การจำแนกคุณลักษณะและการใช้ประโยชน์ของสารยับยั้งเอนไซม์โปรติเนสในส่วนของซาร์โคพลาสมิกจากกล้ามเนื้อปลาเขตร้อน

นางสาวศิริพร ศิริอังคณากุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
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ปีการศึกษา 2556

CHARACTERIZATION AND UTILIZATION OF PROTEINASE INHIBITOR IN SARCOPLASMIC FRACTION FROM TROPICAL FISH MUSCLE

Siriphon Siriangkanakun

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

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CHARACTERIZATION AND UTILIZATION OF PROTEINASE INHIBITOR IN SARCOPLASMIC FRACTION FROM TROPICAL FISH MUSCLE

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

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ศิริพร ศิริอังคณากุล : การจำแนกคุณลักษณะและการใช้ประโยชน์ของสารยับยั้งเอนไซม์โปรติเนสในส่วนของซาร์โคพลาสมิกจากกล้ามเนื้อปลาเขตร้อน (CHARACTERIZATION AND UTILIZATION OF PROTEINASE INHIBITOR IN SARCOPLASMIC FRACTION FROM TROPICAL FISH MUSCLE) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 210 หน้า.

ปลาไนมีกิจกรรมการย่อยสลายตัวเองต่ำกว่าปลาหนวดฤาษี ($P < 0.05$) กิจกรรมของเอนไซม์โปรติเนสและทรานสกลูตามิเนสในปลาไนและปลาหนวดฤาษีในตัวอย่างที่ผ่านการเตรียมด้วยกระบวนการสกัดด้วยด่างมีค่าต่ำกว่าตัวอย่างที่ผ่านการล้างด้วยน้ำ ($P < 0.05$) ซึ่งบ่งชี้ว่ากระบวนการสกัดด้วยด่างมีผลยับยั้งกิจกรรมของเอนไซม์ทั้งสอง เจลปลาไนที่ผ่านการล้างด้วยน้ำมีค่าแรง ณ จุดแตกหักสูงที่สุด ($P < 0.05$) ในขณะที่การสกัดด้วยด่างส่งผลให้เจลปลาหนวดฤาษีมีค่าแรง ณ จุดแตกหักสูงที่สุด ($P < 0.05$) นอกจากนี้การบ่มเจลที่อุณหภูมิต่ำ (เซตติ้ง) ของปลาทั้งสองชนิดที่ผ่านการเตรียมด้วยกระบวนการสกัดด้วยด่างไม่มีผลเพิ่มค่าเนื้อสัมผัสของเจล เจลจากปลาไนที่ผ่านการเตรียมด้วยการล้างด้วยน้ำและสกัดด้วยด่างมีค่าแรง ณ จุดแตกหักสูงกว่าเจลจากปลาหนวดฤาษีที่เตรียมภายใต้สภาวะเดียวกัน

โปรตีนซาร์โคพลาสมิกจากปลาไนมีความสามารถยับยั้งกิจกรรมของเอนไซม์ทริปซินเมื่อทดสอบด้วยเทคนิคการยับยั้งทริปซิน (trypsin inhibitory activity staining) โดยโปรตีนที่มีมวล 35, 41, 47, 52 และ 69 กิโลดาลตันแสดงความสามารถในการยับยั้งกิจกรรมเอนไซม์ทริปซิน เมื่อเติมโปรตีนซาร์โคพลาสมิก 0.18 เปอร์เซ็นต์ ลงในซูริมิปลาทรายแดงส่งผลให้เนื้อสัมผัสเพิ่มสูงขึ้น และพบการคงอยู่ของมัยโอซินสายหนักเพิ่มขึ้น จากผลการศึกษานี้แสดงว่าโปรตีนซาร์โคพลาสมิกสามารถยับยั้งเอนไซม์ทริปซินและเอนไซม์โปรติเนสในซูริมิปลาทรายแดง เพื่อระบุชนิดของโปรตีนที่มีคุณสมบัติเป็นสารยับยั้งเอนไซม์โปรติเนส จึงทำบริสุทธิ์โดยการให้ความร้อน การตกตะกอนด้วยแอมโมเนียมซัลเฟต การแลกเปลี่ยนไอออน การแยกแบบจำเพาะ และการแยกตามขนาด ทำให้ได้โปรตีนบริสุทธิ์ที่มีมวล 47 (inhibitor I) และ 52 (inhibitor II) กิโลดาลตัน เมื่อระบุชนิดของโปรตีนทั้งสองด้วยเทคนิค LC-MS/MS พบว่าคือแอลฟ่าวัน-โปรติเนสอินฮิบิเตอร์ (α1-PI) สารยับยั้ง inhibitor I และ inhibitor II เป็นไกลโคโปรตีน มีน้ำตาลเชื่อมต่อกับกรดอะมิโนแอสพาราจีนที่ตำแหน่ง 214 (N214) และตำแหน่ง 226 (N226) การเติม α1-PI สามารถลดการเสื่อมสลายของมัยโอซินสายหลักและโทรโปมัยโอซินของซูริมิปลาตาหวาน

ปลาทรายแดง ปลาตาหวานและปลาจวดเหลืองเป็นวัตถุดิบสำคัญในการผลิตซูริมิปลาเขตร้อน โดยโปรตีนซาร์โคพลาสมิกของปลาทั้ง 3 ชนิดที่ผ่านการทำบริสุทธิ์บางส่วนด้วยการตกตะกอนแอมโมเนียมซัลเฟตที่ความเข้มข้น 50-70 เปอร์เซ็นต์ และอยู่ในรูปผงด้วยการระเหิดแห้ง

สามารถยับยั้งเอนไซม์ทริปซินได้ 92, 80 และ 85 เปอร์เซ็นต์ตามลำดับ เมื่อใช้เทคนิค GeLC-MS/MS พบโปรตีน α1-PI ในซาร์โคพลาสมิกโปรตีนผงจากปลาจวดเหลืองที่มีมวล 45-50 กิโลดาลตัน ความสามารถในการยับยั้งเอนไซม์ทริปซินของซาร์โคพลาสมิกผงของปลา 3 ชนิดมีความเสถียรต่อความร้อนในช่วงของอุณหภูมิ 30-60 องศาเซลเซียสและมีความเสถียรต่อโซเดียมคลอไรด์จนถึงระดับ 0.2 โมลาร์ การเติมซาร์โคพลาสมิกโปรตีนผงจากปลาทรายแดงเข้มข้น 0.5 และ 1 เปอร์เซ็นต์ลงในซูริมิปลาทรายแดงและบ่มที่อุณหภูมิ 37 และ 65 องศาเซลเซียสส่งผลให้ความแข็งแรงของเจลจากซูริมิปลาทรายแดงเพิ่มขึ้น

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา _____

SIRIPHON SIRIANGKANAKUN : CHARACTERIZATION AND UTILIZATION OF PROTEINASE INHIBITOR IN SARCOPLASMIC FRACTION FROM TROPICAL FISH MUSCLE. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D. 210 PP.

ALKALI pH-SHIFT PROCESS/INHIBITOR/SARCOPLASMIC PROTEINS/ GLYCOSYLATION/LC-MS/MS/GELC-MS/MS

Autolytic activity of common carp was lower than that of goatfish ($P < 0.05$). The alkali-extracted fish protein isolate (FPI) of both common carp and goatfish showed lower autolytic and transglutaminase activity than thrice-washed samples ($P < 0.05$), implying that these enzymes were largely inactivated during alkali pH-shift process. Breaking force of common carp gels was the highest when prepared by conventional washing ($P < 0.05$). In contrast, gels prepared from FPI of goatfish exhibited the highest breaking force ($P < 0.05$). Setting did not enhance textural properties of FPI gels from both species. All gels prepared by conventional washing and alkali pH-shift process of common carp showed higher textural properties than those of goatfish under the same heating conditions.

Sarcoplasmic proteins (SP) from common carp were able to inhibit trypsin. Protein bands with molecular mass of 35, 41, 47, 52 and 69 kDa appeared on trypsin inhibitory activity staining. Textural properties of threadfin bream surimi were improved with addition of SP. In addition, retention of myosin heavy chain (MHC) increased when SP was added at 0.18%. Therefore, common carp SP showed inhibitory activity against trypsin and endogenous proteinases of threadfin bream

surimi. The proteinase inhibitor from common carp SP was purified using heat treatment, ammonium sulfate precipitation, anion exchange, affinity chromatography and gel filtration. Two protein bands with molecular mass of 47 (inhibitor I) and 52 (inhibitor II) kDa were observed. The inhibitors I and II were considered to be alpha-1-proteinase inhibitor (α1-PI), based on LC-MS/MS. They are glycoproteins with N-linked glycans attached to asparagines at N214 and N226. Moreover, addition of α1-PI was able to reduce degradation of MHC and tropomyosin of bigeye snapper surimi.

Threadfin bream, bigeye snapper and yellow croaker are the main species used as raw material for tropical surimi production. SP from three species were fractionated by 50-70% ammonium sulfate precipitation. Lyophilized fractionated SP of threadfin bream (TB-SP), bigeye snapper (BS-SP) and yellow croaker (YC-SP) exhibited 92, 80 and 85% trypsin inhibitory activity, respectively. Based on GeLC-MS/MS, YC-SP possessed α1-PI with molecular mass ranging from 45 to 50 kDa. Trypsin inhibitory activity of three species showed good stability at 30-60 °C and up to 0.2 M NaCl. In addition, textural properties of threadfin bream surimi were improved with addition of 0.5 and 1% TB-SP in conjunction with setting at 37 °C.

