]	213	-	32.1	-	Zhang (2009)
Nile tilapia[b]	210	-	32.0	-	Zeng et al. (2009)
Brown backed toadfish	170	-	-	28.0	Senaratne et al. (2006)
Grass carp[b]	186	-	24.6	-	Zhang et al. (2007)

[a]Imino acids include proline and hydroxyproline

[b]Thermal stability was expressed as Td

[c]Thermal stability was expressed as Tm

ASC is acid-soluble collagen and PSC is pepsin-soluble collagen.

2.2.3 Collagen cross-linking

During maturation, collagen fibers can be strengthened and stabilized by covalent cross-linkages (Belitz et al. 2004). Thus, the cross-links confer mechanical strength to collagen fiber. Collagen is also covalently cross-linked which accounts for its poor solubility and that covalent cross-link is unlikely to be disulfide bond because it is almost devoid of cysteine residues. Belitz et al. (2004) proposed that cross-links formation of collagen involves (i) enzymatic oxidation of lysine and hydroxylysine to the corresponding ω-aldehydes, (ii) conversion of these aldehydes to aldols and aldimines, and (iii) stabilization of these primary products by additional reduction or oxidation reactions.

Lysine and hydroxylysine are necessary for natural intramolecular and intermolecular cross-linking. Cross-linking is based upon aldehyde formation from the single telopeptide lysine or hydroxylysine residues, which the lysyl oxidase deaminates. Lysyl oxidase binds to a highly conserved sequence (Hyl-Gly-His-Arg) opposite the N- and C-terminals of an adjacent quarter-staggered molecule. These hydroxylation is related with divalent cross-links which are formed between the collagen telopeptide region and the helical region and trivalent which cross-links connect together two telopeptide chains and one helical domain in a separate collagen molecule (Kuboki et al. 1981). This lysine cross-link does not form randomly trend to occur near the N- and C- terminal of the collagen molecule. The degree of cross-linking in particular tissue increases with age. That is why meat of older animals is tougher than meat form younger animals.

2.3 Source of collagen

2.3.1 Land animals

Collagen from different sources generally has the varying properties, especially the susceptibility to thermal denaturation or cleavage by proteases (Nalinanon et al. 2010). The most common raw materials for collagen extraction are skin or hides, bones, tendon and cartilages. Pig skin was the first raw material used for the manufacture of collagen and gelatin in the 1930s and continues to be the most important material for large scale industrial production (Gómez-Guíllén et al. 2011). It has a wide applicability in various branches of industry. In the non-denatured form, it has pharmaceutical and biomedical uses, such as tissue engineering for implants in humans, inhibition of angiogenic diseases, treatment of hypertension, urinary incontinence and osteoarthritis (Lee et al., 2001). It is used for many fragrance and cosmetic applications (Tzaphlidou, 2004). In the denatured form (gelatin), it is widely used in the food industry (Slade and Levine, 1987). The main sources of collagen nowadays are still the skins of cows and pigs. However, the recent outbreak of bovine spongiform encephalopathy (BSE) is the turning point for alternative sources of safe collagen for industrial uses (Gareis, 2002).

2.3.2 Aquatic animals

Currently, the increasing attention of alternative source for replacement of mammalian collagen has been paid, especially from seafood processing byproducts. Fish skin is a byproduct from fish processing, which serves as an alternative source for collagen extraction. Several studies dealt with the practical utilization of marine animals for collagen production (Koladziejaka, Silorski, and Niecikowska, 1999: Nagai, Araki, and Suzuki, 2002; Nagai and Suzuki, 2000; Ogawa et al, 2004;

Sadowska, Koladziejaka and Niecikowska, 2003). Nevertheless, fish from different species and environment can have different properties of collagens.

Skin of ocellate puffer fish have been extracted by acetic acid and pepsin with the yield of 10.7 and 44.7% respectively (Nagai, Araki and Suzuki, 2002). The balloon fish covered with thick skin and is underutilized species. Huang, Shiau, Chen and Huang, (2011) extracted collagen from this species, with yield of only 4% using acetic acid and 19% with pepsin extraction. Skin of unicorn leatherjacket showed high amount of cross-links at the telopeptide region leading to non- solubilized part after extracting by acetic acid (Ahmad, Benjakul, Nalinanon, 2010).

Collagen derived from marine fish have been investigated whereas collagens from freshwater fish have been less studied (Muyonga, Cole, Duodu, 2004; Zhang et al., 2007; Liu, Li and Guo, 2007; Duan et al., 2009; Zeng et al., 2009; Zhang, Liu and Li, 2009). However, some collagens from freshwater fish have been reported. Collagen from silver carp (*Hypophthalmichthys molitrix*) showed high thermal stability at 34.5-34.8 °C (Motowidło et al., 2008). Yield of carp (*Cyprinus carpio*) skin collagen was higher than those obtained from scale (Duan, Zhang, Du, Yao and Konno, 2009). Channel catfish and largefin longbarbel are freshwater fish without scale. Channel catfish skin was extracted using acid and pepsin. Pepsin showed higher yield with 22.8%, while acid extraction yielded 16.8% (Zhang, Liu and Li, 2009).

Several invertebrate animals have been studied for collagen extraction. Giant red sea cucumber has been considered as a potential source of collagen for nutraceutical and pharmaceutical applications (Liu, Oliveria and Su, 2010). Type I collagen in skin and connective tissue of giant red sea cucumber were isolated by pepsin digestion with yield of 20.8% and 24.3% respectively. Kimura and Matsuura

(1974) reported that collagen extracted from abalone *(Haliotis discus)* consisted of α3 chains, while collagen from skins of octopus and squid had $(\alpha 1)_2\alpha 2$ which were commonly found in various vertebrate collagens. Outer skin waste of cuttlefish (*Sepia lycidas*) was extracted by acetic acid with only 2% yield (dry weigh basis) while 35% yield was obtained after adding 10% of pepsin, and denaturation temperature of this collagen was 27°C (Nagai et al. (2000). Glycosylated hydroxylysines content in squid and lobster was higher than most vertebrate collagen (Kimura an Matsuura. 1974). Collagen from jellyfishs has been extensively studies. The primary structure of rhizostomous jellyfish was similar to collagen from edible jellyfish mesogloea and was different from those from edible jellyfish exumbrella. The rhizostomous jellyfish contained a large amount of the fourth subunit designated as α4 with the denaturation temperature of 28.8 °C (Mizuta et al. 1998).

Alligator bones present a highly significant part of the wastage from alligator meat processing. Most of collagen from alligator bone was identified as type I collagen. The imino acid contents were close to sub-tropical fish species and slightly lower than that of calf collagen (Wood et al., 2008).

2.4 Isolation of collagen

Isolation of collagen is generally separated into three main steps, including sample preparation, extraction and recovery.

2.4.1 Preparation of raw materials.

Generally, collagen can be extracted from animal skin, bone, scale and so on. Those materials contain a number of contaminants including noncollagenous proteins, lipids, pigment calcium and other inorganic matters are found in scale and bone. Size

reduction and cleaning are used to perform before further chemical pretreatments. Extraction process is able to inactivate proteases responsible for collagen degradation (Regenstein and Zhou 2007). Alkaline pretreatment is widely used for removing noncollagenous proteins and pigment at 0.1 M NaOH (Nagai and Suzuki 2002). Furthermore, alkaline can attack the telopeptide region of the collagen molecule during pretreatment, leading to lower yield. Yang et al (2007) reported that high concentration of alkaline pretreatment with extended time decreased the yield of collagen and gelatin of channel catfish. Raw materials, such as bone and scale, contain high amount of calcium. Thus, decalcification step is necessary for those raw materials. Decalcification can be achieved by using inorganic acid as hydrochloric acid or chelating agents such as ethylenediaminetetraacetic acid (EDTA) (Kittiphattanabawon et al. 2005).

2.4.2 Extraction

The extraction carried out by acid yields collagen termed acid-soluble collagen (ASC). This process results in relative low yield. Pepsin has been developed to improve the extraction yield and the resultant collagen is named pepsin soluble collagen (PSC).

2.4.2.1. Acid solubilization process

Amino acid composition of collagen indicates that collagen is a hydrophilic protein because of the greater content of acidic, basic and hydroxylated amino acid residues than lipophilic residues. Therefore, it swells in polar liquids (Johnstion-Banks 1990). Consequently, acid solution is used for collagen extraction. Collagen from total tissue can be isolated by direct extraction with organic acids such as acetic, citric and lactic or inorganic acid, such as hydrochloric. Moreover, type and

concentration of acid affected yield and properties of collagen. Cheng, Hsu, Chang, Lin and Sakata (2009) studied the influence of different acids on the extractability of collagen: citric acid, hydrochloric acid, acetic acid and lactic acid. Among four acids studied, acetic acid showed the highest extraction yield, whereas hydrochloric acid exhibited the least efficacy for collagen extraction. In acidic condition, the positive charge of collagen polypeptides becomes dominant. As a consequence, repulsion among tropocollagen is enhanced, leading to the increased solubilization. In case of hydrochloric acid extraction, anions (Cl^-) can bind with positive charged amine groups of protein reducing electrostatic repulsion forces between charge groups. This leads to the close contact of collagenous fibers and decrease in bonds with water and swelling capacity (Skierka and Sadowska 2007). Additionally, acid can partially inactivate endogenous proteases involved in collagen degradation. As a result, the enzymatic breakage of intrachain peptide bonds of collagen during extraction decreased (Zhou and Regenstein 2005).

Swelling capacity is an important factor of collagen extraction because it can weaken the binding ability between collagen interior molecular structure and increase protein unfolding by disruption of non-covalent bonds (Cheng, Hsu, Chang, Lin and Sakata, 2009). Swelling process is related to ionization constant (K_a) of various acids. The K_a value of hydrochloric acid, citric, acetic acid and lactic acid are -8, 7.4×10^{-4}, 1.7×10^{-5} and 3.5×10^{-8}, respectively. Strong acid (high K_a value) was effective to raise collagen extraction rate. However, it was known that an extremely low pH value resulted in denaturation and digestion of collagen during extracting. Consequently, acetic acid is frequently used as a solvent for collagen extraction. The small molecular size and low ionization constant of acetic acid are a key factor

inducing swelling (Gómez-Guillén, Sarabia, and Montero, 2001).

2.4.2.2. Pepsin solubilization process

Raw materials with high amount of molecular cross-links result in the lower extraction yield and only acid solubilization process cannot improve the yield of collagen. To increase the extraction yield of collagen, some proteases have been applied to cleave peptides specifically in telopeptide region of collagen, leading to increased extraction efficiency (Nagai et al 2002; Nalinanon et al. 2007). Recently, acid combined with proteases such as pepsin, trypsin or papain was carried out to

Figure 2.4 The cleavage of telopeptide region of collagen by pepsin (a). Pepsin-soluble collagen (b)

Source : Leo et al. (2005)

increase the yield of collagen extraction (Lin and Liu, 2007). Acid extraction with pepsin hydrolysis was widely used for collagen extraction from skin of brownstripe red snapper, fish waste material, albacore tuna and silver line-grunt skin, bone and scale of black drum and sheepshead seabream and black drum and sheepshead skin (Jongjareonrak, 2006; Nagai and Suzuki, 2000; Noitup, Garnjanagoonchorn and

Morrissey, 2005; Ogawa, Moody, Portier, Bell, Schexnayder and Losso, 2003; Li, Liu, Gao and Chen, 2004). Pepsin can remove non-helical telopeptide regions (Figure 2.4) of collagen without damaging their triple helix integrity which leads to an increase in collagen solubility in acid solvent (Nalinanon, Benjakul, Visesanguan and Kishimura, 2007).

Yields of acid-solubilized collagen and pepsin-solubilized collagen from various sources are compared in Table 2.4. Collagen yield obtained from balloon fish skin was estimated to be 4% and19.5% (dry weight basis) based on ASC and PSC, respectively. Yield of other species from different skins has been reported; largefin

Table 2.4 Yields of acid-solubilized collagen and pepsin-solubilized collagen from various sources

Raw materials	% Yield (Based on wet basis)		References
	ASC	PSC	
Brownstrip red snapper *(Lutjanus vitta)*	9.0[b]	4.7[b]	Jongjareonrak et al. (2005)
Brownstrip red snapper *(Lutjanus vitta)*	9.0[b]	4.7[b]	Jongjareonrak et al. (2005)
Channel catfish *(Ictalurus punctaus)*	25.8[b]	-	Liu et al. (2007)
Largefin longbarbel carfish skin (*Mystus macropterus*)	16.8[b]	28.0[b]	Zhang et al. (2009)
Deep-sea redfish *(Sebastes mentella)*	47.5[b]	92.2[b]	Wang et al. (2007)
Grass carp *(Ctenopharyngodon idella)*	46.6[b]	-	Zhang et al. (2007)
Skate skin (*Raja kenoiei*)	8.9 [C]		Hwng et al. (2007)
Nile tilapia skin (*Oreochromis niloticus*)	39.4[b]	-	Zhang et al. (2009)
Carp skin (*Cyprinus carpio*)	41.3[b]		Duan et al. (2009)
Unicorn leatherjacket *(Aluterus monoceros)*	4[b]	19.5[b]	Ahmad et al. (2011)
Rhizostomous jellyfish mesogloea (*Rhopilema asamushi*)	-	32.5[b]	Nagai et al. (2000)
Balloon fish *(Diodon holocanthus)*	4.19	-	Huang et al. (2011)
Cuttlefish outer skin (*Sepia lycidas*)	2[b]	35[b]	Nagai et al. (2001)
Common minke whale (Balaenoptera acutorostrataunesu)	0.9	28.4	Nagai et al. (2008)
Arabesque greenling skin (*Pleurogrammus azonus*)	30.3[b]	14.0[b]	Nalinanon et al. (2010)

[a]All procedures were performed at 4ºC
[b] Based on dry basis.
[c] Based on hydroxyproline content.

longbarbel catfish (ASC: 16.8%, PSC: 20.8%), channel catfish (ASC: 25.8%, PSC: 38.4%) and black drum (ASC:2.3%, PSC: 15.8%). (Hung, Shiau, Chen and Huang, 2011). Drake et al. (1996) reported that most of intra-and intermolecular cross-links found in collagen occur through the telopeptide region and these intramolecular cross links are broken on pepsin digestion. Cleavage of telopeptide region by pepsin is the conversion of cross links such as one β-chain (dimer) to two α-chains (Nagai et al 2001) or γ-chain (trimer) to α-chains, which can be ease for extraction. Collagen solubility has enhanced after cross links are cleaved by pepsin, consequently the yield of collagen was significantly increased.

Kolodziejska et al., (1999) reported that temperature of solution correlated with the collagen extraction yield. The solubilization of collagen increased with increasing temperature and completely solubilized at 45°C. Aukkanit and Gjanjanagoonchon (2010) studied the effect of four extraction temperatures: 4, 10, 20 and 28°C, on collagen properties and yield. They reported that extraction at 10°C gave the highest yield of collagens whereas higher temperatures at 20 and 28°C resulted in lower yield due to low molecular weight peptide fragments. Furthermore, low temperature extraction resulted in high thermal stability of collagens. Collagen extracted 4 and 10°C showed denaturation temperature 39.5 and 37.5°C respectively. Extraction temperatures played a crucial role on collagen yield and properties.

Moreover, yield and properties of collagen would be influenced by the source of the raw material, nature and concentration of acid, alkali or protease used during pretreatment and the temperature and time of pretreatment and extraction (Regenstein and Zhou 2007).

Animal age is one of factors affecting collagen properties. The number of cross-links in collagen increased with age of the animal. Generally, muscle from younger animals contains more collagen (Foegeding et al., 1996). Stability of collagen increased with the amount and stability of cross-link, which increased with age of animal. Collagen with more extensive crosslinks are required more severe process to break the crosslinks and induce collagen denaturation and solubilization into solution.

The amount of collagen cross linking increases with starvation of fish. Foegeding et al. (1996) reported that starving fish contain more collagen with a greater degree of cross-linking than fish which are fed well. This was because myocommata was thickened during starvation.

2.4.3 Recovery of collagen

Salt precipitation is widely used to recover collagen after extraction. Sodium chloride a final concentration of 2.6 M in the presence of 0.05 M Tris (hydroxylmethyl) aminomethane, pH 7.5, is generally used for collagen precipitation. However, the concentrations of NaCl used in different methods are varied from 0.9-2.6 M (Nagai and Suzuki 2000) and salt concentration can be adjusted to maximize the collagen recovery and removal of impurities.

2.5. Characterization of collagens

2.5.1 Fourier transform infrared (FT-IR) spectroscopy

FT-IR is an optical technique that provides information about the molecule vibration of any stable electronic state of the molecule system (Cárcamo et al., 2012). FT-IR is a nondestructive tool for the structural or conformational characterization of biological system with several advantages and it has been widely used to elucidate

protein structure and monitor changes in the secondary structure of protein. The intensities of IR absorption provide quantitative information about the sample contents, depending on the nature of the molecular bond, structure and environment. This technique is based on the absorption of IR radiation with specific frequencies that are characteristic of structure (Belbachir et al., 2009).

The infrared spectrum of a sample is recorded by passing a beam of infrared light through the sample. When the frequency of IR is same as the vibration frequency of a bond, then absorption of that IR radiation occur. Thus, the frequency of the vibrations can be associated with a particular bond type. Mid-infrared wave number range of 4,000-500 cm^{-1} is widely used for monitoring collagen structure (Bellbachir et al. 2009). Table 2.5 shows the IR absorption wave number of amide band and their associated functional groups.

Table 2.5 FT-IR peak locations and assignment for structure of collagens.

Region	Peak wave number (cm^{-1})	Assignment	References
Amide A	3400-3440	N-H stretching vibration and the existence of hydrogen bonds.	Woo et al. 2008
Amide B	2924-2926	CH_2 asymmetrical stretching vibration.	Muyouga et al. 2008
Amide I	1600-1700	Carbonyl groups (C=O bond) stretching vibrations. It is used to determine the polypeptide secondary structure, - 1650-1658 : α-helix - 1625-1640 : β-sheet - 1641-1648 : random coil	Payne and Veis 1988; Belbachir et al. 2009
Amide II	1531-1555	N-H bending coupled with C-N stretching.	Muyonga et al. 2004
Amide III	1300-1180	C-N stretching and N-H bending from amide linkages and wagging vibrations from CH_2 groups from glycine backbone and proline side chains.	Belbachir et al. 2009

The collagen triple helix is a unique protein motif defined by the supercoiling of three polypeptide chains in a polyproline II conformation (Shoulders, and Raines, 2009). Generally, collagen from fish skins showed similar FT-IR spectra, such as carp skin, largefin longbarbel catfish, Nile perch, channel catfish (Duan et al. 2009; Zhang, Liu and Li 2009; Muyonga et al 2004; Liu et al 2007). Wang et al. (2007) and Nalinanon et al. (2010) showed similar conclusion that FT-IR spectra between PSC and ASC collagen of deep-sera red fish and arabesque greenling were different. Zhang et al (2009) found similar spectra of largefin long barbel catfish extracted by acid and pepsin. Amide A band is associated with the N-H stretching frequency (Abe and Krimm, 1972). A free N-H stretching vibration occurs in the range 3400-3440 cm^{-1} and this position will be shifted to lower frequencies when that N-H group of peptide is involved in hydrogen bonding. (Muyonga et al., 2004). Duan et al (2009) reported that amide A band of carp skin was shifted to 3324 cm^{-1} indicated that hydrogen bond existed in carp collagen was similar to collagen from channel catfish which was detected at 3330 cm^{-1} (Liu et al., 2007). Furthermore, collagen extracted by high temperature exihibited a broader amide A band than collagen extracted at low temperature (Muyonga et al., 2004).

The amide I band are characteristic frequencies in the range of 1600-1700 cm^{-1}which is mainly associated with the stretching vibrations of the carbonyl groups (C=O bond) along the polypeptide backbone and is the most useful for infrared spectroscopic analysis of the secondary structure of protein (Payne and Veis 1988, Wang et al. 2007, Kittiphattanabawon et al. 2010). Amide II (~ 1550 cm^{-1}) is associated with N-H bending couple with C-N stretching (Muyonga et al. 2004, Woo et al. 2008). Amide III band of collagens is observed at 1234-1238 cm^{-1}, which is

related to C-N stretching and N-H of CH_2 of glycine backbone and proline side chain (Mohd Nasir et al. 2006).

Amide I band, amide II band and amide III band, which are known to be related to the degree of molecular order and involved with the triple helical structure of collagen. Changing of those amide bands are associated with loss of triple helix state (Muyonga et al. 2004). Furthermore, they have been used to study collagen cross-linking and denaturation (Paschlis et al., 2001; Brown, Huston, Fink and Veis, 1983). The spectral changes are indicative of change in collagen secondary structure. An increasing in intermolecular interaction by hydrogen bonds leads to a changing in shift to lower wave number of amide I, amide II, and increase in intensity of amide III. (Jakobsen et al., 1983).

2.5.2 Protein component

SDS-PAGE is the most widely used for analyzing protein mixture, monitoring protein purity and determining their molecular weight. It is based on the separation of proteins according to size. Collagen cross-link and collagen molecular weight distribution can be elucidated by SDS-PAGE. Table 2.6 shows the molecular weight distribution often found in collagen, β-chain is intermolecular cross-links of two α chains whereas γ-chain is intramolecular cross links of three covalently crosslinked α-chains.

Zhang (2007) reported that a high amount of cross-links at the telopeptide region as well as other inter-molecular cross-links leads to low solubility in acid and high thermal stability. Commonly, composition of type I collagen from animal skin consists of two identical α1-chains and one α2-chain, and occasionally α3 chain. The α3, if present, cannot be separated under the electrophoretic conditions because α3 (I)

Table 2.6 Molecular weight distribution showing the major structure components of collagen

Moleclar fraction	Description
Q	Very high molecular weights, of 15-20 × 10^6 daltons and thought to be branched in chracter owing to thrir inabilityto penetrate the gel successfully
1 - 4	Oligomer of α-chains, level of five to eight
X	Oligomer of four α-chains
γ	285 kDa
β	190 kDa
α	95 kDa
α-peptide	86 kDa
α-,β- and γ-peptide	Seen as tailing their parent peaks

From; Banks (1990).

migration is the same position as α1(I) (Kimmura and Ohno. 1987). The α1-chain and α2-chain could be dimerize covalently into β-component as β12-dimer (one α1 and α2-chain) or β22-dimer (two α2-chains) or polymerize into higher MW components. High molecular weight cross-link leads to low band intensity of α1and α2 (Kimura et al., 1981). Intensity ratios of α1/α2 also showed more than two fold. Collagen molecular weight pattern from arabesque greenling showed high content of β12 and β22 dimer and intensity ratios of α1/α2 showed twofold ((Nalinanon et al. 2010). The similar result was also observed in collagen of underutilized fishes (tiger puffer, dusky spinefoot, sea chub, eagle ray, red stingray and yanti stingray) showing that collagens were type I formed as a heterotrimer by two α1-chain and one of α2-chain. However, the band intensity of α1-chain was not twofold greater than that α2-chain. It was suggested that these collagens might contain inter-and intra-molecular crosslinked components, β (dimer) or γ (trimer) components (Bae et al. 2008). Previous studies indicated that the electrophoretic pattern of collagens from skins and bone did not contain disulfide bonds as they showed similar molecular weight pattern under non reducing and reducing conditions (Benjakul et al 2010).

2.5.3 Thermal stability

Differential scanning calorimetry (DSC) is the most widely used techniques to analyze thermal characteristic of the materials. It is used in many applications, such as polymers, glasses, ceramics, food, proteins and life science materials (Paul 2008). DSC principle is a measure of energy change that occurs in transition temperature. The maximal transition temperatures (*T*m) or denaturation temperatures (Td) of collagen from different sources vary with many factors, such as imino acid contented and living environment. Thermal stability of the collagen triple helix is attributed to the hydrogen bonded networks, mediated by water molecules, which connect the hydroxyl group of hydroxyproline in one stand to the main chain amide carboxyl of another chain (Babu and Ganesh 2001) Hence, difference in hydroxyproline content might determine the denaturation temperature of collagens (Benjakul et al 2012). Furthermore, Muyonga et al (2004) elucidated that denaturation temperature of collagen rather depended on the amount of hydroxyproline than proline residue even both of them are the same imino acid.

The living environment of animal has been reported to affect collagen thermal stability. Collagens derived from fish species living in cold environment have lower thermal stability than those living in warm water. Imino acid content is related with thermal stability of collagens, which is correlated with habitat temperature (Foegeding et al. 1996). Kimura and Ohno, (1987) and Sadowska et al (2003) reported that Td of collagen from Alaska pollack and Baltic cod skin was 16.8 and 15ºC, respectively. In addition, cold water fish species, such as arabesque greenling, deep sea redfish and cod skin showed Td around 15-16ºC (Nalinanon et al. 2010; Wang et al. 2007; Duan et al. 2009). Several collagens from cold water fish showed Td at 25-28ºC including

ocellate and tiger puffer fish, japanese sea bass, chub mackerel and blue head shark (Duan et al. 2009; Zhang et al. 2007; Nagai et al. 2002; Bae et al. 2008; Nagai and Suzuki, 2000). Fish collagen from tropical and sub tropical water, such as young and adult Nile perch, showed T_d of 36ºC, which was higher than most other fish collagens (Muyonga et al., 2004).

2.6 Anigotensin I-converting enzyme (ACE)

2.6.1 Renin Angiotensin System

Rennin angiotensin system (RAS) is the main regulator of blood pressure homeostasis (Eriksson et al., 2002). The rennin-angiotensin system is activated after loss of blood volume or a drop in blood pressure. Angiotensin is a glycoprotein that is synthesized from the juxtaglomerular apparatus of the kindney and secreted into the bloodstream by liver. Renin converts the plasma protein angiotensin to inactive peptide angiotensin I. Angiotensin I converting enzyme (ACE) converts angiotensin I into angiotensin II by cleaving at the C-terminal, removing the dipeptide His-Leu (Figure 2.5).

ACE is a trans-membrane dipeptidly peptidase which occurs in two different isomers, somatic and testicular which is transcribed from a single gene from different initiation sites (Oscar, 2005). Angiotensin II also acts on the kindney to promote sodium re-absorption and decreases water excretion, by stimulating aldosterone secretion from adrenal cortex and by acting on AT1 receptor (Kovacs et al., 2002). An increase in extracellular fluid volume results in an increase in constricts arteries and elevates blood pressure (Barbosa-Filho et al., 2006). ACE inhibitors lower blood pressure by inhibiting the formation of angiotensin II, thus relaxing the arteries,

allowing more flexibility. Furthermore, ACE inhibitor can improve the pumping efficiency of a failing heart.

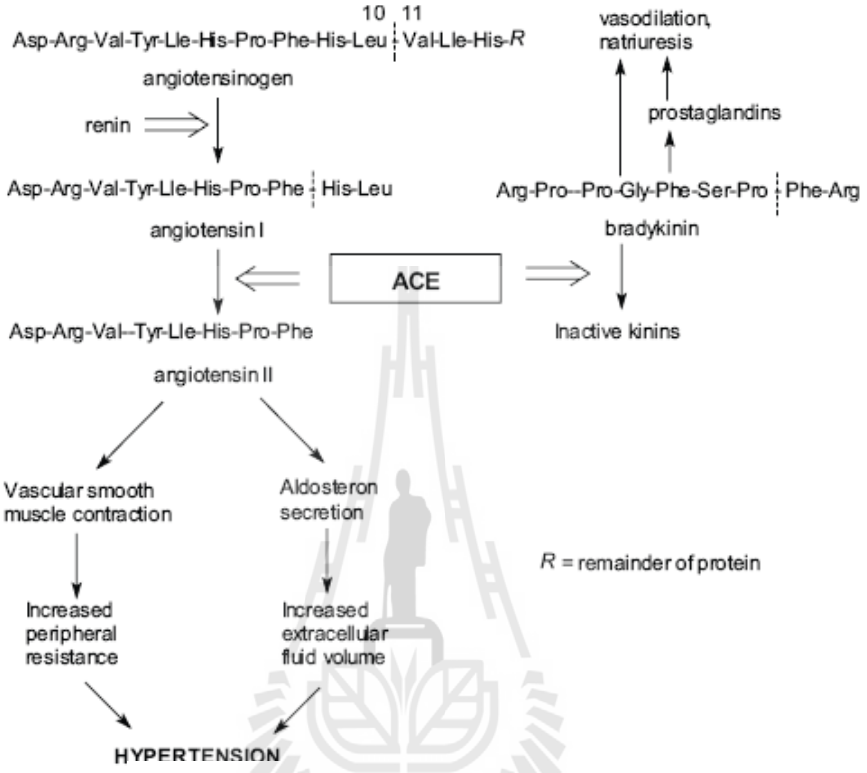


Figure 2.5 Hypertensive mechanism of angiotensin

From: Barbosa-Filho (2006).