School of Food Technology Student's Signature_____

Academic Year 2013 Advisor's Signature_____

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Siriphon Siriangkanakun

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LIST OF ABBREVIATIONS

AC	=	Actin
ACN	=	Acetonitrile
ANOVA	=	Analysis of variance
Asn	=	Asparagine
Asp	=	Aspartic acid
α1-PI	=	Alpha-1-proteinase inhibitor
α_2-M	=	Alpha2-macroglobulin
BPP	=	Beef plasma protein
BS-SP	=	Lyophilized fractionated bigeye snapper sarcoplasmic protein
°C	=	Degree Celsius
CD	=	Circular dichroism
CK	=	Creatine kinase
Con A	=	Concanavalin A
CPP	=	Chicken plasma protein
Da	=	Dalton
DEAE	=	Diethylaminoethyl
DMRT	=	Duncan's multiple range test
DTT	=	Dithiothreitol
ESI-MS	=	Electrospray ionization mass spectrometry

LIST OF ABBREVIATIONS (Continued)

EW	=	Egg white power
FA	=	Formic acid
FPI-C	=	Fish protein isolate of common carp
FPI-G	=	Fish protein isolate of goatfish
GAPDH	=	Glyceraldehy-3-phosphate dehydrogenase
GeLC-MS/MS	=	Gel-based liquid chromatography-tandem mass spectrometry
g	=	Gram
×g	=	Relative centrifugal force
HCl	=	Hydrochloric acid
h	=	Hour
IAA	=	Iodoacetic acid
LC-MS/MS	=	Liquid chromatography with tandem mass spectrometry
kDa	=	Kilodalton
M	=	Molar
MALDI-TOF	=	Matrix assisted laser desorption/ionization-time of flight
M-C	=	Mince of common carp
M-G	=	Mince of goatfish
Met	=	Methionine
MHC	=	Myosin heavy chain
MP	=	Myofibrillar protein
MS	=	Mass spectrometry

LIST OF ABBREVIATIONS (Continued)

MS/MS	=	Tandem mass spectrometry
MWCO	=	Molecular weight cut-off
mg	=	Milligram
min	=	Minute
mL	=	Milliliter
mM	=	Millimolar
mm	=	Millimeter
m/z	=	Mass-to-charge ratio
NaCl	=	Sodium chloride, salt
µl	=	Microliter
µm	=	Micrometer
N	=	Asparagine
NCBI	=	The National Center for Biotechnology Information
OC	=	Ovomucoid
pI	=	Isoelectric point
PMF	=	Peptide mass fingerprinting
PNGase F	=	Peptide -N-Glycosidase F
PP	=	Potato powder
ppm	=	Parts per million
PPP	=	Pig plasma protein
%	=	Percent
SDS	=	Sodium dodecyl sulfate

LIST OF ABBREVIATIONS (Continued)

SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	=	Serine
SH	=	Sulfhydryl
SP	=	Sarcoplasmic protein
SpC	=	Spectral count
SPI	=	Soy protein isolate
TB	=	Threadfin bream
TB-SP	=	Lyophilized fractionated threadfin bream sarcoplasmic protein
TCA	=	Trichloroacetic acid
TGase	=	Transglutaminase
TM	=	Tropomyosin
TN	=	Troponin
Tris	=	Tris (hydroxymethyl) aminomethane
U	=	Unit activity
WM-C	=	Washed mince of common carp
WM-G	=	Washed mince of goatfish
YC-SP	=	Lyophilized fractionated yellow croaker sarcoplasmic protein

CHAPTER I

INTRODUCTION

1.1 Introduction

Common carp (*Cyprinus carpio*) is an economically important aquacultured freshwater fish around the world. Global aquaculture production for common carp was estimated to be 3.7 million metric tons in 2011 (FAO, 2013). However, they are not widely consumed due to pinbones. This leads to its low value. Goatfish (*Upeneus* spp.) is one of important tropical marine fish used for surimi production. However, gel-forming ability of goatfish surimi is poor because of its high endogenous proteolytic activity (Rawdkuen and Benjakul, 2008). Appropriate washing is demanded to achieve high quality and recovery of fish mince. Conventional washing process refines the myofibrillar proteins using 3 water washing cycles. The major objectives of this washing are to concentrate myofibrillar protein and to remove water-soluble matters, resulting in a relatively low yield. The pH shift process is a novel method for recovery fish protein. This process involves either acid or alkali solubilization of muscle proteins, followed by isoelectric precipitation and adjusting pH to neutral condition. Several studies reported that textural properties of fish muscle proteins prepared by alkali pH-shift process were greater than conventional washing and acid pH-shift process (Kim, Park, and Choi, 2003; Kristinsson and Hultin, 2003; Yongsawatdigul and Park, 2004).

Autolytic degradation is one of the most important factors for quality determination of fish muscle proteins. It has been recognized as a result of endogenous proteinases. Some proteinases, especially cathepsin L, cannot be completely removed by conventional washing process. As a result, autolytic degradation increased after washing and caused the loss of surimi gel strength (Kinoshita, Toyohara, and Shimizu, 1990; Toyohara, Sakata, Yamashita, Kinoshita, and Shimizu, 1992). However, several studies reported that proteinases were inactivated during alkali pH-shift process (Kristinsson, Theodore, Demir, and Ingadottir, 2005). Information about autolytic activity of both common carp and goatfish has rarely been investigated. Understanding about autolytic activity of fish muscle proteins would lead to a proper means for improving its gelation.

Setting in the conventional surimi process has been known to increase the textural properties of surimi gels after pre-incubation at a certain temperature below 40 °C before cooking to form gel at 90 °C (Lanier, 2000). Endogenous transglutaminase (TGase) is responsible for the formation of covalent non-disulfide cross-linking under setting condition, leading to an increase in gel strength. (Joseph, Lanier, and Hamann, 1994; An, Peters, and Seymour, 1996; Yongsawatdigul, Worratao, and Park, 2002). Tadpitchayangkoon and Yongsawatdigul (2009) reported that textural properties of striped catfish (*Pangasius hypophthalmus*) prepared by alkali pH-shift process under setting condition was higher than those prepared from a direct heating method. However, information related to setting phenomenon of tropical fish species prepared by alkali pH-shift process is still limited.

Proteolytic degradation of myofibrillar proteins has an adverse effect on gel-forming ability of surimi. The presence of endogenous proteinases in fish muscle

inhibits the development of three-dimensional gel networks, resulting in gel softening as well as quality loss of surimi products. Trypsin-like serine proteinase is a major enzyme responsible for degradation of myofibrillar protein and gel-weakening of fish muscle proteins. Martone, Busconi, Folco, and Sanchez (1991) found trypsin-like serine proteinase in hake skeletal muscle. This proteinase degraded the major contractile and cytoskeletal constituent proteins of myofibrils. Choi, Cho, and Lanier (1999) reported that trypsin-like proteinase was the major proteinase in Atlantic menhaden muscle and caused poor textural properties. To alleviate the softening of surimi gel caused by endogenous proteinases, some food grade protease inhibitors such as egg white powder and whey protein concentrate have been used.

Sarcoplasmic proteins (SP) are proteins extracted from muscle tissue by water or low ionic strength solution. Fish muscle protein is composed of 20-40% SP, depending on the species (Okada, 1999). These proteins contain many enzyme involved in muscle metabolism. SP are previously known to interfere with gelation of myofibrillar proteins so they are typically removed during surimi production by conventional washing process. Recently, various studies revealed that SP positively contribute to gel formation. Morioka and Shimizu (1990) reported that addition of mackerel SP to threadfin bream surimi resulted in an increase of gel formation. Some studies have shown that endogenous transglutaminase (TGase) contained in SP and catalyzed cross-linking of myosin heavy chain, resulting in a more elastic gel (Yongsawatdigul and Piyadhammaviboon, 2007). In addition, serine proteinase inhibitors have also been found in SP. Piyadhammaviboon and Yongsawatdigul (2010) reported that SP from threadfin bream showed inhibitory activity towards serine proteinases, especially trypsin. Therefore, the presence of proteinase

inhibitor(s) could also partly contribute to the gel-enhancing effect of sarcoplasmic proteins. Understanding on the gel-enhancing role of proteinase inhibitor from sarcoplasmic protein would lead to more efficient strategies to recover and utilize sarcoplasmic protein as a functional ingredient.

The proteinase inhibitors that exhibit selective interaction of serine proteinases are referred to as serpins. The main serpin is alpha-1-antitrypsin that can be found in blood plasma, seminal plasma, serum of mammalian and fish species (Mickowska, 2009; Wojtczak, Całka, Glogowski, and Ciereszko, 2007; Aranishi, 1999). Various proteinase inhibitors have been purified from fish muscle proteins (Cao et al., 2000; Sangorrín, Folco, Martone, and Sánchez, 2001). However, purification and identification of proteinase inhibitor from common carp SP have not been investigated.

Endogenous muscular proteinase inhibitors have been studied in freshwater fish including crucian carp and catfish (Sun et al., 2009; Nurhayati et al., 2013), whereas proteinase inhibitors from marine fish have rarely been investigated. Threadfin bream (*Nemipterus* spp.), bigeye snapper (*Priacanthus* spp.) and yellow croaker (*Larimichthys* spp.) are important tropical marine fish for surimi production in the southeast Asian region. It was reported that 50 kg of sarcoplasmic proteins are removed for every 100 kg of surimi produced (Morrisey, Lin, and Ismond, 2005). In order to maximize utilization of sarcoplasmic proteins, their efficacy as proteinase inhibitors should be investigated.

Proteomic is a powerful and direct approach for protein identification analysis. Shotgun proteomics have been widely used to identify protein because it can identify several proteins within one analysis. However, the complexity of the peptide mixture

limits the use of shotgun proteomics (Maksup, Roytrakul, and Supaibulwatan, 2012). Gel-based liquid chromatography-tandem mass spectrometry (GeLC-MS/MS) is designed to overcome problems caused by protein complexity. The complex protein mixtures are pre-fractionated by one-dimensional sodium dodecyl sulfate-PAGE (SDS-PAGE), and entire gel lanes are excised and further subdivided into smaller sections. Subsequently, the proteins in these gel sections are digested in-gel with trypsin, and the generated peptides are analyzed to LC-MS/MS (Ye et al., 2010). Slicing the gel lane into smaller bands separates the protein mixtures into narrow molecular weight range and each gel band is analyzed separately, resulting in reduced protein complexity in each analysis (Rezaul, Wu, Mayya, Hwang, and Han, 2005). Thus, this approach can isolate some low abundant proteins from the high abundant ones. In contrast, tryptic peptides analyzed by shotgun proteomics from high abundant proteins could be detected across most of the fractions, making low abundant ones ignored. Therefore, GeLC-MS/MS would be a powerful tool for identification of complex protein mixtures like SP.