2.6.2 Structure of angiotensin-I converting enzyme.

ACE (dipeptidyl carboxpeptidase, EC 3.4.15.1) is classified as a zinc and chloride dependent metallopeptidase (Li, Le, Shi and Shrestha, 2004). It is a membrane-bound protein located at the cell surface. The membrane-bound ACE may be cleaved from the cell surface to become a soluble form. Soluble ACE is found in the blood, urine, lung edema, amniotic fluid, cerebrospinal fluid, lymph, seminal plasma and prostate (Sturrorock et al., 2004). ACE is a two domain enzyme with an

N-terminal domain (612 amino acids) and C-terminal domain (650 amino acids). Each domain contains an active zinc-binding motif, His-Glu-Met-Gly-His, that is a characteristic of zinc site and it is activated by chloride and has a broad in *vitro* substrate. (Sturrock et al., 2004).

Figure 2.6 shows the binding model between active site of ACE and inhibitor peptides. The zinc ion of ACE is located between subsites S_1 and S_1' to participate in hydrolytic cleavage on the peptide bond of the substrate, resulting in a release of the dipeptide product. Hydrogen bond donating group are located between subsite S_1' and S_2' which can form ionic interaction with the C-terminal negativity charged amino acid carboxy group of the substrate (Ondetti and Cushman, 1982). Monovalent anions, particularly chloride ions, can enhance the activity of ACE. The activation of the C-domain is highly dependent on chloride concentration, whereas the activation of the N-domain is not dependent on that chloride concentration (Wei et al., 1991).

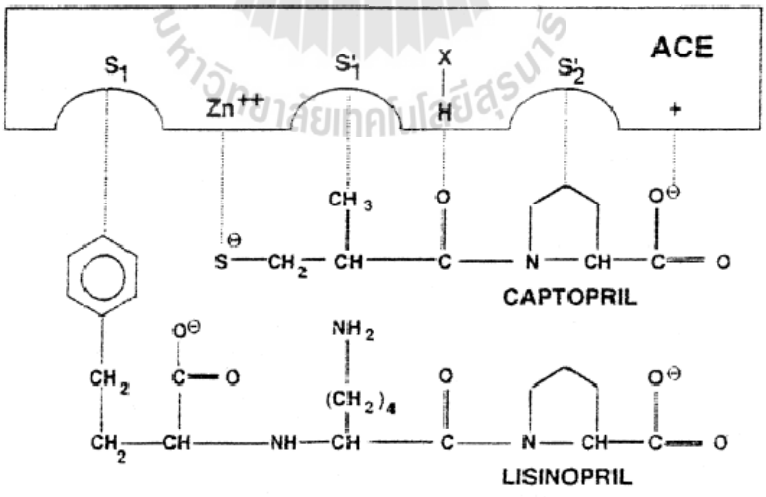


Figure 2.6 Propose of binding model for interaction between substrate and the active site of angiotensin I converting enzyme. From: Li, Le, Shi and Shrestha (2004).

2.6.3 *In vitro* assay for ACE inhibitory activity

ACE inhibitory activity is estimated by measuring ACE activity on cleaving the synthetic substrate hippuryl-L-histidyl-L-leucine (HHL) to form hippuric acid (HA) and His-Leu. The amount of hippuric reflecting the amount of His-Leu cleaved is measured by a spectrophotometric method (Figure 2.7). (Cushman and Cheung, 1971). This method has been widely used by pharmaceutical and food industries and modified by some researchers by changing concentration of ACE, substrate types and substrate concentration. Spectrophotometric measurement has been modified to high performance liquid chromatography (HPLC) (Wu and ding, 2002). Instead of HA extraction and spectrophotometric quantification, HPLC-column has been used for separation of HHL and HA.

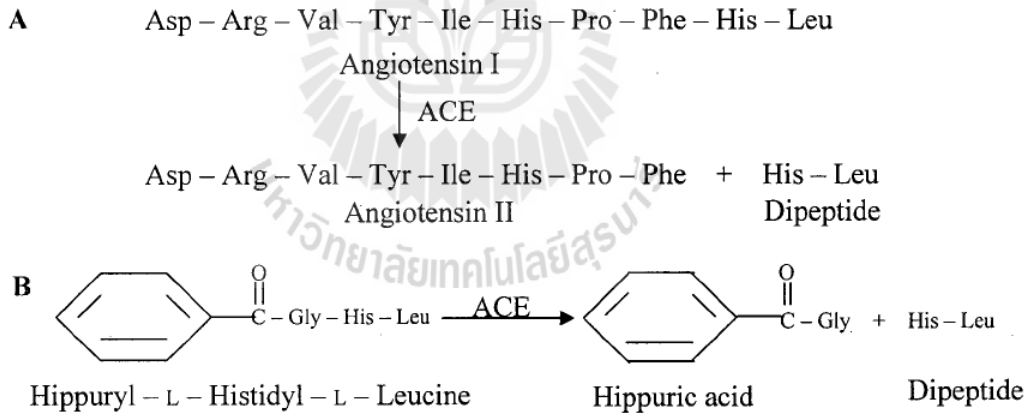
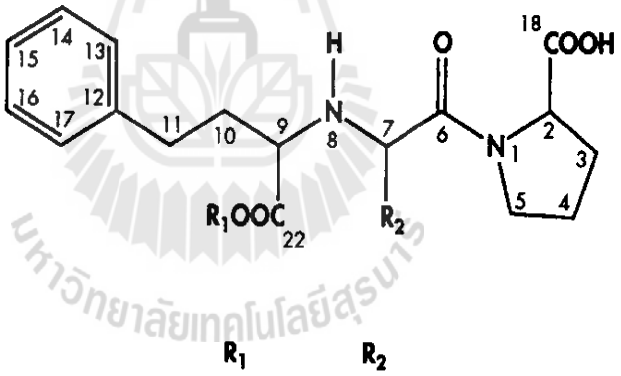


Figure 2.7 Activity of ACE on (A) angiotensin I and (B) hippuryl-L-histidyl-L-leucine From; Cushman and Cheung, (1971)

2.7 Angiotensin I-converting enzyme inhibitory peptides.

2.7.1 ACE commercial inhibitor.

ACE inhibitors are one of several classes of pharmacological agent that have been widely used in hypertensive therapy. Captopril and lisinopril are known as potent synthetic ACE inhibitors (Ondetti et al., 1971; Cohen, 1985). Figure 2.8 shows the structure of commercial inhibitors, namely enalaprilat, enalapril and lisinopril. Enalapril is an ester prodrug of the pharmacologically active enalaprilat. Following oral administration of enalapril, the parent compound (Enalaprilat) is formed by bioconversion of enalapril. Enalalaprilat is poorly absorbed from the gastrointestinal tract.



	R_1	R_2
Enalapril	$-C_2H_5$	$-CH_3$
Enalaprilat	—H	$-CH_3$
Lisinopril	—H	$-(CH_2)_4NH_2$

Figure 2.8 Chemical structures of the ACE-inhibitors enalaprilat, enalapril and lisinopril

From ; Swaan, Stehouwer and Tukker (1995)

Lisinopril is a structural homologue of enalaprilat, differing only in the second amino acid side chain. Lisinopril inhibitor ACE is also poorly absorbed in gastrointestinal. Its oral bioavailability is only 25-30%, but is has a longer duration of action than enapril (Swaan, Stehouwer and Tukker ,1995). Captopril is a commonly prescribed ACE inhibitor drug. It converts to an active di-acid form in the liver or intestine (Sica, 2003). Captopril binds tightly to ACE at its active site and competes with angiotensin I (Cushman et al., 1987). However, high level inhibitor activity of these 3 drugs is usually accompanied by significant side-effects such as dry cough, skin rashes, taste disturbances, agioedema and hyperkalemia (Atkinson and Robertson, 1979; Sica, 2003)

2.7.2 Angiotensin-I converting enzyme inhibitory peptides from food

In recent years, some food proteins have been identified as sources of ACE inhibitory peptides and are currently known an bioactive peptides. (FitzGerald, Murray and Walsh, 2004). Bioactive peptides are inactive within the primary sequence of parent protein and can be degraded into the animal or human body through either in *vitro* or in *vivo* by proteolysis of the parent protein molecule, such as during gastrointestinal digestion or during food processing (Meisel, 1997). Consequently, they are liberated in the bodies. Bioactive peptides can affect numerous physicological functions of organisms, such as they may act as antihypertensive agents (Vercruysse, Camp and Amagghe, 2005). Bioactive peptides have been studied in many different food sources and among the different classes of bioactive peptides, the anitypertensive peptides are the best known (Yamamoto, Ejiri and Mizuno, 2003). The main sources of ACE inhibitory peptides are dairy products, fish and plant proteins.

2.7.2.1 ACE inhibitory peptide derived from plants

Plant proteins with antihypertensive activity are mung bean, rice, zein , sesame, spinach and soybean (Hong, Ming, Yi, Zhanxia , Yongquan and Chi, 2008). ACE inhibitory properties of different enzymatic hydrolysates of glycinin soybean have been studied by Mallikarjan et al., (2007). Glycinin peptide showed IC_{50} of 169 μM. The peptide which was isolated from wheat gliadin hydrolysates were inhibited the hypertensive activity (Motoi et al., 2004). Soybean peptide isolated from Korean fermented soybean paste has been reported to exert angiotensin I converting enzyme (ACE) inhibitory activity in vitro peptide sequence of His-His-Leu (HHL) It was powerful ACE inhibitory peptide and decreased blood pressure after 5mg/kg of body weight have been injected to rat. (Shin et al., 2001). Table 2.7 shows the plant protein sources with ACE inhibitory activity.

Table 2.7 ACE inhibitory peptide derived from plant.

Amino acid sequence	IC_{50} (μM)	Origin
VLIVP	169	Glycinin
LVY	1.80	Seasame
LSA	7.81	Seasam
LKY	0.78	Seasame
LQP	1.04	Seasame
IVVA	315.3	*Cholorella vulgaris, spirulina platenis*
AEL	63.8	*Cholorella vulgaris, spirulina platenis*
FAL	26.3	*Cholorella vulgaris, spirulina platenis*
IAPG	11.4	*Cholorella vulgaris, spirulina platenis*
KDYRL	26.5	Mung bean
LRY	0.15/0.23	Pea
MRWRD	2.1	Spinach Rubisco
MRW	0.6	Spinach Rubisco
VW	1.4	Sake lees
LRP	0.27	α-Zein
LSP	1.7	α-Zein
TQVY	18200	Rice
RDHP	-	Rice

Modified from: Li et al. (2008)

2.7.2.2 ACE inhibitory peptide derived from milk proteins

Bioactive peptides of milk are latent inactive form and proteolysis may be required to release of bioactive peptides. Meisel, Goepfert and Guenther (1997) reported that bioactive peptide can be released by fermentation. Furthermore, activity of gastrointestinal proteinases (pepsin, trypsin and chymotrypsin) can release ACE inhibitory peptides (Maruyama et al., 1987). Therefore, hydrolysate of whole milk, whey protein are potentially good source of ACE inhibitory peptide (Hong et al., 2008). Fermented milk drinks, cheese or yoghurt have been reported to contain ACE inhibitory peptides that are produced by proteinases from various bacterial strains (Gobbetti et al., 2000; Meisel et al., 1997). The majority of milk protein peptides that have potency for ACE inhibitor usually showed IC_{50} of 100-500 μmol/L, such as β-lactoglobulin derived 2 types of peptide sequences, IPA (β-lactosin A) and ALPM (β-lactosin B) with IC_{50} of 141 and 928 μmol/L, respectively (Meihel, 2005).

2.7.2.3 ACE inhibitory peptide derived from egg

Ovalbumin from egg showed antihypertensive peptide after hydrolysis by enzymes (Cheung et al., 1980). Crude egg hydrolysate obtained from pepsin, trypsin and chymotrypsin showed ACE inhibitory activity. Miguel et al., (2004) have been reported that the most active hydrolysates were obtained after treatment with pepsin and the highest ACE inhibitory activity was found at molecular weight lower than 3 kDa.

2.7.2.4 ACE inhibitory peptide derived from animals

Some ACE inhibitory peptides are derived from animals, such as fish, chicken, and cattle. The ACE inhibitory peptide, Hyp-Gly-Leu-Hyp-Gly-Phe, was isolated from chicken breast muscle extract (Saiga et al., 2006). Chicken leg bone

protein were hydrolyzed by Alcalase and ACE inhibitory. The skate skin hydrolysates were obtained by enzymatic hydrolysis using Alcalase, α-chymotrypsin, neutrase, pepsin, papain and trypsin. Peptides derived by α-chymotrypsin had the highest ACE inhibitory activity compared to other hydrolysates (Lee, Jeon and Byun, 2011).Byun and Kim (2001) isolated peptides from Alaska pollock skin with the sequence of Gly-Pro-Leu and Gly-Pro-Met and IC_{50} of 2.6 and 17.13 μM, respectively.

2.8 Structure requirement of ACE inhibitory peptides

Binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the inhibitor (Ondetti and Cushman, 1982). This fact had been confirmed by elongating the dipeptide with certain amino acid and it was found that tripeptides are more potent than dipeptides (Li et al., 2004). Amino acid residues with bulk side chains as well as hydrophobic side chain were preferred for dipeptides, whereas the most favorable residues for tripeptide at the carboxyl terminus were aromatic amino acid. Di- and tri- peptides can be absorbed directly from the digestive tract into blood circulatory showing the ACE inhibitor action. (Mathews et al., 1976). Furthermore, the long chain peptide is highly susceptible to proteolytic enzymes in the gastrointestinal tract (Walter et al., 1996)

The binding model between peptide and ACE is proposed in Fig 6. It shows that ACE has three active sites, S_1, S_1' and S_2'. Consequently, amino acid sequence is important factor affecting inhibitory activity. ACE appears to prefer a substrate or competitive inhibitors containing hydrophobic (aromatic or branched side chain) amino acid residues at C-terminal positions (Li et al., 2004). Many studies reported that peptides containing tryptophan, phenylalanine, tyrosine or proline at the C-

terminal have a high potent inhibitory activity. Furthermore, hydrophobic-hydrophilic property of peptide is an important factor affecting the inhibitory peptide. Highly hydrophilic property usually gives weaker or no activity because it makes the peptide inaccessible on the active site of ACE.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Raw material

Tilapia skins (TS) were donated by Khun-Serb Industry (Samutsakorn, Thailand) and hybrid catfish (HS) were obtained from a local market at Nakhon Ratchasima (Thailand). Samples were packed in a polystyrene foam box filled with ice and transported to Suranaree University of Technology laboratory. Upon arrival, scales and residual meat were removed from tilapia skins and descaled skins were washed with cold tap water (≤ 10°C). Hybrid catfish was cleaned with cold water and skin was removed then placed in ice water. Thereafter, both skins was cut into small pieces (0.2×0.2 cm^2) using a pair of scissors and were lyophilized, kept frozen at -20°C throughout the study.