1.2 Research objectives

The objectives of this research are:

1. To investigate the effect of conventional washing process and alkali pH-shift process on autolysis and gel-forming ability of freshwater fish (common carp) and marine fish (goatfish).

2. To study proteinase inhibitory activity of sarcoplasmic protein extracted from common carp and effect of sarcoplasmic protein on surimi gelation.

3. To purify and identify proteinase inhibitor from common carp sarcoplasmic proteins.

4. To investigate the proteinase inhibitory effect of threadfin bream, bigeye snapper and yellow croaker sarcoplasmic proteins.

1.3 Research hypothesis

Common carp exhibits higher gel properties than goatfish and can be used as an alternative raw material for surimi-based products. Gels of common carp and goatfish prepared by various conditions including unwashed mince, conventional washing process, and alkali pH-shift process exhibit different textural properties. In addition, sarcoplasmic protein of tropical freshwater fish, common carp, contains proteinase inhibitor which is able to minimize proteolytic activity and improve textural properties of surimi. The proteinase inhibitor from common carp sarcoplasmic protein can be purified by chromatographic techniques. Purified proteinase inhibitor would reduce autolytic activity of surimi. Furthermore, tropical marine fish including threadfin bream, bigeye snapper, and yellow croaker might contain proteinase inhibitors in sarcoplasmic proteins which could have a gel enhancing effect on textural properties of surimi gel.

1.4 Scope of the study

Freshwater fish (common carp) and marine fish (goatfish) were prepared under various conditions including unwashed mince, conventional washing process, and alkali pH-shift process. Autolytic and transglutaminase activity were assayed. In addition, gel-forming ability of two species prepared by various treatments was

investigated. Sarcoplasmic proteins from common carp were assayed for proteinase inhibitory activity. A proteinase inhibitor in common carp sarcoplasmic proteins was purified, identified, and characterized. Moreover, marine fish species including threadfin bream, bigeye snapper, and yellow croaker which are important species for surimi production were investigated for proteinase inhibitory activity of sarcoplasmic proteins. Proteinase inhibitor(s) from sarcoplasmic proteins of these species was identified using GeLC-MS/MS approach.

1.5 Expected results

Textural properties of common carp and goatfish will be improved through alkali pH-shift process. Common carp is probably as an alternative source of raw material for mince fish production. In addition, more understandings about proteinase inhibitor of fish sarcoplasmic proteins will be obtained. Sarcoplasmic proteins which are byproducts from surimi processing could be fully utilized based on the proteinase inhibitory function.

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CHAPTER II

LITERATURE REVIEWS

2.1 Surimi

Surimi is the stabilized myofibrillar protein from fish muscle. It is mechanically deboned fish flesh and is washed with water to remove most of lipids, blood, enzymes and sarcoplasmic proteins, and then blended with cryoprotectants to provide a better frozen shelf life (Lanier, 1992). It is an intermediate product, which is used for a variety of foods from the traditional kamaboko products of Japanese to the recent shellfish substitutes (Kumazawa, Numazawa, Seguro, and Motoki, 1995).

2.2 pH shift process

The pH shift is a new method for fish protein recovery. This method has several advantages, such as high yield, high protein quality, and efficient removal of insoluble impurities (Hultin and Kelleher, 1999; Yongsawatdigul and Park, 2004; Choi and Park, 2002; Undeland, Kelleher, and Hultin, 2002*;* Hultin, Kristinsson, Lanier, and Park, 2005; Park and Lin, 2005). In addition, neutral lipids and membrane lipids can be efficiently removed by the pH-shift process, something which minimizes the risk for lipid oxidation (Park, 2009). Park (2009) reported that the recovered proteins by pH-shift process exhibited a high yield of 35-40% while conventional washing process obtained 25-30% protein recovery. Yield of conventional washing process is low since this process involves several washing steps

(Kristinsson, Theodoure, Demir, and Ingadottir, 2005). Diagram of the pH-shift process is shown in Figure 2.1. Muscle proteins are mixed with 6-9 volumes of cold water and extracted at extreme pH (either pH 2 or 11) using either 2 N HCl or NaOH. The proteins gain a positive and negative charge, respectively, which cause the proteins to solubilize due to electrostatic repulsion. At acidic pH, the proteins gain a net positive charge due to neutralization of the negative charges of the carboxylate side chains of aspartic and glutamic acids. While the proteins gain a net negative charge at alkaline pH because of the deprotonation of basic groups, such as the imidizole side chains of histidine, guanidyl side chains of arginine, and the amino side chains of lysine, and from deprotonation of the phenolic side chains (Kristinsson, Theodore, Demir, and Ingadottir, 2005; Hultin, Kristinsson, Lanier, and Park, 2005; Yongsawatdigul and Park, 2004). The viscosity of the solution increases due to the swelling and elongation (increased hydrodynamic volume) of the dispersed myofibrillar proteins at these pH values (Undeland, Kelleher, and Hultin, 2002). Under these conditions, the insoluble proteins can be separated from the soluble proteins using high speed centrifugation. After centrifugation step, a three phase system is obtained: a) upper layer with neutral lipid, b) sediment with membranes, insoluble proteins, bones, skin, and parts of the phospholipids, and c) middle layer with soluble protein (Hultin, Kristinsson, Lanier, and Park, 2005). Subsequently, the solubilized proteins are collected and adjusted to a pH close to the isoelectric precipitation (pI) of the muscle proteins, generally between pH 5 and 6. Almost all the muscle proteins become insoluble at this point. This is due to lack of electrostatic repulsion which allows aggregation and precipitation of the proteins via hydrophobic interactions (Chang, Feng, and Hultin, 2001; Chang, Hultin, and Dagher, 2001). This

protein precipitation and aggregation facilitates subsequent dewatering by screening or centrifugation. Product from pH-shift is called fish protein isolate (FPI). This includes sarcoplasmic proteins, which are mostly washed away during conventional surimi manufacture, and myofibrillar proteins (Hultin, Kristinsson, Lanier, and Park, 2005).

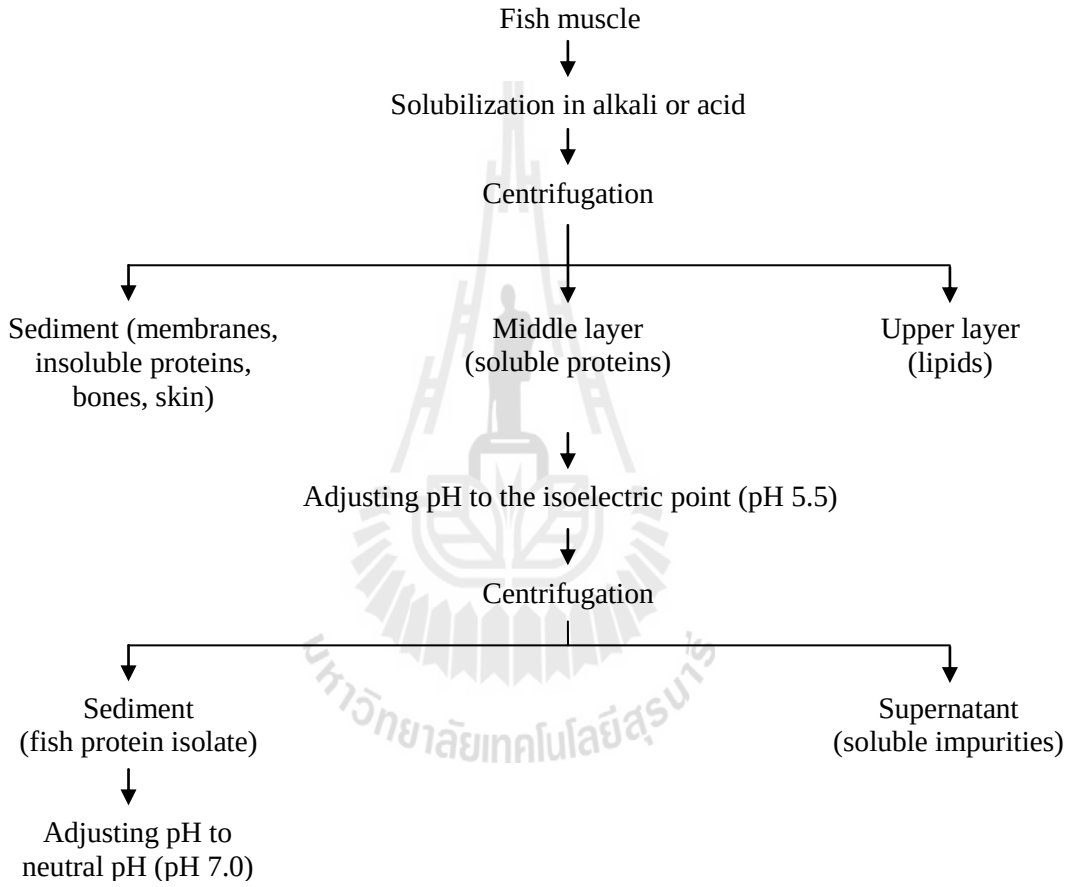

Figure 2.1 The process of fish protein recovery using pH shift process.

Adapted from: Hultin et al. (2005)

2.2.1 Endogenous enzymes in fish protein isolate

Endogenous enzymes, including proteinases and transglutaminase, in fish muscle proteins are important factors that partly govern the gel-forming ability.