3.1.2 Chemicals

Calf skin type-I acid-soluble collagen, proteinase K, angiotensin I converting enzyme (ACE) from rabbit lung, hippuryl-histidyl-leucine (HHL), pepsin from porcine stomach mucosa, Proteinase K and trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma Co. (St. Louis, MO, USA). All other reagents used were of analytical grade. Reagents used for gel electrophoresis were purchased from Promega (Madison, WI, USA) Alcalase 2.4 L was gifted from Novozymes (Bagsvaerd, Denmark). Trypsin from hog pancreas was purchased from Biochemika

(Buchs, Switzerland). All other reagents used were of analytical grade.

3.2 Extraction of collagen

Tilapia and hybrid catfish collagen were extracted according to the method of Kittiphattanabawon et al. (2005) with slight modifications. All processes were performed at 4°C with continuous stirring. Lyophilized skins were soaked in 0.1 M NaOH at a ratio 1:30 (w/v) for 48 h with a change of solution every 8 h for removing non-collagenous proteins and pigments. Fat was removed using 10% (v/v) butyl alcohol at a ratio 1:30 (w/v) for 24 h with a change of solution every 8 h. The samples were then washed with distilled water until pH of water became neutral.

The defatted skins were added 0.5 M acetic acid at a ratio 1:50 (w/v), and homogenized for 3 min and stirred for 72 h at 4°C. The homogenates were centrifuged at 10,000×g for 30 min at 4°C.The precipitate were re-extracted with 10 volume of 0.5 M acetic acid for 12 h, and supernatants were centrifuged at 10,000×g for 30 min at 4°C. Supernatants were combined and NaCl was added to a final concentration of 2.6 M in the presence of Tris-HCl 0.05 M to precipitate collagen, which was collected by centrifugation at 10,000×g for 50 min at 4°C. The collected collagen was dissolved in 0.5 M acetic acid and dialyzed against deionized water using 10-kDa molecular weight cut off (MWCO) for 3 days at 4°C with changes of deionized water every 24 h until neutral pH was obtained. The precipitates were lyophilized and kept frozen at -20°C throughout the study.

3.3 SDS-PAGE

SDS-PAGE was performed by the method of Laemmli (1970). Collagens were

dissolved in 5% SDS and heated at 60°C for 5 min. Samples were mixed with sample buffer (0.5M Tris-HCl, pH 6.8 containing 5% sodium dodecyl sulfate (SDS) , 20% glycerol) at the ratio 1:2. Electrophoresis was performed on 7.5% running gel and 4% stacking gel. Fifteen µg protein were loaded, which were separated electrophoretically at a constant voltage of 120 V using a Mini protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). Gels were stained with 0.125% (w/v) Coomassie Blue R-250 for 1 h and destained in a solution containing 25% (v/v) methanol and 10% (v/v) acetic acid.

3.4 Amino acid content

Determination of amino acid profiles was followed AOAC (2000). In brief, 0.05-0.10 g of lyophilized collagen was mixed with performic acid for 16 h in ice bath in order to oxidize cysteine and methionine to cysteic acid and methionine sulfone, respectively. Sodium metabisulfite was added to decompose performic acid. Subsequently, samples were digested with 6 N HCl at 110°C for 24 h under nitrogen. The digested samples were derivertized with propyl chloroformate. Quantitative analysis of total amino acids was achieved using gas chromatography–mass spectrometry (GC–MS, Agilent 6890N GC/5973 Inert MSD, Agilent Technologies, Darmstadt, Germany) equipped with a ZB-AAA column (10 m x 0.25 mm I.D., 0.25 µm film thicknesses, Phenomenex, Torrance, USA). The amino acid was expressed as residue/1000 residues as equation below

$$\left(\frac{Amino\ acid\ (g)}{molecular\ e\ weigh\ of\ amino\ acid}\right) \times 6.02\ \times 10^{23}\ \times \frac{1}{1000}$$

3.5 Fourier-transform infrared spectroscopy (FTIR)

FTIR measurement was carried out by mixing 2 mg lyophilized collagen and 100 mg potassium bromide (KBr) HOW. The mixture was mechanically pressed s to form a translucent tablet. All spectra were record using an FTIR spectrophotometer (PE spectrum GX, Maryland, USA) from 4000 to 500 cm^{-1} at a data acquisition rate 2 cm^{-1} per point in the mode of transmittance.

3.6 Peptide mapping

The lyophilized TS and HS collagen and commercial calf skin type I collagen (2 mg protein) were dispersed in 1 ml of 20 mM Tris-maleate, pH 7 containing 0.5% (w/v) SDS at 4°C. The sample was heated at 100°C for 5 min and then cooled in an ice bath. The digestion was carried out at 0°C for 60 min by adding proteinase K at the enzyme to sample ratio of 1:2500 (v/w). The digestion was terminated by adding 10 µl of 10 mM PMSF (Sigma Co., USA). The samples were mixed with a treatment buffer and subjected to boiling water for 3 min. SDS-PAGE was performed by the method of Laemmli (1970), using 4% stacking gel and 17% resolving gel.

3.7 Effect of pH and NaCl on collagen solubility

Collagen solubility was carried out according to (Montero, Jimennez-Col-menero, and Borderias, 1991) Collagen solutions were prepared at concentration of 3 mg/ml and 6mg/ml in 0.5 M acetic acid and homogenized for 3 min at maximum speed (IKA Labortechnik, Selangor, Malaysia). The homogenates were shaken gently at 4°C for 12 h and centrifuged at 10,000×g for 30 min at 4°C. Protein content in the supernatant

was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as a standard.

Effect of pH on collagen solubility. The solubilized collagen (3 mg/ml) which was prepared from the above was transferred to a centrifuge tube (8 ml) and the pH was adjusted with 6 N NaOH or 6 N HCl to obtain a final pH ranging from 1 to 10. Total volume was made up to 10 ml using distilled water. The mixture was shaken gently for 30 min at 4°C and centrifuged at 10,000×g for 30 min at 4°C. Protein in the supernatant was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as a protein standard. Relative solubility was calculated by comparing to that obtained at the pH rendering the highest protein content.

Effect of NaCl on collagen solubility. Collagen at concentration of 6 mg/ml (5 ml) was mixed with 5 ml of cold NaCl in acetic acid of various NaCl concentrations of 0, 2, 4, 6, 8, 10 and 12% (w/v) to obtain the final NaCl concentrations of 1, 2, 3, 4, 5 and 6% (w/v) at the pH 2.5, respectively. The mixtures were shaken gently for 30 min at 4°C and centrifuged at 10,000×g 30 min at 4°C. Proteins in the supernatant and relative solubility were determined as described above.

3.8 Thermal denaturation of collagens

Thermal denaturation was carried out according to Kittiphattanabawon et al. (2005) with slight modifications. Lyophilized TS and HS collagens were accurately weighed into aluminium pans (8 mg protein) and either deionized water (DI) or 0.05 M acetic acid was added at the ratio 1:40 (w/v) and sealed. The mixtures were allowed to stand for 2 days at 4°C. Denaturation temperatures were evaluated on a Pyris

Diamond DSC (Perkin Elmer Connecticut, USA.) The instrument was calibrated using indium and the samples were scanned over the range of 10-80°C at a heating rate of 10°C/min. The denaturation temperature (T_d) was estimated from the maximum peak of DSC thermogram and total denaturation enthalpy (ΔH) was determined from the peak area and expressed in J/g protein.

3.9 *Vigibacillus* sp. SK 39 proteinases production

Inoculum was prepared by transferring a loopful of pure culture into Y-broth (5% NaCl, 1% yeast extract, 0.3% trisodium citrate, 0.2% KCl and 2.5% $MgSO_4 \cdot 7H_2O$, pH 7.0) and incubated at 35°C for 24 h (Sinsuwan, Rodtong & Yongsawatdigul, 2008). Optical density (OD) of the inoculum measured at 600 nm was about 0.3 which was equivalent to 10^7 CFU/ml. Fifty ml of inoculum was transferred to 450 ml of the same medium and incubated at 40°C with a shaking speed of 120 rpm. Crude extracellular proteinase was collected by centrifugation at 8,000× g, 4°C for 30 min (RC 28S, Sorvall Co., Newtown, Conn., USA).Supernatants were collected and filtered through a 0.45-μm followed by ultrafiltration 30-kDa using a MWCO membranes indicate of by the manufacturer. Activity was assayed by using HS collagen as a substrate. HS collagen (25 mg) was mixed with 0.7 ml of 0.2 M McIlvain buffer at pH 6 and pre-heated for 20 min at 55°C.Subsequently, 0.3 ml of enzyme was added and further incubated at 55°C for 90 min. The reaction was terminated by the addition of 500 μL of cold 50%TCA and stand in the ice bath for 15 min. The mixtures were collected by 10,000× g, 4°C for 5 min (RC 28S, Sorvall Co., Newtown, Conn., USA) and supernatants were collected. α-Amino acid contents were determinated according to Alder-Nissen (1979) method. Fifty μl of sample 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 in water bath. One ml of 0.1 N HCl was added to stop reaction and left at room temperature for 30 min. Absorbance was monitored at 420 nm. Glycine was used as a standard. Unit activity was defined as the amount of enzyme required to produce 1 mmol of glycine equivalents per minute crude enzyme was kept at 4°C throughout the study.

3.10 Preparation of fish skin collagen hydrolysates

TP and HS collagens were hydrolyzed using various proteinases. The reaction mixture (5ml) contains 36 mU of proteinase (trypsin, pepsin, Alcalase or *Virgibacillus* sp. SK39 proteinase) and 2.75 mg protein/ ml in 0.2 M McIlvain buffer under their optimal condition. Unit activity was defined as the amount of enzyme required to producing one mmole of glycine equivalents per minute under the optimum conditions: pH 8, 50°C for Alcalase and trypsin, pH 2, 37°C for pepsin and pH 6, 55°C for *Vigibacillus* sp. SK 39. Hydrolysis was carried out in a shaking water bath for 6 h (Memmert model WNB22, memmert GmbH+Co.KG, Schwabach, Germany). To terminate the enzymatic reaction, the mixtures were heated in a water bath at 100°C for 10 min and cooled immediately. Samples were adjusted to pH 7 using either 1 M NaOH or 1 M HCl. The hydrolysates were centrifuged at 10,000×g for 15 min and supernatants were collected and determined for ACE inhibition activity. As pepsin-hydrolyzed collagen from HS showed the highest ACE-inhibiotry activity, it was selected for further studies. The effect of degree of hydrolysis (DH) on ACE-inhibitory activity of pepsin-hydrolyzed HS collagen was investigated at various time intervals of 1, 2, 3, 4, 6, and 8 h.

Hybrid catfish collagen hydrolysates were fractionated using an ultrafiltration unit with the 30- kDa MWCO membrane. The retentate was collected and defined as >30-kDa fraction whereas the permeate was fractionated by a 5-kDa MWCO membrane. The resulting retentate and permeate were collected and defined as 5–30 kDa and <5 kDa fraction, repectively. The three fractions, >30 kDa, 5–30 kDa and <5 kDa fractions , were stored at -80°C until further use.

3.11 Determination of the degree of hydrolysis (DH)

DH was determined according to Alder-Nissen (1979). Fifty µl of each hydrolysate was mixed with 0.5 ml of 0.215 M phosphate buffer, pH 8.2 and 0.5 M of 0.05% TNBS reagent. The mixture was incubated at 50°C for 1 h in a water bath. One ml of 0.1 N HCl was added to stop reaction and left at room temperature for 30 min. Absorbance was monitored at 420 nm. Glycine was used as a standard. To determine total amino acid content, lyophilized TS and HS collagens was hydrolyzed with 6 N HCl (sample to acid ratio 1:100) at 120°C for 24 h using an autoclave. The degree of hydrolysis was calculated following the equation

$$DH = [(h_s - h_o)/ h_t] \times 100\%$$

Where h_s is the amount of α-amino content of hydrolysates released at time, h_o is the amount of α-amino content of sample at time 0, and h_t is the total amount of α-amino obtained after acid hydrolysis.

3.12 ACE inhibitiory activity assay

In vitro ACE inhibitiory activity was performed by the method of Cushman and Cheung (1971). Collagen hydrolysates (25 µL) was added to 50 µL of 25 mU/mL

ACE, 25 μL of deionized water, and pre-incubated for 5 min at 37°C. Subsequently, 150 μL of substrate (8.3 mM HHL in 50 mM sodium borate buffer containing 500 mM NaCl at pH 8.3) was added and further incubated at 37°C for 60 min. The reaction was terminated by the addition of 250 μL of 1 M HCl. Hippuric acid was extracted with 1.5 mL of ethyl acetate. After centrifugation (800×g, 15 min), the released HA in the upper layer was transferred into a glass tube and was dried in a 80°C-sand bath. The dried sample was dissolved in 1 mL of deionized water and filtered through a 0.45-μm membrane filter before being subjected to a HPLC equipped with a photodiode array detector (DAD) (Agilent Technologies, Santa Clara, CA, USA). Samples (20 μL) were injected to a Zorbax Eclipse XDB-C18 column (4.6×150 mm, Agilent Technologies, Santa Clara, CA, USA). The column was eluted with mobile phase (A) of 0.05% TFA in water and (B) of 0.05% TFA in acetonitrile at a flow rate of 0.5 mL/min. The separation condition was carried out using 20–60% of mobile phase B for the first 13 min, maintained for 2 min at 60% of mobile phase B, and then returned to 20% of mobile phase B for 2 min. The eluate was followed at 228 nm. External standard of HA was used. Blanks were prepared by adding HCl before adding ACE. ACE inhibition (%) was calculated as follows:

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

Where C_o is HA content of reaction of the control (without collagen hydrolysates), C_B is HA content of the control blank, I_0 is HA content of reaction with collagen hydrolysates, and I_B is HA content of reaction blank. The IC_{50} defined as the concentration of hydrolysate required to inhibit 50% of the ACE activity was also determined.