Proteolytic degradation during acid-treated fish muscle proteins was studied by Choi and Park (2002). They reported that breaking force and deformation values of acid pH-shift process from Pacific whiting were lower than those of conventional surimi process. The lower gel prepared by acid pH-shift process resulted from activity of proteinases, especially cathepsin L. Kim et al. (2003) found cathepsin L-like activity in Pacific whiting treated at low and high pH. The highest cathepsin L-like activity was observed in fish proteins at pH 10.5, while prominent reduction in activity was found at pH 11 and 12. A possible explanation for this sudden change is that cathepsin L-like enzymes might be largely inactivated at pH 11 or higher. Normally, stability of sarcoplasmic proteins, including proteolytic enzymes, are found under mild alkaline condition. Presumably, pH 10.5 was not high enough to maintain stable cathepsin L-like activities and the enzyme could be reactivated when the pH was readjusted to 7.0 and further activated at 5.5, which is known as the optimum pH for cathepsin L-like enzyme. Therefore, fish proteins treated at pH 10.5 exhibited lower breaking force than that at pH 11, resulting from endogenous proteinases. However, Yongsawatdigul and Park (2004) reported that the highest gel quality was obtained when rockfish was treated by alkali-pH shift process. This result indicated that alkali treated rockfish muscle proteins did not promote proteolysis. Proteolytic activity of mince, washed mince and alkali-treated sample from striped catfish (*Pangasius hypophthalmus*) was maximun at 65-70 °C (Tadpitchayangkoon and Yongsawatdigul, 2009). Washed mince showed higher proteolytic activity than mince, suggesting the presence of myofibril-bound proteinase(s). In addition, the lowest autolytic activity was observed in alkali-treated fish muscle proteins, implying that proteinases were inactivated during alkali-pH shift process.

Endogenous transglutaminase (TGase) is an enzyme catalyzing acyl-transfer reaction, resulting in ε-(γ-glutamyl) lysine cross-links. The γ-carboxyamide groups of peptide-bound glutamine residues in proteins act as acyl donors, while the primary amino groups, including the ε-amino of lysine can be acyl acceptors (Folk 1980). When the ε-amino group of lysine acts as acyl acceptor, crosslinking of proteins is mediated through the formation of ε-(γ-glutamyl) lysine linkages. Endogenous TGase has been reported to be responsible for proteins cross-links in setting phenomenon. Setting in the conventional washing process has been known to increase the textural properties of surimi gels after pre-incubation at a certain temperature below 40 °C before cooking to form gel at 90 °C (Lanier, 2000). The optimum temperature for setting among species may be determined by the heat stability of myosin (Morales, Ramirez, Vivanco, and Vazquez, 2001). Textural properties of gels from Atlantic menhaden prepared by acid and alkali solubilization under setting condition were investigated by Perez-Mateos and Lanier (2006). They reported that TGase activity was only evident in alkali-treated fish muscle proteins without added salt and incubated at 40 °C. Breaking force and deformation values of acid-treated fish muscle proteins were lower than those of alkali-treated fish muscle proteins, implying that acid-treated fish muscle proteins exhibited no apparent TGase activity under setting condition. Tadpitchayangkoon and Yongsawatdigul (2009) reported that breaking force of alkali pH-shift treated striped catfish (*Pangasius hypophthalmus*) which was subjected to setting process was higher than those prepared from a direct heating method. In addition, low temperature setting did not promote textural properties of acid- and alkali-extracted fish protein isolate of Alaska

Pollock, implying that endogenous TGase might be damaged during acid or alkali extraction (Kim and Park, 2008).

2.2.2 Gelation of fish protein isolate

The pH shift process almost always produces FPI with better textural properties than conventional washing according to several studies. The greatest textural properties were obtained when Pacific whiting mince was treated at pH 11, probably due to enhanced disulfide bond formation and decreased proteolytic activity (Kim, Park, and Choi, 2003). Breaking force and deformation values of gels from acid- or alkali-produced rockfish muscle proteins, rockfish mince, and rockfish mince washed three times were investigated by Yongsawatdigul and Park (2004). They found that alkali-extracted FPI exhibited the highest textural properties followed by the washed mince gel and mince gel. Acid extraction process produced the lowest breaking force and deformation values. This was because disulfide linkages occurred at a greater extent in gel prepared by alkaline pH-shift process, resulting in higher breaking force and deformation. In addition, they found protein bands with molecular mass of 42 and 120 kDa in alkali-treated paste of rockfish but almost disappeared in alkali-treated gels, suggesting that these proteins interact with myofibrillar protein and sarcoplasmic protein through disulfide linkages during gelation. Kim et al. (2003) reported that the gelling mechanism of protein recovered from pH-shift process was different from that of conventional serimi process. The conventional surimi method avoids any denaturation during process to prevent protein damage and maintain protein quality while pH-shift process induces fish proteins to be denatured through strong acid or alkaline treatments. The highest textural properties of gels from Atlantic croaker was prepared by alkali pH-shift process, compared to acid pH-shift

process and conventional surimi (Perez-Mateos, Amato, and Lanier, 2004). Kristinsson and Ingadottir (2006) also found that alkali-extracted FPI made from tilapia gelled better than conventional surimi. FPI treated by the alkali-extracted process from frozen Atlantic croaker showed higher structural fracture stress and fracture strain than conventional surimi gels (Kristinsson and Liang, 2006). Textural properties of alkali-extracted FPI from Alaska Pollock showed higher than those of acid--extracted FPI (Kim and Park, 2008). Almost all studies suggested that alkali-extracted FPI produced better gels than acid- extracted FPI.

2.3 Muscle proteins of fish

Fish muscle proteins can be divided into three groups based on their solubility. They are myofibrillar proteins (salt-soluble protein), sarcoplasmic proteins (water-soluble protein) and stroma proteins (insoluble proteins).

2.3.1 Myofibrillar proteins

Myofibrillar proteins are the major proteins in fish muscle which consist of myosin, actin, tropomyosin, troponin and actinin. The proteins in fish account 66-77 % of the total protein content in muscle, compared with 52-56 % in mammals (Mackie, 1994). These proteins are extracted with neutral salt solutions of ionic strength above 0.15 usually ranging from 0.30 to 0.70. It has been known that myofibrillar proteins are mainly responsible for the functional properties of muscle tissues, including gelation and water-binding (Yasui, Ishioroshi, and Samejima, 1980). Myosin and actin are important components of myofibrillar proteins that are responsible for muscle contraction in the living animal, as well as many functional characteristics in processed meat products (Xiong, 2000).

2.3.2 Stroma proteins

Stroma proteins are connective tissues and are composed primarily of collagen, elastin, and lipoproteins of the cell membrane. These proteins represent approximately 3-5% of total protein content of fish muscle (Suzuki, 1981). Stroma proteins are insoluble in water, acid, or alkali solution and neutral salt solution of 0.01-0.1 M concentration (Lawrie and Ledward, 2006). When surimi is made, these proteins are retained along with the myofibrillar proteins. However, they are removed during refining step.

Collagen is denatured and converted to gelatin by heating, depending on the structure of the collagen present. This soluble gelatin can interfere with the gelation of myofibrillar proteins (Park, 2000). In addition, Ziegler and Acton (1984) reported that the stroma proteins did not exhibited gelation ability as the fraction only coagulated upon heating to 80 °C. Elastin is very resistant to moist heat and cooking. It is normally a reflection of the different structural arrangements of muscle cells in fish, compared to mammals (Mackie, 1994).

2.3.3 Sarcoplasmic proteins

Sarcoplasmic proteins are globular in tertiary structure and soluble in water or dilute salt solution. Fish muscle protein is composed of 20-40% sarcoplasmic proteins, depending on the species (Okada, 1999). The sarcoplasmic proteins are a large family of proteins that consist of many types of proteins including heme, enzymes and proteins.

The heme proteins of red muscle and blood which are responsible for the pigmentation of muscle are myoglobin and hemoglobin, respectively. For surimi processing, both myoglobin and hemoglobin have an essential role in the whiteness

which is one of factors determining the quality of surimi. Denaturation of the heme proteins can result in their binding to myofibrillar proteins and resulting in discoloration. In addition, heme proteins can induce lipid oxidation which contributes to premature denaturation and aggregation of the myofibrillar proteins. Therefore, reduction of interaction between heme proteins and muscle can prevent discoloration and lipid oxidation.

Major sarcoplasmic proteins of fish are glycolytic enzymes such as phosphorylase, lactate dehydrogenase, enolase, creatine kinase, aldolase, and glyceraldehyde phosphate dehydrogenase (Table 2.1). Several enzymes in sarcoplasmic proteins have native molecular weights ranging from 18 kDa to 360 kDa. Scopes (1970) reported that glyceraldehyde phosphate dehydrogenase showed the highest concentration (20%). Native glyceraldehyde phosphate dehydrogenase exhibites an apparent molecular weight of approximately 146 kDa. However, this enzyme is a homotetrameric protein composed of subunits with an apparent molecular weight of approximately 37 kDa. Ladrat, Verrez-Bagnis, Noel, and Fleurence (2003) reported that sarcoplasmic proteins of sea bass contained various proteins with molecular weight ranging from 12 to 97 kDa as determined by SDS-PAGE. Major proteins were: a 97-kDa protein identified as phosphorylase; a 51-kDa protein identified as enolase; a 41–39 kDa huge band, which was assumed to be creatine kinase and aldolase, and a 36-kDa component assumed to be glyceraldehyde-3-phosphate dehydrogenase. Sarcoplasmic proteins with molecular mass of 12 and 13 kDa were presumed to be parvalbumins. In addition, a 17-kDa protein from seabass was identified to be nucleoside diphosphate kinase.

Table 2.1 Relative proportions of sarcoplasmic proteins.

Types	Quantity (mg/g)	Molecular weight (kDa)
Glyceraldehyde phosphate dehydrogenase	12	146
Aldolase	6	160
Creatine kinase	5	82
Enolase	5	47.3
Lactate dehydrogenase	4	140
Pyruvate kinase	3	237
Phosphorylase b	2.5	180
Triose phosphate isomerase	2	45
Phosphoglucomutase	1.5	62
"F protein"	1.5	35
Phosphoglycerate kinase	1.2	94
Phosphoglucose isomerase	1	130
Phosphofructokinase	1	360
Phosphoglycerate mutase	1	65
α–Glycerophosphate dehydrogenase	0.5	60
Myokinase	0.5	21
Myoglobin	~0.5	18
AMP deaminase	0.2	300
Others	6.6	Variable molecular weights
Total	55	

Adapted from: Scopes (1970)

This protein was able to be an indicator of freshness quality since it disappears upon cold storage (Terova, Cattaneo, Preziosa, Bernardini, and Saroglia, 2011). Sarcoplasmic proteins of mackerel include phosphorelase (94 kDa), enolase

(50 kDa), creatine kinase (43 kDa), aldolase (40 kDa) and glyceraldehyde phosphate dehydrogenase (35 kDa) (Toyohara et al., 1999).