3.13 Purification of hybrid collagen hydrolysate

The HS collagen hydrolysate at MWCO <5 kDa exhibiting the strongest ACE inhibitory activity was purified using fast protein liquid chromatography (FPLC, AKTA, Amersham Bioscience Co., Uppsala, Sweden). The lyophilized fraction was dissolved in 10 mM sodium acetate buffer (pH 4) and loaded onto a carboxymethyl (CM)-Sepharose cation exchange column equilibrated with 10 mM sodium acetate buffer (pH 4.0) and eluted with a linear gradient of NaCl (0-1.0 M) in the same buffer at a flow rate of 1.0 ml/min. Each fraction was monitored by ultraviolet (UV) absorbance at 215 nm and collected by auto collector at a volume of 1 ml. The pooled fraction showing the strongest ACE inhibitory activity was lyophilized and dissolved in deionized water before loading into a Superdex peptide 10/300 GL column (10 mm×300 mm, Amersham Biosciences Co., Uppsala, Sweden). The column was equilibrated and eluted with deionized water in isocratic mode at flow rate of 0.3 ml/min. Each fraction was monitored by UV absorbance at 215 nm.The pooled fraction was lyophilized and dissolved in deionized water before loading into to Superdex 30 prep grade column (2.6 × 100 cm)equilibrated with deionized water and then eluted with the same flow rate of 0.3 ml/min. the pooled fractions were determined for ACE inhibitory activity. Fraction showing the highest ACE inhibitory activity was further analyzed amino acid sequence by LC-MS/MS.

3.14 LC-MS/MS

Amino acid sequences of partially-purified peptides were determined using Ultimate 3000 LC System (DionexLtd., USA) coupled to ESI-Ion Trap MS (HCT Ultra PTM Discovery System, Bruker, Germany) with electrospray. Peptides were

separated on a nanocolumn (Acclaim PepMap 100 C18, 3 mm, 100A, 75 mm id × 150 mm). Eluent A was 0.1% formic acid in water. Eluent B was 80% acetonitrile in water containing 0.1% formic acid. Elution was performed using a linear gradient from 0 to 70% of B for 13 min at a flow rate of 300 nl/min. De novo sequencing was carried out with Pepnovo which can be accessed by the URL http://proteomics.ucsd.edu/LiveSearch/.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Proximate composition

Crude protein of lyophilized tilapia skin (TS) and hybrid catfish skin (HS) was 80 and 46% (dry basis), respectively (Table 1). HS contained significantly higher amount of fat than TS ($p<0.05$). Ash and moisture content of skin from both species were comparable. Proximate composition indicated that both skins are a rich source of protein that can be utilized by a more efficient means to produce product with higher value.

Table 4.1 Proximate composition (% dry basis) of dried tilapia skin and hybrid catfish skin.

Composition	Tilapia	Hybrid catfish
Crude protein	80.13±1.11	46.23±1.70
Crude fat	20.01±1.09	53.14±0.34
Ash	0.53 ±0.05	0.67±0.05

Yield of TS and HS collagen was 58.9±1.57 and 48.0±7.33% of dried skin, respectively. This result was very similar to those previously reported. In general, yield of collagen from marine fish skin among various fish species ranged from 9 to 54.3% (dry weight basis) (Nagai et al., 2000;Nagai and Suzuki, 2000; Nagai, Yamashita, Tanigushi, Kanamori, and Suzuki, 2001; Nagai, Araki, and Suzuki, 2002;

Senaratne, Park, Kim 2006), whereas collagen from freshwater fish have been reported in the range of 16.8 to 46.6%.(Zhang, Liu, Li, Shi, Miao and Wu 2007; Zhang, Liu, and Li, 2009; Zhang, Liu, and Li, 2009).

Extraction yield depends on many factors, such as extraction method (acid type, concentration of acid) and the nature of source. Acetic acid enhances skin swelling process. In acidic condition, the positive charge of collagen polypeptides becomes dominant, which enhances repulsion among tropocollagen molecule. This eventually leads to disruption of non-covalent bonding and increased solubilization (Zhou and Regenstein 2005).

Both skins were not completely solubilized with 0.5 M acetic acid even with two repetitions of extraction. This could be because TS and HS skin contained high amount of molecular cross-links at the telopeptide region. The telopeptide is the end region of collagen containing non-helical parts and plays an important role in the cross-link structure. If telopeptide is highly cross-linked, the solubility of collagen in the acid solution is low (Foegeding et al., 1996). TS and HS collagen might contain different molecular arrangement, leading to different yield of collagen.

4.2 SDS-PAGE

SDS-PAGE is the most widely used for analyzing protein mixture, monitoring protein purity and determining their molecular weights. It is based on the separation of proteins according to size and then locating by binding to a dye. SDS-PAGE patterns are shown in Figure 4.1. TS and HS collagens showed γ-component, β-component and α bands (α_1 and α_2), which were similar to calf skin type I collagen (lane 2). This result suggested that both TS and HS collagens were type I collagen which consisted of two

identical α_1 chains and one α_2 chain (Burghangen, 1999; Foegeding et al., 1996;Wong, 1989). This was similar to fish collagens previously reported in largefin longbarbel catfish, silver carp, grass carp fish skin, common horse mackerel skin, yellow sea bream and tiger puffer (Zhang, Liu and Li, 2009; Rodziewicz-Motowidło et al., 2008; Zhang, Liu, Li, shi, Miao and Wu, 2007; Yata, Yoshida, Fujisawa, Mizuta and Yoshinaka, 2001).

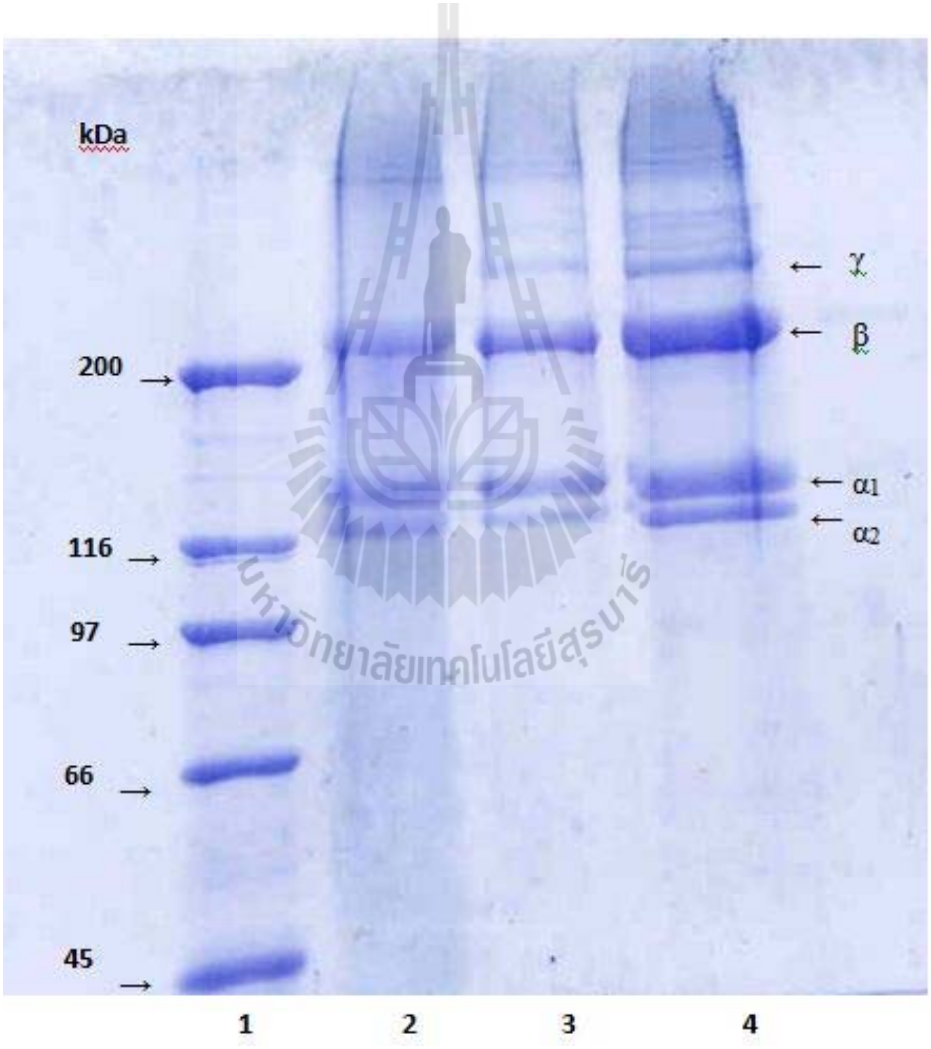


Figure 4.1 SDS-PAGE patterns of collagen from skin of tilapia and hybrid catfish on 7.5% acrylamide. Lane 1: protein markers; Lane 2: Type I calf skin collagen; Lane 3: TS collagen; Lane 4: HS collagen.

A typical SDS-PAGE pattern of type I collagen exists in 2 forms, $[\alpha_1(I)]_2\alpha_2(I)$ and $\alpha_1(I)\alpha_2(I)\alpha_3(I)$. The α3, if present, could not be separated under the electrophoretic condition because α3 (I) migration is the same position as α1(I) (Kimmura and Ohno. 1987). α_3–Chains were found in collagen from Alaska pollack (Kimura and Ohno., 1987) and brown backed toadfish (Senaratne, Park and Kim 2006). High molecular weight β-chain and γ-chain were observed in both collagens. β-Chain is intermolecular cross-links of two α chains, whereas γ-chain is intra-molecular cross links of three covalently cross linked α-chains. Zhang (2007) reported that high amount of cross-links of β and γ chain are formed at the telopeptide region as well as other inter-molecular cross-links, leading to low solubility. Molecular cross-link of collagen varies with environment of fish. Foegeding et al. (1996) reported that starving fish contain more collagen with a greater degree of cross-linking than well fed fish. Myocommata are thickened during starvation with a mechanically stronger collagen than normal fish.

Skins of numerous fish species have been reported to contain type I existing in β and γ components (Guille et al, 2000; Kittiphattanabawon et al., 2005) and high amount of β and γ chain was repeated in Japanese sea-bass, chub mackerel, blue head shark fish collagens (Nagai and Suzuki (2000), black drum sea bream, sheep head sea bream (Ogawa et al., 2003) and Nile perch (Muyonga et al., 2004).

4.3 Amino acid composition

Amino acid composition directly influenced physico-chemical properties of collagen, including solubility and thermal stability. Table 4.2 shows amino acid composition of collagen from TS and HS. Major amino acids of both collagens were

glycine, proline, alanine and lysine. They were poor of cysteine, indicating that there were no trace of other types of collagen or procollagen, which usually contained disulfide linkages. (Angele et al., 2004). Glycine is typically found at every third position throughout collagen molecule and is the most abundant amino acid in collagen (Brazel et al., 1987). The presence of glycine residues is a critical requirement for collagen superhelix structure because glycine contains no side chain. (Regenstein and Zhou, 2007). It allows a close packing structure along the central axis of the triple helix molecule (Gelse, Pöschl and Aigner, 2003). Both collagens contained trace amount of tyrosine which is an indicator of the remaining telopeptide (Angele et al., 2004).

Table 4.2 Amino acids composition of TS and HS collagen (residues/1000 residues).

Amino acid	**Tilapia**	**Hybrid catfish**
Alanine	122.1	142.1
Arginine	<0.1	<0.1
Aspartic Acid	36.9	37.2
Cysteine	<0.1	<0.1
Glutamic Acid	76.5	53.2
Glycine	202.7	230.1
Histidine	30.2	21.4
Hydroxylysine	<0.1	<0.1
Hydroxyproline	36.7	38.1
Isoleucine	29.6	32.3
Leucine	60.4	51.3
Lysine	124.1	90.7
Methionine	8.8	10.5
Phenylalanine	62.9	57.2
Proline	162.7	188.9
Serine	<0.1	<0.1
Threonine	<0.1	<0.1
Tryptophan	<0.1	<0.1
Tyrosine	14.8	9.7
Valine	31.3	36.8
Imino acid	**199.4**	**227**
Proline hydroxylation (%)	18.4	16.8

Lysine was found in TS collagen to a higher content than in HS collagen. This amino acid plays an important role for natural inter-molecular and intra-molecular cross-links (Angele et al., 2004). Lysine hydroxylation correlated with high thermo stability of collagen (Notbohn et al., 1992). Proline, hydroxyproline and hydroxylysine are also unique amino acids found in collagen. Imino acid content, which is the sum of proline and hydroxyproline of HS collagen, were higher than that of TS collagen (Table 4.2). Imino acid content of fish collagen ranged 135-220 residues/1000 residues, depending on species (Kittiphattanabawon et al., 2005; Motowidło et al., 2008; Bae et al., 2008; Zhang. et al 2009). Imino acid content of calf skin and porcine skin collagen is 215 and 220 residues/1000 residues, respectively (Zhang et al., 2007). Marine fish and freshwater fish collagen contained imino acid content of 150-217 residues/1000 residues (Kimura and Ohno, 1987; Kittiphattanabawon, et al., 2005; Senaratne, Park, and Kim, 2006; Hwang, Mizuta, Yokoyama, and Yoshinaka, 2007; Bae et al., 2008; Huang, Shiau, Chen and Huang, 2011; Muyonga, Cole and Duodu, 2004; Liu, Li, and Guo, 2007; Zhang et al., 2007; Zhang, Liu, and Li, 2009; Duan, Zhang, Du,Yao and Konno, 2009;Zeng, Zhang, , Lin, Yang, Hong and Jiang 2009).

Imino acid plays an important factor in the formation of triple helix strands. The higher imino acid is more stable the helix. Pyrrolidine ring of proline and hydroxyproline impose restrictions on the conformation of the polypeptide chain (Riesleet al., 1998). However, the degree of hydroxylation of proline residues plays an important role in stabilizing the triple helix of collagen (Ramachandarn, 1988). Proline hydroxylation was calculated to be 18.4 and 16.8% in TS and HS collagen, respectively, which was lower than that of collagens from other freshwater fish skins that was reported in the range of 34-40% (Zhang, Liu and Li, 2009; Zhang et al., 2007;

Zeng et al., 2009; Liu, Li and Guo, 2007; Muyonga, Cole and Duodu, 2004; Duan, Zhang, Du,Yao and Konno, 2009). The degree of proline hydroxylation of marine fish varied widely between 25-47% (Nagai et al., 2002; Sadowska, Kolodziejska and Niecikowska. 2003; Jongjareanraket al., 2005; Kittiphattanabawon et al., 2005; Senaratne, Park and Kim, 2006; Nalinanon, Benjakul, Visessanguan, and Kishimura, 2007; Hwang, Mizuta, Yokoyama and Yoshinaka, 2007; Bae et al., 2008; Huang, Shiau, Chen, and Huang, 2011).This result indicated that collagen extracted from TS was different from HS collagen in terms of amino acid compositions.