Bottom fish (red sea bream), pelagic fish (Pacific mackerel) and freshwater fish (carp) exhibited similar patterns consisting of 10 components whose molecular weights were 94, 60, 49-51, 43, 40, 35, 33, 26, 25 and 23 kDa (Nakagawa, Watabe, and Hashimoto, 1988) as shown in Table 2.2.

Table 2.2 Composition of sarcoplasmic proteins from fish muscle analyzed by SDS gel electrophoresis and densitometry.

Component	**Relative amount (%)**		
(kDa)	**Red sea bream**	**Pacific mackerel**	**Carp**
94	8.9 ± 0.9	10.4 ± 0.9	7.6 ± 0.4
60	7.4 ± 0.2	7.5 ± 0.4	6.6 ± 0.2
49-51	11.9 ± 1.6	12.7 ± 0.3	9.4 ± 0.1
43	12.6 ± 0.5	13.7 ± 0.4	19.5 ± 0.3
40	13.7 ± 0.8	18.7 ± 0.6	12.3 ± 0.2
35	13.5 ± 1.6	11.6 ± 0.6	10.7 ± 0.3
33	7.0 ± 0.4	5.7 ± 0.3	4.9 ± 0.1
26	3.1 ± 0.5	2.6 ± 0.1	2.4 ± 0.1
25	3.0 ± 0.4	3.1 ± 0.1	2.3 ± 0.1
23	1.2 ± 0.2	1.0 ± 0.1	2.4 ± 0.1
Others	17.7 ± 2.4	13.0 ± 0.9	21.9 ± 1.2

Adapted from: Nakagawa, Watabe, and Hashimoto (1988)

The patterns in three major components of sarcoplasmic proteins differed from one fish to another. Red sea bream, Pacific mackerel, and carp were determined for relative amounts using SDS-gel electrophoresis and densitometry of 43-, 40- and

35- kDa protein in the total sarcoplasmic proteins. It was found that 12.6, 13.7 and 13.5% were in red sea bream, 13.7, 18.7 and 11.6% were in Pacific mackerel, and 19.5, 12.3, and 10.7% were in carp, respectively. Therefore, a different quantitative estimation of three bands was used to classify fish species into three groups: pelagic fish rich in 43, 40 and 35 kDa, bottom fish rich in 43 and 35 kDa and freshwater fish rich in 43 kDa.

2.3.3.1 Effect of sarcoplasmic proteins on gel formation

Sarcoplasmic proteins are previously known to have adverse effect in the formation of heat-induced gel by interfering with myosin cross-linking during gel formation. In addition, heat-stable proteinases containing in sarcoplasmic proteins can have a negative effect on the gelation of myofibrillar proteins due to their ability to cleave proteins and thereby weaken the gel structure (Choi, Kang, and Lanier, 2005). Therefore, sarcoplasmic proteins are normally removed during surimi production by washing. Minced fish is washed with chilled water and dewatered to concentrate myofibrillar proteins, resulting in high-quality surimi. Pelagic fishes have high sarcoplasmic protein content so it is difficult to obtain elastic gels (Park and Lin, 2005). Recently, various studies demonstrated that sarcoplasmic proteins positively contribute to gel formation of myofibrillar proteins. Morioka and Shimizu (1993) found that gels prepared from sarcoplasmic proteins with molecular weight of 94, 40, and 26 kDa displayed high gel strength. Karthikeyan, Mathew, Shamasundar, and Prakash (2004) reported that some of sarcoplasmic proteins from oil sardine (*Sardinella longiceps*) fractionated by ammonium sulfate increased the storage modulus of washed sardine meat gel during heating regime over that of washed meat alone. Morioka and Shimizu (1990) found that kamaboko gel was improved when

sarcoplasmic protein (SP) from mackerel was added into threadfin bream myofibrillar protein (MP) at a ratio of 1:3 (SP:MP). Gel forming ability of lizardfish and Pacific mackerel in combination with their sarcoplasmic protein components was dependent upon the ratio of sarcoplasmic protein to myofibrillar proteins (Morioka, Nishimura, and Obatake, 1998). However, textural properties were not increased significantly when sarcoplasmic proteins was less than 9%. This result was similar to Morioka, Kuashima, and Shimizu (1992) who reported that the addition of 10-20% sarcoplasmic proteins to the mackerel surimi paste resulted in an increase of breaking strength. However, Kim, Yonsawatdigul, Park, and Thawornchinsombut (2005) found that addition of 2% freeze-dried sarcoplasmic proteins from rockfish (*Sebastes flavidus*) into Alaska pollock surimi increased breaking force, indicating that sarcoplasmic proteins did not interfere with gelation of myofibrillar proteins, but enhanced gel strength. Moreover, addition of 1% milkfish sarcoplasmic proteins obtained from ultrafiltration improved the thermal gelation and promoted the setting effect of myofibrillar proteins, resulting in higher gel strength (Ko and Hwang, 1995). Most sarcoplasmic proteins and myofibrillar proteins are not removed by a pH shift process. However, several studies reported that textural properties of fish muscle proteins prepared by a pH shift process were greater than those of conventional surimi (Kim, Park, and Choi, 2003; Kristinsson and Hultin, 2003; Yongsawatdigul and Park, 2004).

The physicochemical properties of the thermal gel of water-washed pork meat in the presence of the soluble fraction of porcine sarcoplasmic proteins were observed by Miyaguchi, Hayashi, and Sakamoto (2007). Three fraction of porcine sarcoplasmic proteins was obtained at 75% with ammonium sulfate. Two

precipitated fractions of sarcoplasmic proteins were obtained at 0-50% (SP-f1) and 50-75% (SP-f2) saturation, whereas the soluble fraction was obtained at 75% saturation (SP-f3). They found that a thick protein band with MW of 35 kDa of SP-f3 contained mainly glyceraldehyde-3-phosphate dehydrogenase (GAPDH), while SP-f1 and SP-f2 had other sarcoplasmic proteins, such as phosphorylase b, enolase, actin and phosphoglycerate mutase. The gel strength of water-washed pork meat was highest when SP-f3 was added. In contrast, Shimizu and Ikeda (1979) reported that GAPDH and aldolase bound with fish myofibrils, limiting proper myofibrillar cross-linking and this interaction resulted in weaker gel formation. Therefore, the role of GAPDH on gel formation of myofibrillar proteins varied with species.

The role of transglutaminase (protein-glutamine γ - glutamyltransferase, TGase; EC 2.3.2.13) on gelation has been investigated by several researchers. Nowsad, Katch, Kanoh, and Niwa (1995) found that breaking force of the suwari gel made from Alaska Pollock, carp, horse mackerel, sardine, and Spanish mackerel increased with adding 3% to 4% sarcoplasmic proteins. In addition, they also demonstrated that cross-linking of myosin heavy chain was accelerated by the addition of sarcoplasmic proteins. This was mainly the result of activity of transglutaminase contained in the sarcoplasmic protein fraction. Benjakul, Visessanguan, and Chantarasuwan (2004) reported that higher cross-linking of myosin heavy chain was observed when sarcoplasmic fraction from bigeye snapper was added into natural actomyosin. The effect of gel-enhancing and protein cross-linking ability of sarcoplasmic proteins from tilapia were observed by Yongsawatdigul and Piyadhammaviboon (2007). They demonstrated that sarcoplasmic proteins appeared to be a potential ingredient for improving textural properties of fish protein gels. This

was because sarcoplasmic proteins from tilapia contained TGase activity, resulting in cross-linking of lizardfish actomyosin. Addition of common carp srcoplasmic protein powder enhanced the textural quality of threadfin bream surimi and it was hypothesized that transglutaminase was a major factor contributing to an enhancing effect of sarcoplasmic proteins (Jafarpour and Gorczyca, 2009). Besides the role of TGase, some researchers found that sarcoplasmic proteins contained proteinase inhibitor. Piyadhammaviboon and Yongsawatdigul (2010) found that textural properties of lizardfish surimi enhanced with addition of threadfin bream sarcoplasmic protein mainly because of endogenous proteinase inhibitor in sarcoplasmic proteins. In addition, Siriangkanakun and Yongsawatdigul (2012) found that common carp sarcoplasmic protein strongly inhibited trypsin and contributed to improving textural properties of threadfin bream surimi.

2.4 Proteinases in surimi

Endogenous proteinases are known to be responsible for textural properties of surimi. The presence of endogenous proteinases in surimi inhibits the development of three-dimensional gel networks. These enzymes cleave polypeptides/proteins into small fragments, resulting in gel softening as well as quality loss of gel products. However, gel weakening of surimi or mince induced by proteinases depends on fish species. Proteinases are divided into four major groups according to the characteristic of their catalytic active site, including serine, cysteine, aspartic, and metallo proteinases (The International Union of Biochemistry Nomenclature Committee, 1984). However, serine and cysteine proteinases have been reported to be a major problem for surimi products.

2.4.1 Aspartic proteinases

Aspartic proteinases contain aspartic residues in their active sites. The enzymes are fully active at acidic pH and inhibited by pepstatin. Eukaryotic aspartic proteases include pepsins, cathepsins (D and E), and renins. Howerver, only cathepsins participated in fish muscle degradation. Cathepsin D was purified from carp (Goldman-Levkovitz, Rimon, Rimon, 1995), herring (Nielsen and Nielsen, 2001) and cuttlefish (Balti et al., 2010) with molecular mass of 36, 38-39, and 37.5 kDa, respectively. Higher molecular mass of cathepsin D was found in tilapia (55 kDa) with optimal condition at pH 3.5 and 37 °C (Capasso et al., 1999). In addition, cathepsin D from carp (Makinodan, Akasaka, Toyohara, and Ikeda, 1982) and grey mullet (Bonete, Manjon, Llorca, and Iborra, 1984) showed maximum activity at pH 3-4. Cathepsin E from salmon displayed activity at pH 2.8 (Yamashita and Konagaya, 1992). Both cathepsin D and cathepsin E might not be predominant proteinases during surimi gelation since their activities exhiblted at acidic pH.