4.4 Fourier transform- infrared spectroscopy (FT-IR)

FT-IR is an optical technique that provides information about the molecule vibration of any stable electronic state of the molecule system (Cárcamo et al., 2012). FT-IR is a non-destructive tool for the structural or conformational characterization of biological system with several advantages. It has been widely used to elucidate protein structure and monitor changes in the secondary structure of protein. (Belbachir et al., 2009).

Infrared spectrum of a sample is recorded by passing a beam of infrared light through the sample when the frequency of IR is same as the vibration frequency of a bond, then absorption of that IR radiation occurs. Thus, the frequency of the vibrations can be associated with a particular bond type. Mid-infrared wave number in the range of 4,000-500 cm^{-1} is widely used for monitoring collagen structure (Bellbachir et al., 2009). Collagen triple helix is a unique protein motif defined by the supercoiling of three polypeptide chains in a polyproline II conformation (Shoulders. and Raines, 2009). The regions of Amides A band (3400-3440 cm^{-1}) is related to N-H stretching

vibrations between intramolecule hydrogen bonds of helix (Bryan et al., 2007; Akobsen et al., 1983). This Amide A band was found in all collagens tested (Figure 4.2). Amide I band (1600-1660 cm^{-1}) is associated with stretching vibration of carbonyl group (C=O) in peptide, which has been extensively used in analysis of protein secondary structure (Belbachir et al., 2009). The Amide I band position of calf skin, TS and HS collagens were found at 1633, 1642 and 1643 cm^{-1}, respectively, which indicated that TS and HS collagen contained α-helix as the main secondary structure similar to calf skin. Amide II band (1550-1600 cm^{-1}) is associated with C-H stretching, while Amide III band (1235-1450 cm^{-1}) is related to C-N and N-H stretching and wagging vibrations from CH_2 group from the glycine backbone and

Figure 4.2 Fourier transform-infrared spectra of collagen from calf skin type I, tilapia skin (TS) and HS skin.

proline side chain which are involved with the triple helix structure of collagen (Muyonga et al., 2004; Wang et al., 2007; Woo et al., 2008). Calf skin, TS and HS collagen illustrated similar spectra, suggesting that all collagens exhibited comparable conformation.

Fourier transform-infrared spectra pattern of both collagens were not different from other fish collagens, including carp skin, largefin longbarbel catfish, Nile perch, channel catfish (Duan et al. 2009; Zhang, Liu and Li 2009; Muyonga et al 2004; Liu et al 2007). Difference in FT-IR spectral was due to an increase of intermolecular hydrogen bonds which led to a shift to lower wave number of Amide I, Amide II, and an increase in intensity of Amide III. (Jakobsen et al., 1983). Furthermore, collagen extracted at high temperature exihibited a broader Amide A band than collagen extracted at low temperature. (Muyonga et al., 2004).

4.5 Thermal stability

DSC thermograms of TS and HS collagen are shown in Figure 4.3. TS and HS collagen in the presence of 0.05 M acetic acid showed T_d at 36.42 ± 0.12°C and 35.15 ± 0.71°C, respectively, while those dissolved in DI were higher. Enthalpy (ΔH) values of samples solubilized in 0.05 M acetic acid were lower than those of collagens solubilized in DI. Similar results were also reported by Kittiphattanabawon et al., (2005) and Jongjareonrak et al (2005).

A decrease in denaturation temperature of acetic acid solubilized collagen might be caused by the unfolding of proteins due to electrostatic repulsion helical structure of collagens and destruction of hydrogen bonds. In contrast, collagen in the presence of water is more intact requiring higher temperature and energy to unfold. For this

reason, denaturation temperature and enthalpy of collagens in the presence of DI were higher than in acid.

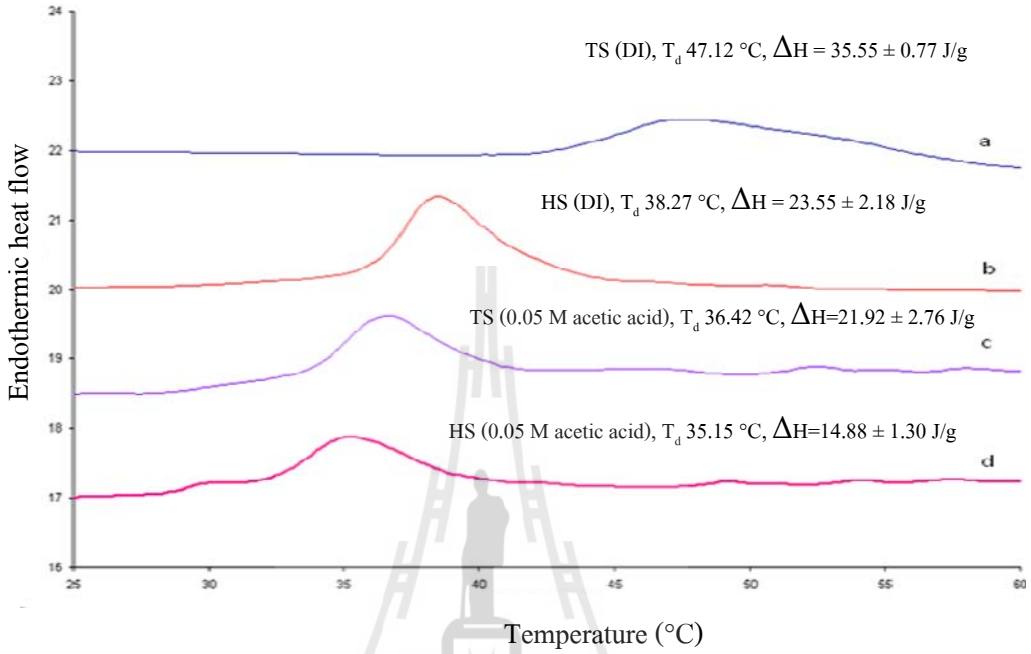


Figure 4.3 DSC thermograms of (a) tilapia collagen in deionized water (b) tilapia collagen in 0.05 M acetic acid (c) hybrid catfish collagen in deionized water (d) hybrid catfish collagen in 0.05 M acetic acid.

TS collagen showed higher T_d than HS collagen in both DI and 0.05 M acetic acid. Denaturation temperature of animal collagen well correlated with imino acid content and the amount of lysine or tyrosine (Angele et al., 2004). Zhang (2007) reported that a high amount of cross-links at the telopeptide region as well as other inter-molecular cross-links leads to a high thermal stability. Based on amino acid composition in Table 4.2, tyrosine and lysine contents of TS collagen was higher than those of HS collagen. Tyrosine is an indicator of telopeptide remained in collagen, while lysine relates to inter-molecular and intra-molecular cross-linkings of collagen.

Therefore, TS collagen likely contained more molecular crosslinks, leading to high thermal stability.

The relationship between amino acid composition of collagen structure and its collagen stability have been reported. Amino acid sequence and length of amino acid sequence are main factors affecting thermal stability. Denaturation temperature (T_d) of triple helices increased in the order of $(GlyProPro)_{10}$< $(GlyHypHyp)_{10}$ < $(GlyProHyp)_{10}$ with T_d of 41, 65 and 69°C, respectively. Furthermore, length of GlyProHyp also affected thermal stability. Denaturation temperature of $(GlyProHyp)_7$ and $(GlyProHyp)_{10}$ sequcence was 36 and 69°C, respectively (Holmgren, Bretscher, Taylor and Raines, 1999; Bretscher, Jenkins, Taylor, DeRider and Raines, 2001). Amino acid sequences of TS and HS collagen could be different, leading to differences in thermal stability.

Zhang et al. (2009) reported that collagen extracted from fish with scale showed higher T_d than that extracted from fish without scale. It seemed that T_d of collagen were also probably influenced by the presence of scale. Calcium in the dermis is used to generate scale, which may affect denaturation temperature of collagen. This may explain why TS collagen showed higher T_d than HS collagen.

Previous studies showed that thermal stability of fish collagen is typically lower than calf collagen. Denaturation temperature of animal collagen was associated with the environment and body temperature of animal. T_d of land animal collagen is generally higher than that of aquatic animal (Komsa et al., 1999). Collagens derived from fish species living in cold environments typically contain lower content of hydroxyproline than those from fish living in warm environment (Foegeding et al., 1996).

T_d of collagen from cold water fish is lower than that of warm water fish collagen. T_d of porcine skin was about 37°C (Ikoma et al., 2003) and 40.8°C for calf skin (Kimmura et al., 1998). Collagen of cold water marine fish exhibited T_d in a range of 15-29.6°C, such as Japanese sea-bass:26.5°C; chub mackerel:26.5°C; salmon:19.4°C; Baltic cod:15°C and Alaska pollack: 16.8°C (Kim and park, 2004; Nagai and Suzuki, 2000; Kimura, Zhu, Matsui, Shijoh and Takamisawa,1988; Huang, Shiau, Chen and Huang, 2011; Sadowska , Kolodziejska, and Niecikowska, 2003; Kimura et al., 1987). Denaturation temperature of deep sea marine fish, such as Baltic cod, was lower than other marine fish around 5°C. In contrast, warm water marine fish collagen showed higher denaturation temperature than those from cold water species. (Bae et al., 2008; Jongjareanrak et al., 2005; Nalinanon et al., 2007). Most of marine fish collagens denature at temperatures below 30°C. Denaturation temperature of freshwater fish ranged 24.6-36.5°C, such as young and adult of Nile perch: 36.3 and 36.5°C; Nile tilapia: 32.0°C; carp:28°C; grass carp:24.6°C; Largefinlongbarbel catfish:32.1°C and channel catfish:32.5°C (Muyonga et al., 2004; Zeng et al., 2009; Duan, Zhang, Du, Yao, and Konno, 2009; Zhang, et al., 2007; Zhang, Liu and Li, 2009;Liu, Li and Guo, 2007).

4.6 Peptide mapping

Patterns of TS and HS collagen digested by Proteinase K are shown in Figure 4.4. After digestion, high molecular weight of TS and HS collagens were degraded into small peptides with molecular mass in the range of 8-24 kDa. Calf skin collagen was not hydrolyzed at 0°C, indicating that it did not unfold to allow proteinase accessibility. Consequently, calf collagen hydrolysate still showed native structure of

β and α component with molecular weight (MW) ranging from 45-200 kDa. This result was similar to that of Duan et al., (2009) who compared hydrolytic pattern of calf collagen and carp skin, bone and scale collagen by proteinase K. Furthermore, the peptide maps of bigeye snapper collagen digested by V8 proteinase and lysyl endopeptidase revealed different pattern from calf skin collagen (Kittiphatanabawon et al., 2005).

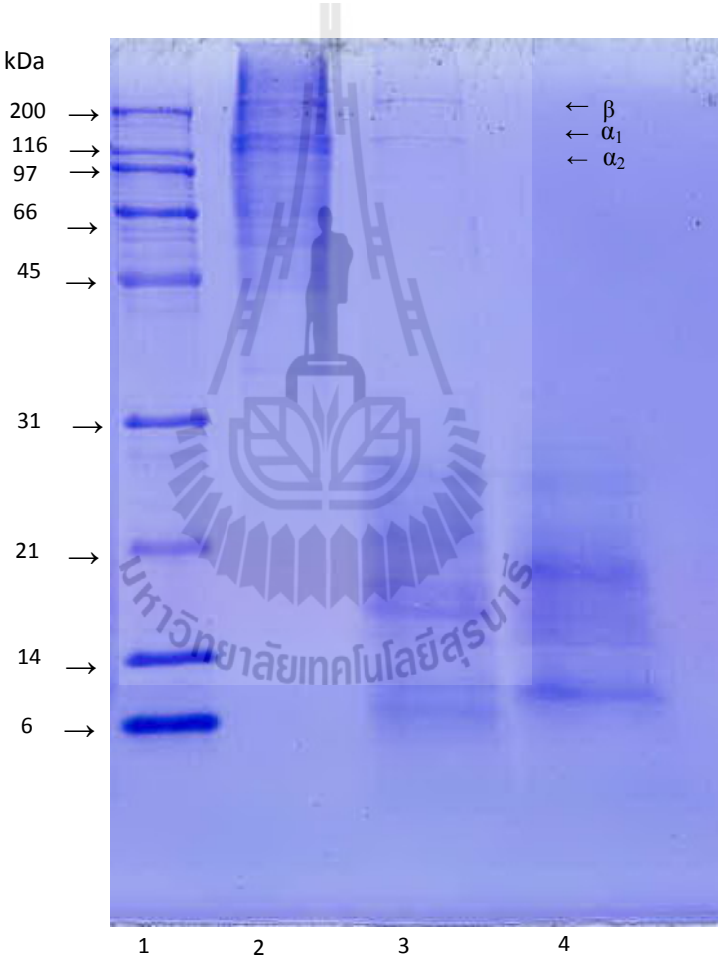


Figure.4.4 Peptide mapping of proteinase K digests at 0°C. Lane 1 protein marker; Lane 2 peptide fragments of calf skin; Lane 3 peptide fragments of tilapia; Lane 4 peptide fragments of hybrid catfish.

Based on peptide mapping, structural stability and primary structure of calf skin type I collagen was different from that of TS and HS collagens. TS peptide fragments still contained high molecular weight of β and α component, while pattern of HS collagen peptides did not show high molecular weight component. TS and HS collagen could be presumed to have different sequences of amino acids as well as degree of cross-linking.

4.7 Solubility of collagens

4.7.1 Effect of pH

The effect of pH on the solubility of TS and HS collagens are shown in Figure 4.5. The maximum solubility of both collagens was found at pH 3. At extremely low pH, collagen carries a large excess of positive charges, resulting in electrostatic repulsion across individual molecules in acid solution. This would induce unfolding and increase interaction with water molecules.

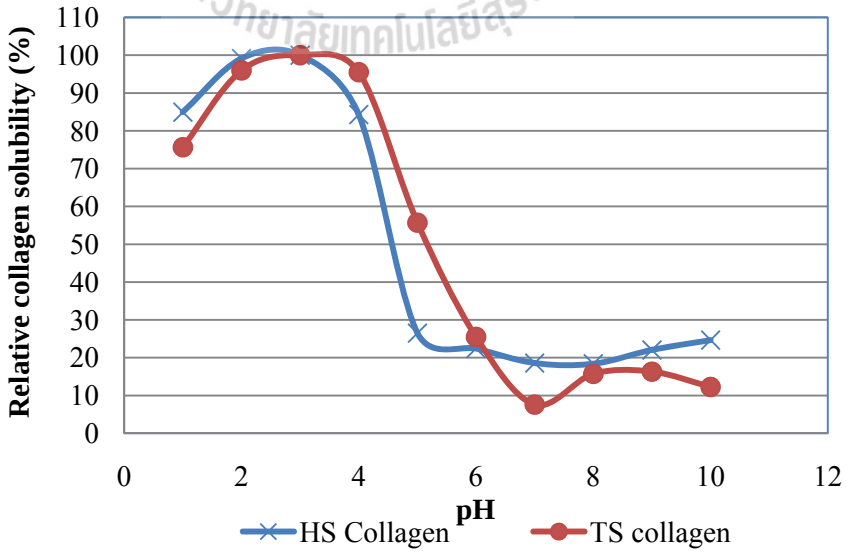


Figure 4.5 Solubility of collagens from TS and HS at different pHs.