2.4.2 Cysteine proteinases

Cysteine proteinases contain the essential residues (histidine and cysteine) at active sites as called “catalytic dyad”. The proteinases have molecular mass about 21-30 kDa. The highest hydrolytic activity at pH4-6.5 (Grzonka et al., 2001). Cysteine proteinases have been reported to contribute to gel softening in many types of fish species. Arrowtooth flounder softening was caused by cathepsin L proteinase with maximum proteolytic activity at 50-60 °C (Greene and Babbitt, 1990; Visessanguan, Menino, Kim, and An, 2001). Cathepsin B and L caused softening in chum salmon (Yamashita and Konagaya, 1991b). An, Weerasinghe, Seymour, and Morrissey (1994) reported that cathepsin L exhiblted the highest activity at 55°C in

both Pacific whiting fish fillets and surimi, while cathepsin B was also found to be active in fish fillet. Cathepsin B was removed during the washing step in conventional surimi process. However, cathepsin L was not removed during washing. Therefore, cathepsin L was the most predominant proteinase in Pacific whiting surimi and caused a decrease of surimi gel strength. Erickson, Gordon, and Anglemier (1983) reported cathepsin C was found in Pacific whiting. This proteinase showed optimum activity at pH 7.0, which was higher than the optimum activity at pH 6.0 in true cod. Some proteinases, especially cathepsin H, can be removed by washing process, resulting in undetectable activity in washed mince (An, Weerasinghe, Seymour, and Morrissey, 1994). Calpains are family of cysteine proteinase. Purified calpains were reported from several fish species including carp muscle (Taneda, Watanabe, and Seki, 1983; Toyohara and Makinodan, 1989), tilapia (Jiang, Wang, and Chen, 1991; Wang, Ma, Su, Chen, Jiang; 1993), and Chinook salmon (Geesink, Morton, Kent, and Bickerstaffe, 2000). However, calpain, cathepsin C and H were unlikely to be responsible for degradation of fish muscles.

2.4.3 Serine proteinases

Serine proteinases contain serine residues in their active sites. Most serine proteinases are fully active in the alkaline pH. Thus, they are called “alkaline proteinase”. Myofibril-bound fraction and sarcoplasmic fraction contained serine proteinases. Myofibril associated serine proteinase in lizardfish with a molecular weight of 60 kDa hydrolyzed myosin heavy chain at 55-60 °C (Cao, Osatomi, Hara, and Ishihara, 2000). Yongsawatdigul and Piyadhammaviboon (2004) reported that the hightest autolytic activity of lizardfish (*Saurida tumbil*) mince and surimi was at pH 6 and 7, respectively, with optimum temperature at 65 °C. Serine proteinases in

lizardfish were tightly bound to myofibril and were not removed by washing. Serine proteinases were found to be responsible for textural breakdown of threadfin bream (*Nemipterus bathybius*) (Toyohara and Shimizu, 1988), and oval-filefish (*Navodon modestus*) (Toyohara, Sakata, Yamashita, Kinishita, and Shimizu, 1992). Trypsin-like is a major proteinase causing the degradation of myofibrillar protein and gel texture of surimi. Martone, Busconi, Folco, and Sa´nchez (1991) reported that hake skeletal muscle contained trypsin-like proteinases which were able to degrade the major contractile and cytoskeletal constituents of myofibrils. In addition, major proteinase found in sarcoplasmic fraction of bigeye snapper was a serine protease with molecular mass of 62 kDa (Benjakul, Leelapongwattana, and Visessanguan, 2003).

2.5 Proteinase inhibitors

Proteinase inhibitors are proteins in the body that regulate the catalytic activity of proteinases. The proteinase inhibitors mimic the usual protein substrate by binding to the active site resulting in a decrease of proteolytic activity. Proteinase inhibitors can be divided into 2 general categories based upon their specification of activity: nonspecific proteinase inhibitors and class-specific proteinase inhibitors.

2.5.1 Nonspecific proteinase inhibitors

Nonspecific inhibitors are able to inactivate an enormous variety of proteinases. α_2-Macroglobulin (α_2-M) is a predominant inhibitor of this group. Human α_2-M is a glycoprotein composed of 4 identical subunits bound together by -S-S- bonds, each with a molecular mass of approximately 185,000 Da (Chaudhuri, 1993). This inhibitor has the ability to inhibit members of all 4 major classes of proteinases (the aspartic, cysteine, serine, and metallo proteinases) due to a unique

mechanism of action, referred to as the trap mechanism (Figure 2.2). A target proteinase binds to a generic bait region on α_2-M, resulting in a conformational change in α_2-M. The active site of the proteinase is not bound directly and retains some very limited proteolytic activity (Hibbetts, Hines, and Williams, 1999).

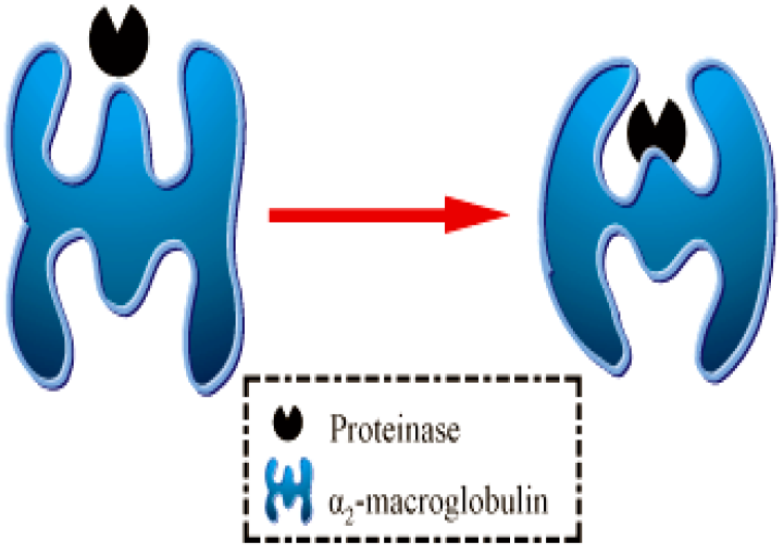

Figure 2.2 α_2-Macroglobulin mechanism of action.

Adapted from: Hibbetts et al. (1999)

2.5.2 Class-specific proteinase inhibitors

Specific proteinase inhibiotors can be separated into 4 groups including aspartic proteinase inhibitors, cysteine proteinase inhibitors, metalloproteinase inhibitors and serine proteinase inhibitors. Specific proteinase inhibitors have lower molecular mass and higher specificity when compared with α_2-M (Hibbetts et al., 1999). Most of these inhibitors abolish all enzymatic activity toward all substrates, have strictly competitive inhibitory activity, and have inhibitory sites that can each inhibit proteinases belonging to only 1 of the 4 classes of proteolytic enzymes. All proteinase inhibitors prevent access of substrates to the proteinases' active sites

through steric hindrance (Figure 2.3). Many act in a substratelike manner by binding directly to the active site of the proteinase, whereas others bind to surface sites adjacent to the actual active site. Both methods prevent interaction between the proteinase and substrate.

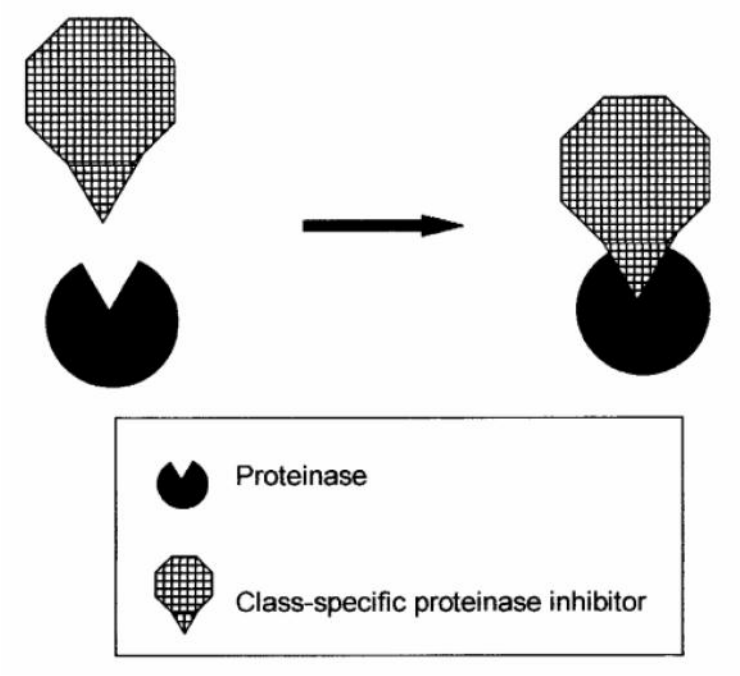

Figure 2.3 Class-specific proteinase inhibitor mechanism of action.

Adapted from: Hibbetts et al. (1999)

2.5.2.1 Aspartic proteinase inhibitors

Pepstatin A is an inhibitor of aspartic proteinase inhibitors. Molecular mass of pepstatin A is 685.9 Da. It forms a 1:1 complex with aspartic proteinases such as pepsin, rennin, cathepsin D, bovine chymosin, and proteinase B (*Aspergillus niger*). Pepstatin A was able to inhibit pepsin at picomolar concentrations (Marciniszyn, Hartsuck, Tang, 1976). The inhibitor is highly selective and does not inhibit thiol proteases, neutral proteases or serine proteases. This inhibitor was found

to be a potent competitive inhibitor of most aspartic proteinase but a weak inhibitor of renin. Autolysis of goatfish mince and washed mince were inhibited by pepstatin A at pH 4 (Yarnpakdee, Benjakul, Visessanguan, and Kijroongrjana, 2009). Furthermore, pepstatin A inhibited proteolysis of lizardfish surimi (Yongswatdigul, and Piyadhammaviboon, 2004).