Low solubility of TS and HS collagens was observed at pH 7 and 8, respectively. As pI of collagen is at pH 6–9 and minimum solubility is typically observed at this pH range. At pI, hydrophobic interactions increase, promoting the precipitation and aggregation of collagen. Various fish species also showed maximum solubility of collagen at pH 2-3 and minimum at pH 6-7 (Zeng et al., 2009; Woo et al., 2008; Jongjareonrak et al., 2005; Kittphattanabawon et al., 2005; Bae et al., 2008).

4.7.2 Effect of salt concentration

Solubility in 0.5 M acetic acid of collagens sharply decreased at NaCl concentrations greater than 3% (Figure 4.6). Solubility of other fish skin collagens at different NaCl concentrations have been reported. Kittphattanabawon et al. (2005) extracted collagen from bigeye snapper skin and reported its lowest solubility at 3% NaCl. Brownstrip red snapper extracted by either pepsin or acetic acid showed a

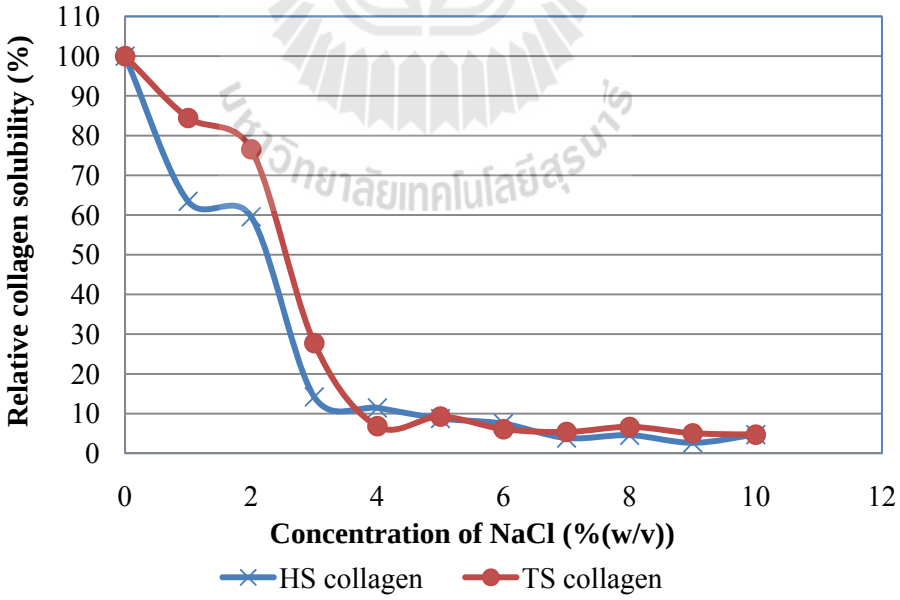


Figure 4.6 Solubility of collagen from TS and HS collagen at different NaCl concentrations.

decrease in solubility when NaCl concentration increased to 3% or 2% (w/v), respectively (Jongjareonrak et al., 2005).

4.8 ACE inhibitory activity of TS and HS collagen peptides

Enzyme unit was controlled at 36 mU using collagen as a substrate. Concentration of collagen in the reaction was 2.75 mg protein/ml with the reaction time of 6 h. The highest DH was found in the hydrolysate prepared from pepsin, followed by *Virgibacillus* sp. SK39 proteinase, Alcalase and trypsin, respectively. Pepsin hydrolysate of both TS and HS demonstrated the highest ACE inhibitory activity (Table 4.3).

Table 4.3 ACE inhibitory activity of TS and HS collagen hydrolysates at 6 h.

Enzymes	Inhibition (%)			
	Tilapia	**%DH**	**Hybrid catfish**	**%DH**
Virgibacillus sp. SK 39	41.65 ± 0.80^{b}	28.25	14.67 ± 1.24^{a}	28.33
Pepsin	93.25 ± 0.50^{g}	31.03	92.57 ± 0.17^{g}	32.19
Trypsin	46.62 ± 5.28^{c}	5.16	50.53 ± 0.65^{d}	7.55
Alcalase	87.43 ± 0.54^{f}	31.18	79.94 ± 0.76^{e}	24.43

Different letters indicate significant differences among mean ($P < 0.05$). Values are expressed as mean ± S.D. ($n = 4$).

Peptides of ACE inhibitor have been reported to contain hydrophobic amino acid residues, such as proline, phenylalanine and tyrosine at the third position from the C-theminal (Cheung et at., 1980). Alcalase is an endoproteinase having broad specificity, and high specificity toward aromatic (Phe, Trp, and Tyr), acidic (Glu), sulfur-containing (Met), aliphatic (Leu and Ala), hydroxyl (Ser), and basic (Lys) residues (Doucet, Otter, Gauthier, and Foegeding, 2003). Trypsin is specific for Lys at the carboxyl side of a peptide bond. *Virgibacillus* sp. SK 39 was isolated from Thai fish

sauce. Proteinase from *Virgibacillus* sp. SK39 has not been widely studied. Pepsin is the most efficient in cleaving peptide bonds between hydrophobic and preferably aromatic amino acids including phenylalanine, tryptophan and tyrosine (Nelson and Cox, 2000). Thus, peptides derived from pepsin hydrolysis contain aromatic and hydrophobic amino acids at the terminal. Pepsin produced peptides exhibiting stronger ACE inhibitory than other enzymes tested. Lee, Qian and Kim (2009) reported that tuna frame protein hydrolysate by pepsin showed the most potent ACE inhibitory activity as compared to five proteolytic enzymes: Alcalase; α-chymotrypsin; papain; Neutrase and trypsin. Cod protein frame hydrolysate produced by papain showed the maximum inhibitory activity than those prepared by chymotrypsin, trypsin, pepsin and pronase E (Jeon, Byun and Kim., 1999). The extent of hydrolysis largely depends on both the nature of the substrate and the type of proteinase. As pepsin was the most potent proteinase for yielding the highest ACE inhibitory activity, pepsin-hydrolyzed sample was chosen for further investigation.

4.9 ACE inhibitory activity of pepsin-hydrolyzed collagen

Degree of hydrolysis (DH) rapidly increased with time during the first 4 h (Figure 4.7). The hydrolysis appeared to slow down after 4 h of digestion. Hydrolysates derived from HS collagen showed higher ACE inhibitory inhibition activity compared to TS collagen at any degree of hydrolysis ($p<0.05$, Figure 4.8). ACE inhibitory activity increased with DH in both collagens and further increase of DH beyond 30% did not increase ACE inhibitory activity. TS and HS hydrolysate by pepsin showed the highest potent of ACE inhibition at DH 30%.

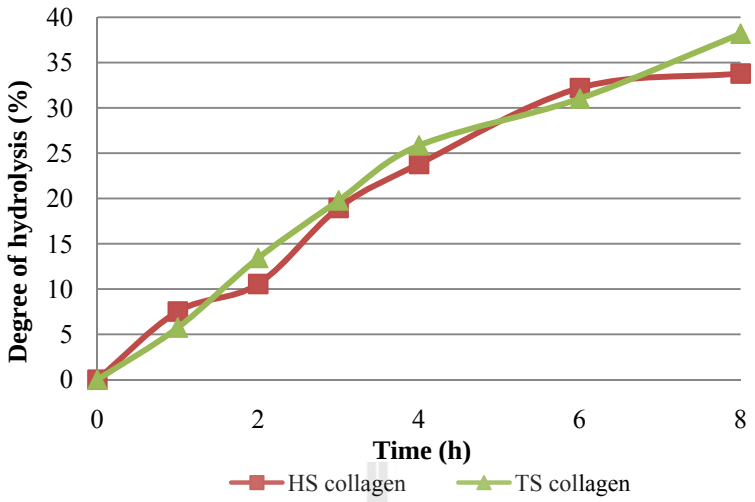


Figure 4.7 Degree of hydrolysis of pepsin-hydrolyzed collagen at varied time.

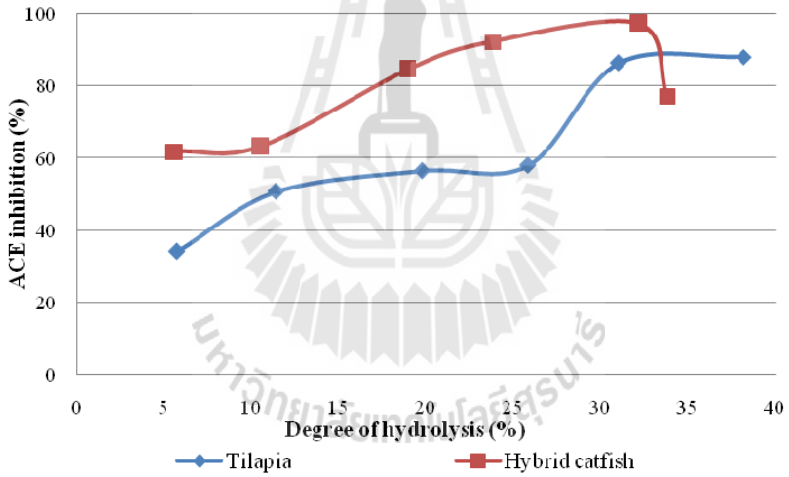


Figure 4.8 ACE inhibitory of pepsin-hydrolyzed collagen TS and HS at varied degree of hydrolysis.

ACE inhibitory activity of TS and HS collagens at 33 and 38% DH was comparable and lower than those at 30% DH ($p<0.05$). This indicates that ACE inhibitory does not necessarily increase with DH. Specific amino acid composition in the peptide sequences is a more important factor manifesting ACE inhibitory activity

of collagen hydrolysate.

The presence of proline, phenylalanine and tyrosine at the C-terminal of peptide can strongly influence binding ability to ACE. Since HS collagen contained high amount of proline than TS collagen, peptides from HS collagen hydrolysate were likely to contain more proline residues. This would increase ACE binding and promote inhibitory activity. HS collagen was selected for further purification.

4.10 Purification of ACE inhibitory peptide

4.10.1 Ultrafiltration

HS collagen hydrolysate was fractionated using ultrafiltration membrane with a molecular weight cut-off 30 kDa, followed by 10 kDa, respectively. Three fractions were collected and ACE inhibition activity of each fraction was evaluated. ACE

Table 4.4 ACE-inhibitory activity of ultrafiltrated pepsin hydrolyzed HS collagen.

MW (Dalton)	Inhibition (%)
Crude hydrolysate	92.57 ± 0.17^{a}
> 30 kDa	54.50 ± 1.45^{b}
5-30 kDa	57.11 ± 1.45^{bc}
<5 kDa	61.41 ± 1.40^{c}

Different letters indicate significant differences ($P < 0.05$). Values are expressed as mean ± S.D. (n = 4).

inhibitory activity of HS collagen hydrolysates varied with molecular mass distribution. Hydrolysate with MW <5 kDa showed the highest inhibitory activity ($p < 0.05$, Table 4.4). Normally, bioactive peptides containing 3-20 amino acid residues and low MW peptides are more potent ACE inhibitor than high MW peptides (Philanto, 2000).

The relationship of ACE inhibitory activity and molecular weight of peptides have been studied. Small peptides showed better ACE inhibition than large peptide. The long chain peptides are highly susceptible to bind with ACE subsites (Gómez-Ruiz Ramos and Recio, 2007). Activity of all fractions showed lower activity than crude peptide. Synergistic effect among all peptides appeared to be important in this case.

It is noteworthy that most small peptides show high ACE inhibitory activity and could be easily absorbed in the intestine by peptide transport. The IC_{50} value of peptide with MW < 5 kDa peptide was 9.01±0.04 μg (glycine equivalent)/ ml. It seems that peptide from pepsin digestion can produce strong ACE inhibitory activity with low IC_{50} value.

Collagen hydrolysate from various source with potent ACE inhibitory activity have been reported. (Byun and Kim, 2001; Fahmi et al., 2004; Ichimura et al., 2009 Saiga et al., 2008; Zhao et al., 2007). Hydrolysate from cod frame and sardine muscle at MW < 3 kDa showed IC_{50} of 83 μg/ml and 80 μg protein/ml, respectively (Matsu et al., 1993; Jeon et al., 1999). Collagen hydrolysates from scallop, oyster, codfish bone, and codfish skin exhibited IC_{50} higher than 10mg/ml (He et al., 2007).

4.10.2 Purification of ACE inhibitory activity of peptide

The HS collagen hydrolysate at MWCO <5 kDa exhibiting the strongest ACE inhibitory activity was purified. Carboxymethyl (CM)-Sepharose cation exchange column was used. There were two peptide peaks. Unbound fraction designated as I; and another one fraction designated as II. Fraction II was eluted at 0.6-1.0 M NaCl and possessed stronger activity than fraction I with inhibition activity of 86.98% at the 0.5 μg (glycine equivalent)/ml (Figure 4.9 (B))

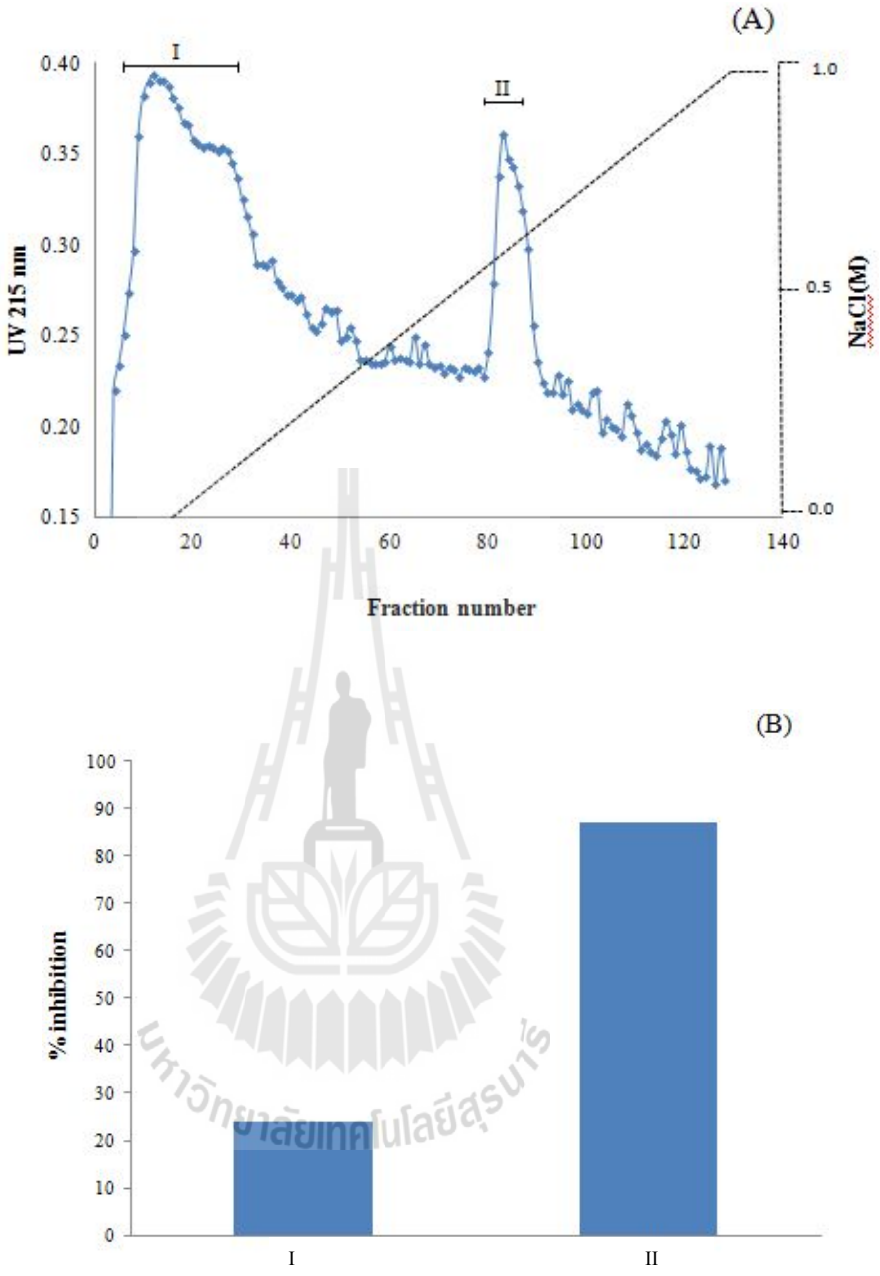


Figure 4.9 Separation of peptides from active fraction < 5 kDa using a carboxymethyl (CM)-Sepharose cation exchange column (A). Bound peptides were eluted with a linear gradient of NaCl (0-1.0 M) in 10 mM sodium acetate buffer (pH 4.0) at a flow rate of 1.0 ml/min and 1-ml fractions were collected. ACE inhibitory activity of peak designated I and II was determined at 0.5 µg peptide (glycine equivalent)/ml (B).

Fraction II was further purified by size exclusion chromatography on a Superdex 30 prep grade and fractionated into two fractions (Figure 4.10 (a)). Fractions were pooled and lyophilized. Fraction G-I exhibited the higher ACE inhibitory activity than G-II with inhibition of 70.34% at 0.2 µg glycine equivalent (Figure 4.10 (B)).

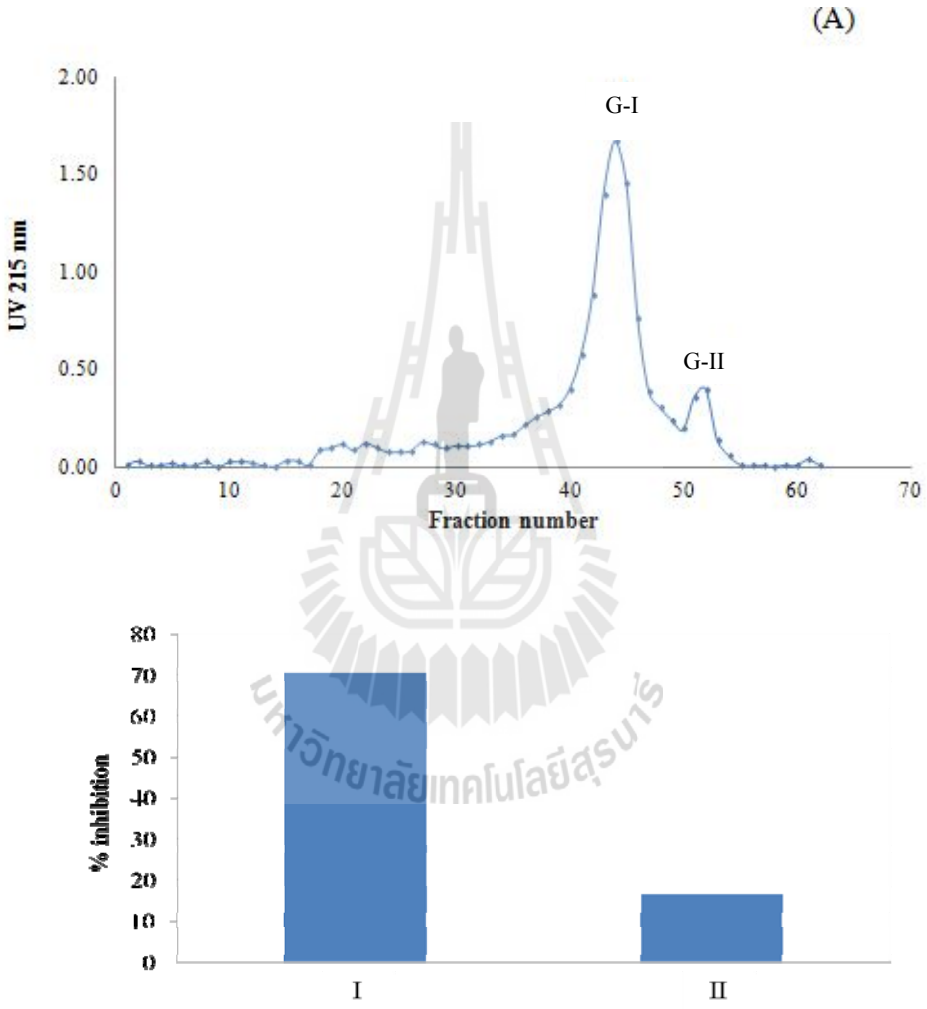


Figure 4.10 Elution profile of active fraction II on Superdex 30 prep grade. (A). Gel column was eluted with deionized water in isocratic mode at flow rate of 0.3 ml/min.Each fraction was monitored at 215 nm and 0.5-ml fractions were collected. ACE inhibitory activity designated as I and II was determined at 0.2 µg peptide (glycine equivalent)/ml (B).

Fraction G-I was dissolved in deionized water before loading into Superdex peptide 10/300 GL The single fraction was eluted as shown in Figure 4.11. The purified peptide from HS collagen was obtained with ACE inhibitory activity of 72.06% at a peptide 0.2 µg glycine (equivalent)/ml.

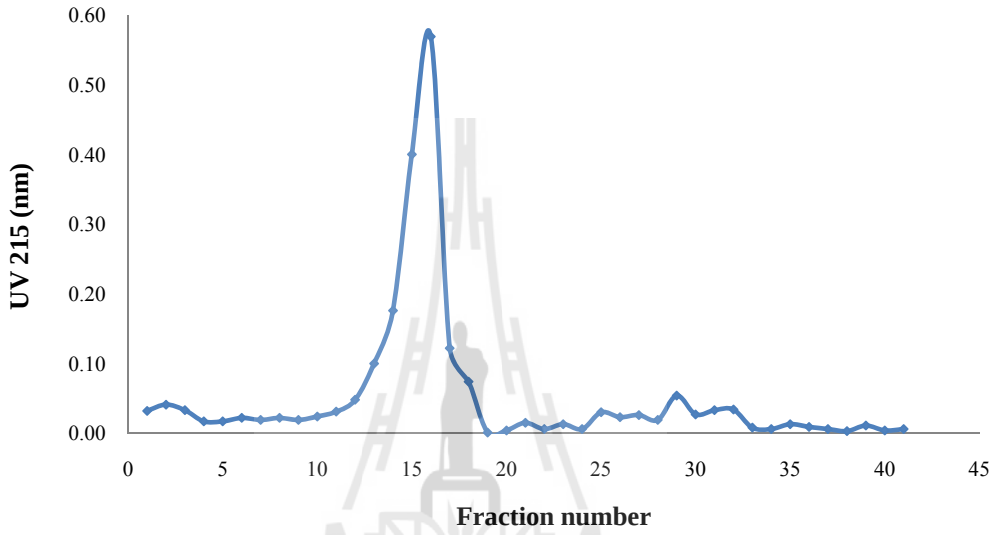


Figure 4.11 Elution profile of active fraction I on Superdex peptide 10/300 GL. Gel column was eluted with deionized water in isocratic mode at flow rate of 0.3 ml/min. Each fraction was monitored at 215 nm.

This purified peptide was further subjected to LC/MS-MS for amino acid sequencing. A total of 18 peptide fragments is shown in Table 4.5. Amino acids of partially- purified HS collagen were leucine (Leu), arginine (Arg), phenylalanine (Phe) and asparagine (Asn). Leu was present in the highest content. C-terminal of peptides contained Arg, while N-terminal contained aliphatic amino acids.

Bioactivity of peptides depends greatly on amino acid sequences. Cheng et al., (1980) reported that aromatic amino acids at the C-terminal and branched-chain

aliphatic amino acids at the N-terminal were required for a peptide binding to ACE as a competitive inhibitor. Ondetti and Cushman (1977) proposed a binding model between substrate or ACE inhibitor and active site of ACE. Three subsites of ACE are S_1 (anterpenultimate), S_1' (penultimate) and S_2' (ultimate). The C-terminus amino acid residue preferably binds with subsites of ACE. Pro and Val at C-termini are reported to be more favorable for the ACE inhibition (Zhao et al., 2007).

Table 4.5 Amino acid sequences of ACE inhibitory peptides obtained from *denovo* peptide sequencing

Sequence no.	PepNovo score	Molecular weight (Da)	Amino acid sequence
1	60.708	1018.626	Leu-Met-Leu-Leu-Leu-Phe-Arg
2	37.147	921.638	Glu-Asn-Leu-Ser-Phe-Arg-Arg
3	98.852	903.664	Glu-Leu-Leu-Leu-Leu-Phe-Arg
4	86.536	790.576	Glu-Leu-Leu-Leu-Phe-Arg
5	48.014	905.657	Pro-Gln-Leu-Leu-Pro-Pro-Leu-Lys
6	43.09	791.66	Asp-Lys-Leu-Leu-Phe-Arg
7	86.861	905.671	Met-Leu-Leu-Leu-Leu-Phe-Arg
8	50.755	1017.667	Glu-Asn-Leu-Leu-Leu-Leu-Phe-Arg
9	43.813	904.795	Asn-Glu-Leu-Leu-Leu-Phe-Arg
10	65.303	2919.462	Asn-Leu-Met-Ala-Gly-Thr-Gln-Gly-Ser-Tyr-Lys
11	64.83	2151.592	Leu-Gln-Val-Gln-Glu-Leu-Gln-Pro-Leu-Lys
12	93.703	1017.864	Leu-Asn-Glu-Leu-Leu-Leu-Phe-Arg
13	7.102	812.443	Glu-Leu-Leu-Asn-Pro-Val-Lys
14	62.685	923.765	Met-Asn-Leu-Asn-Glu-Phe-Arg
15	66.785	790.412	Glu-Leu-Leu-Leu-Phe-Arg
16	70.635	904.757	Leu-Leu-Leu-Glu-Asn-Phe-Arg
17	35.069	905.667	Met-Leu-Leu-Leu-Leu-Phe-Arg
18	65.145	1016.926	Met-Pro-Leu-Leu-Leu-Leu-Phe-Arg

Peptides derived from HS collagen consisted of arginine and lysine at C-terminal. Nakagomi et al., (2000) reported that peptide isolated from human serum albumin and human plasma contained Arg at C-terminal (Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg and Tyr-Leu-Tyr-Glu-Ile-Ala-Arg-Arg) and showed the highest ACE

inhibitory activity. These peptides acted as a noncompetitive inhibitor. Positive charge on the guanidine or ε-amino group contributed to inhibitor potency. Collagen hydrolysate from skate skin had Arg at C-terminal (Gln-Leu-Gly-Phe-Leu-Gly-Pro-Arg) showed strong ACE inhibitory activity with IC_{50} of 148 μM (Lee, Jeon and Byun, 2011). Ala –Trp and Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg were isolated from a tryptic hydrolysate of human serum albumin. Ala –Trp is a competitive inhibitor, while Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg is a noncompetitive inhibitor of ACE (Tauzin et. al., 2002; Fujita et al., 2000; Nakagomi. et al., 2000; Nakagomi et al., 1998; Cheng et al., 1980).

CHAPTER V

CONCLUSIONS

TS and HS skin are rich source of protein. High yield of collagen was obtained after acid extraction approximately 48-60 % (dry basis). Physico-chemical properties of two collagens were slightly different. Both collagens were characterized as type I collagen. HS collagen showed high amount of imino acid than TS collagen, while TS contained higher number of tyrosine, lysine. TS and HS collagen showed slightly different peptide patterns, which were different from calf commercial collagen. Stability of fish collagens was relatively low in the presence of 0.05 M acetic acid and more stable in deionized water (DI). TS collagen containing low imino acid than HS showed higher denaturation temperature than HS collagen in the presence of both acid solution and DI. TS and HS collagen were solubilized at acidic solution. The maximum solubility of TS and HS collagen was observed over the range of pH 2-4. Solubility of both collagens decreased as concentration of salt increased >3% in 0.5 M acetic solution (pH 2.5). FT-IR spectra of both collagens showed predominant helix structure of both collagens.

Our results indicated that TS and HS collagen hydrolysates can be produced to attain different bioactive properties, depending on the type of proteinase used. Pepsin produced the most potent bioactive peptide. The ACE inhibitory activity rapidly increased at the first 4 h of digestion. Peptides with molecular weight <5 kDa showed the highest ACE inhibitory activity than those >10 kDa, 5-30 kDa. Peptides at MW <

5kDa showed IC_{50} at 9.01±0.04 μg/ml (glycine equivalent). After partial purification, peptide with the highest inhibitory contained Leu, Arg, Phe and Asn. C-terminal of peptides contained Arg, while N-terminal contained aliphatic amino acids. Further research is needed to determine the antihypertensive activity of each peptide sequence and the effect of in *vitro* gastrointestinal digestion on ACE inhibitory activity.

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