2.5.2.2 Cysteine proteinase inhibitors

Cystatin is a natural protein inhibitor for regulation of cysteine proteinase activities. Chicken egg white contained cystatin with molecular mass of 12.7 kDa. Ionic strength (up to 0.64) and pH (pH 4-9) exhibited a little effect on its inhibitory activity (Fossum and Whitaker, 1968). The cystatin comprises intracellular cystatins A and B with pI values of 6.5 and 5.6, respectively (Turk and Bode, 1991). Both cystatins A and B were found as glycoproteins. Cysteine proteinases including ficin, papain, cathepsin B, cathepsin H, cathepsin L, and dipeptidyl peptidase were inhibited by chicken egg white cystatin (Anastasi et al., 1983). The molecular mass of the plant cystatins ranged between 5 to approximately 87 kDa and pI 5.6-9.6, depending on sources (Oliveira, Xavier-Filho, and Sales, 2003).

Kininogens belong to the cystatin superfamily and consist of 3 members. All the mammalian species contained two type of kininogens: a high-molecular-mass kininogen (H-kininogen), about 120 kDa; and a low-molecular-mass kininogen (L-kininogen) about 68 kDa (Grzonka et al., 2001). T-kininogen was found only in rats (Dela and Colman, 1991). Both H-kininogen and L-kininogen are single-chain glycoproteins. The highest concentration of kininogens was found in blood plasma and synovial fluid (Abrahamson, Barrett, Salvessen, and Grubb, 1986).

Higashiama et al. (1986) reported that H-kininogen and L-kininogen were strong inhibitors for cathepsin B and L.

2.5.2.3 Metallo proteinase inhibitors

Metallo proteinases are metal (especially zinc)-containing endopeptidases at the catalytic site. The metalloproteinases are synthesize and secreted by connective tissues in mammals. The metalloproteinase inhibitors collectively are called tissue inhibitors of metalloproteinases (TIMPs). In the body, TIMPs can be found in all connective tissues. The balance between matrix metalloproteinases and TIMPs seems to be an important factor in the pathogenesis of tumor invasion and arthritis. In addition, ovostatin (ovomacroglobulin) found in egg white proteins inhibited a wide range of endoproteinases, including thermolysin (a metal-ion requiring proteinase) and collagenase (Nagase, Harris, Woessner, and Brew, 1983). Jiang, Wu, Su, and Tzeng (2000) found that calpastatin, a specific calpain inhibitor from grass prawn (*Penaeus monodon*), revealed four μ-calpain and two m-calpain binding domains.

2.5.2.4 Serine proteinase inhibitors

Serine proteinase inhibitors (serpins) are the largest superfamily of the class-specific proteinase inhibitors involved in many critical biological processes like blood coagulation, fibrinolysis, programmed cell death, development and inflammation (Carrell, Pemberton, and Boswell, 1987; Gettins, 2000; Huntington, Read, and Carrell, 2000). Serpins are single chain proteins containing a conserved domain structure of 350-400 residues, usually flanked by amino- or carboxyl-terminal extensions (Huber, and Carrell (1989). In addition, the majority of the serpins are small glycoproteins composed of single polypeptide chains

with variable members of carbohydrate moieties (Patterson, 1991). These inhibitors are very abundant in plasma. One of these plasma serpins is known as alpha-1-proteinase inhibitor and account for approximately 90% of the tryptic inhibitory capacity of normal plasma.

Previously, alpha1-proteinase inhibitor (α1-PI) was referred to as alpha1-antitrypsin (α1-AT) because it inhibited only trypsin. Later, it was reported to inhibit several serine proteinases. Therefore, α1-AT was renamed to be α1-PI (Travis, 1986). α1-PI is a member of serine proteinase inhibitors (serpin family). Target proteinases of serpins are determined by P1-P1' bond located within a reactive center loop structure of α1-PI (Figure 2.1), 30-40 amino acids from the carboxyl terminus of the inhibitor (Hibbets, Hines, and Williams, 1999). Normally, serpins consist of 350-400 amino acid residues (Patston, 2000). When serpin interacts with their target proteinase by specific binding at the reactive peptide bond P1–P1', the formation of stable, irreversible complexes, between serpin and target enzyme occurs with the cleavage of a single specific peptide bond, P1-P1′ bond, of the α1-PI (Figure 2.2). Subsequently, the peptide is liberated from the carboxyl terminus of the serpin. Therefore, sequence determination of specific peptide bond allows designation of reactive center. All native α1-PI molecules consist of Met-Ser (P1–P1') as a specific peptide bond within reactive center loop (Table 2.3). Human α1-PI contains 394 amino acid residues and has contains Met^{358}-Ser^{359} residues at its reactive center (Patterson, 1991).

Wojtczak et al. (2007) reported that carp α1-PI from seminal plasma formed SDS-stable complexs with trypsin, chymotrypsin and elastase with apparent molecular mass ranging from 75-81 kDa. Molecular weights of complexes

appeared to be lower than the sum of the individual components. This phenomenon may be partially attributed to the loss of the 4 kDa C-terminal peptide from the reactive loop of the inhibitor. Potempa, Korzus, and Travis (1994) reported that stable covalent enzyme-inhibitor complexes are formed during reaction and these complexes are not dissociable upon boiling and in the presence of SDS.

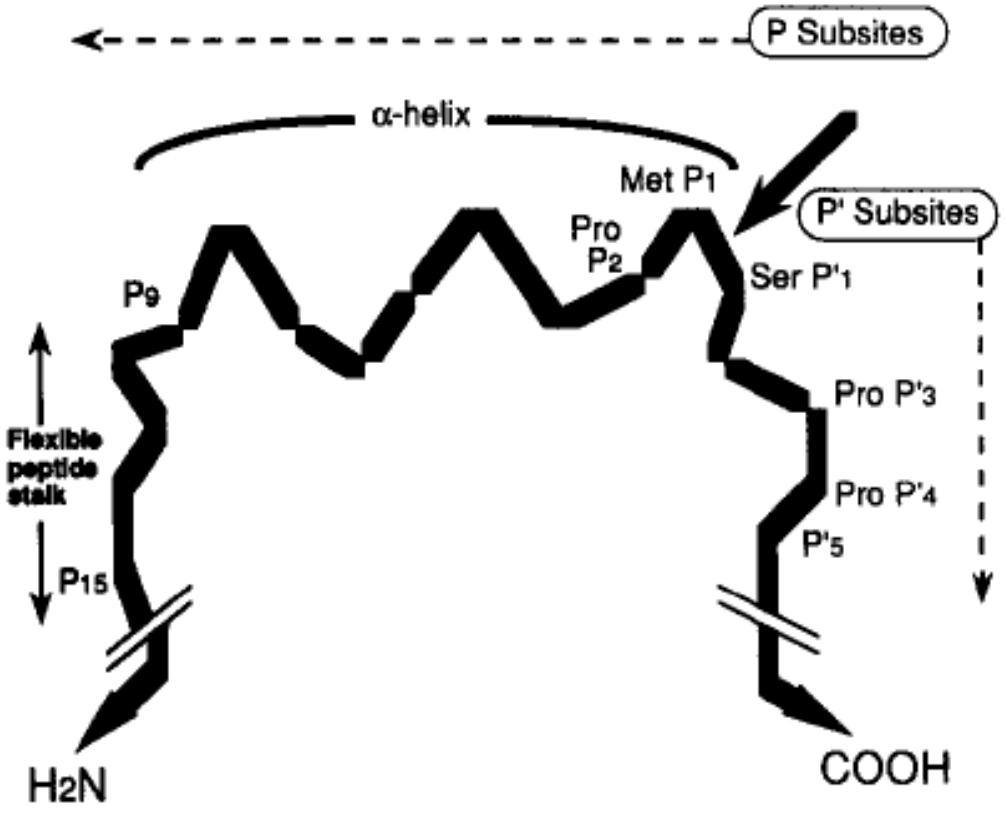

Figure 2.4 Schematic representation of the exposed flexible reactive site loop of the serpins. The loop consists of (1) an N-terminal flexible peptide stalk at residues P15-P9, (2) an α-helix formed by residues P9-P1' and (3) a C-terminal peptide stalk comprised of residues P1'-P5'. The reactive center peptide bond between P1-P1' is arrowed, residues N-terminal to this are referred to as P subsites, those C-terminal as P' subsites (Patterson, 1991).

In mammals, α1-PI is synthesized mainly in the liver. Then it is released into plasma, from where it may reach tissues to protect them against excessive proteolysis (Carrell, Whisstock, Lomas ,1994) while major physiological role of fish serpins is to prevent self-destructive activity of proteinases (Micckowska, 2009). Chinchilla α1-PI was able to inhibit trypsin (Diven, Vietmeier, Hempel, and Chambers, 1990) while α1-PI from carp serum (Aranishi, 1999), horse (Patterson, Bell, and Shaw, 1991b), pig (Gahne and Juneja, 1986), wallaby (Patterson, Bell, and Shaw, 1991a) inhibited both trypsin and chymotrypsin. Mouse α1-PI only inhibited elastase (Takahara and Sinohara, 1983). α1-PI from rat liver (Chao, Chai, Chao, and Chao, 1990) and α1-PI from bighead carp (*Aristichthys nobilis*) perimeningeal fluid (Huang et al.,1995) strongly reduced activity of trypsin, chymotrypsin and elastase. Kuehn, Rutschmann, Dahlmann, and Reinauer (1984) reported that purified α1-PI from rat serum showed molecular mass of 55 kDa and inhibited trypsin, chymotrypsin and elastase. Serpins account for more than 10% of plasma proteins which the main serpin in blood is α1-PI (Mak et al., 2004). α1-PI has been purified from plasma of many mammals, such as rabbit Koj, Hatton, Wong, and Regoeczi (1978), mouse (Takahara and Sinohara, 1982), rat (Roll and Glew, 1981), and canine (Melgarejo, Williams, and Griffith, 1996).

Majority of the serpins are small glycoproteins with molecular mass rang from 40-60 kDa composed of single polypeptide chains with variable members of carbohydrate moieties. Fetz, Ruaux, Steiner, Suchodolski, and Williams (2004) reported that feline α1-PI from serum had molecular mass of 57 kDa. Human α1-PI has a molecular mass of approximately 52 kDa (Carrell et al., 1981). This inhibitor is relatively small and polar, composed of a single polypeptide chain. It has

three carbohydrate attachment sites at asparagines residues chains. Mak et al. (2004) reported that the molecular mass of purified proteinase inhibitor from rainbow trout seminal plasma was determined to be 56 kDa using gel filtration and SDS-PAGE (under reducing and non-reducing condition). Therefore, this inhibitor was composed of a single polypeptide chain. Molecular mass of the inhibitor decreased from 56 kDa to 47 kDa after adding Peptide-N-glycosidase F (PNGase F), suggesting that it was glycoprotein and carbohydrate content was determined to be 16%. From these results, this inhibitor was similar to α1-PI. Patterson (1991) reported that serpins have carbohydrate content ranging from 10-20%.

Table 2.3 Reactive-site amino acid sequences of serpin from various species. The reactive site residues (P1-P1') are denoted by grey color.

Serpins	**Amino acid sequence around serpin reactive site**
Carp α1-PI	IMPM SLPDTVILNRPFLVL
Rainbow trout α1-PI	IMPM SLPDTIMLNRPFLLFILEDSTKSIVFMGKVTNPS
Trout seminal plasma serpin	IMPM SLPDTIMLNRPFLLFILEDSTKSIVFMGKVTNPS
Human α1-PI	AIPM SIPPEVKFNKPFVFLMIEQNTKSPLFMGKVVNPT
Sheep α1-PI	AIPM SLPPDVEFNRPFLCILYDRNTKSPLFVGKVVNPT
Bovine α1-PI	AIPM SLPPDVEFNRPFLCILYDRNTKSPLFVGKVVNPT

Adapted from: Mickowska (2009)

Two isoforms of horse α1-PI were purified by Pellegrini and Fellenberg (1980) and Pellegrini, Zweifel, and Fellenberg (1985). Wojtczak et al. (2007) found two isoforms (isoform A and isoform B) of α1-PI from seminal plasma carp. They had molecular weight of 55.5 (isoform A) and 54.0 (isoform B) kDa. Both

isoforms were glycoproteins. Two isoforms of a proteinase inhibitor were purified from bighead carp perimeningeal fluid (Huang et al., 1995). They had the same molecular mass of 62 kDa and were able to inhibit trypsin, chymotrypsin and elastase. Both inhibitors form SDS-resistant complexes with trypsin, chymotrypsin and elastase. Moreover, both are glycoproteins whose carbohydrate moieties can be removed by PNGase F. This indicated that the presence of N-linked carbohydrate side chain in molecule of the inhibitor. Therefore, these inhibitors were revealed that they were closely related to α1-PI. Patterson (1991) reported that most α1-PI have N-linked glycosylation. Guzdek, Potempa, Dubin, and Travis, (1990) reported that carbohydrate had no any influence on the proteinase inhibitory activity of human α1-PI. However, the carbohydrate played an important role in maintenance of stability. Isoelectric point (pI) of carp blood plasma α1-PI and carp seminal plasma α1-PI were 5.5 and 5.1, respectively (Mickowska, 2009; Wojtczak et al., 2007) which lower pI values have been reported for mammalian α1-PI (Mattes, Matthiessen, Tureck, and Schwarz, 2001). The pI values of human α1-PI (Carrell et al., 1982), cat α1-PI (Fetz et al., 2004), canine α1-PI (Melgarejo, Williams, and Griffith, 1996) and pig α1-PI (Suzuki, Yoshida, Ichimiya, Yamamoto, and Sinohara, 1990) were estimated to be 4.4-4.7, 4.3-3.5, 4.6-4.7, 4.6-4.9, respectively.

2.5.3 Proteinase inhibitor derived from fish

Degradation of fish quality is initiated by the enzyme activity naturally present in a fish so the regulation of proteolytic enzymes in tissues by inhibitors is a prerequisite for the maintenance of homeostasis. Several proteinase inhibitors have been reported from various parts of fish. Atlantic salmon (*Salmo salar* L.) skin contained two cysteine proteinase inhibitor. Both inhibitors were classified as a

member of kininogen (52 kDa) and salarin (43 kDa). They inhibited papain and ficin but not trypsin. In addition, they were both N- and O-glycosylated proteins (Ylonen et al., 1999), whereas N-linked but not O-linked carbohydrate side chains were found in α_1-antiproteinase-like protein from rainbow trout seminal plasma (Mak et al., 2004). Two isoforms of proteinase inhibitor were found in bighead carp (*Aristichthys nobilis*). Both inhibitors inhibited activity of bovine trypsin, bovine chymotrypsin and porcine elastase. Molecular mass of two inhibitors decreased from 62 to 53 kDa with addition of N-glycosidase F (PNGase F), suggesting that they were glycoproteins. Each isoform had two N-linked carbohydrate side chains while O-linked carbohydrate side was not detected.

Cao et al. (2000) found a serine proteinase inhibitor with molecular mass of 55 kDa from white croaker (*Argyrosomus argentatus*) skeletal muscle. This proteinase inhibitor was identified to be phosphoglucose isomerase. It inhibited a myofibril-bound serine proteinase isolated from the skeletal muscle of lizardfish (*Saurida wanieso*) while bovine trypsin, bovine chymotrypsin and a myofibril-bound serine proteinas from carp (*Cyprinus carpio*) were not inhibited by this inhibitor. Similar result was reported by Sun et al., (2009). They found that phosphoglucose isomerase strongly inhibited a myofibril-bound serine proteinase from crucian carp, while no inhibitory activity was indentified toward crucian carp trypsin, crucian carp chymotrypsin, mandarin fish trypsin A, mandarin fish trypsin B, porcine trypsin and white croaker myofibril-bound serine proteinase. Molecular weight of the protein was about 55 and 65 kDa by SDS-PAGE and 120 kDa by gel filtration, suggesting that it was a heterodimer. A proteinase inhibitor with molecular mass of 65 kDa was purified from white croaker skeleral muscle (*Micropogon opercularis*) (Sangorrín, Folco,

Martone, Sánchez, 2001). It can bind to Concanavalin A-Sepharose suggesting that it was a glycoprotein. Moreover, it showed inhibitory activity towards serine protienases (trypsin, chymotrypsin, elastase and subtilisn) but it did not affect cysteine (bromelain and papain) or metallo (thermolysin) proteinases. Nagashima, Takeda, Ohta, Shimakura, and Shiomi (2004) found two trypsin inhibitors from skin mucus of pufferfish, *Takifugu pardalis.* Both inhibitors were acidic glycoproteins with an apparent molecular mass of 57 and 47 kDa. Yamashita and Konagaya (1991a) reported that a 16-kDa proteinase inhibitor from chum salmon eggs was considered to be cystatin family II. Ustadi, Kim, and Kim (2005) reported that a proteinase inhibitor from glassfish (*Liparis tanakai*) egg was able to inhibit cysteine proteinases but not serine proteinases. It was shown to be a noncompetitive inhibitor against papain and classified as a member of cystatin family I due to its lack of disulfide bonding. Cysteine proteinase inhibitors from Pacific herring, chum salmon, pond smelt, glassfish, and Alaska pollock egg had molecular mass of 120, 89, 84.5, 17 and 16.8 kDa, respectively (Ustadi, Kim, and Kim, 2005). Li, Lin, and Kim (2008) reported that a proteinase inhibitor from chum salmon (*Oncorhynchus keta*) plasma was highly specific for cysteine. This inhibitor had molecular mass of 70 kDa based on SDS-PAGE and Sephacryl S-100 gel filtration. In addition, it was also a glycoprotein and classified as a kininogen. The inhibitor was stable at 10-50 °C and at pH 7-9. In addition, cathepsin inhibitory activity increased with addition of Mn^{2+}, while Ca^{2+} and Co^{2+} slightly repressed. Busconi, Fob, Martone, Trucco, and Sgnchez (1984) found two alkaline proteases and a trypsin inhibitor from muscle of white croaker *(Micropogon opercularis).* This result was similar to Martone, Busconi, Folco, and Sa´nchez (1991) who reported that hake skeletal muscle contained a trypsin-like serine

protease and a trypsin inhibitor. Piyadhammaviboon and Yongsawatdigul (2010) reported that sarcoplasmic protein contained trypsin inhibitor with molecular mass of 95, 41 and 37 kDa. Trypsin inhibitory activity decreased at temperatures above 45 °C and completely diminished at 60-65 °C.

α_2-M found in plasma of most mammals, birds, reptiles and amphibians is a glycoprotein composed of 4 identical subunits bound together by -S-S- bonds (Chaudhuri, 1993; Starkey and Barrett, 1982). α_2-M from fish and invertebrates are mainly disulfide-linked dimmers (Starkey and Barrett, 1982a; Gollas-Galván, Sotelo-Mundo, Yepiz-Plascencia, Vargas-Requena, and Vargas-Albores, 2003). The dimeric forms of α_2-M were found in plaice plasma (Starkey and Barrett, 1982b), rainbow trout plasma (Ellis, 1987), brook trout plasma (Freedman, 1991), brook charr plasma (Zuo and Woo, 1997), and gilthead seabream (Funkenstein, Rebhan, Dyman, and Radaelli, 2005). Grass carp plasma α_2-M consisted of two different subunits of molecular mass of 95 kDa and 80 kDa (Li and Lu, 2006). Chuang, Liu, and Lee (2008) reported that molecular mass of purified α_2-M from plasma of grouper *Epinephelus coioides* was about 180 kDa in non-reduced SDS-PAGE, and 97 and 80 kDa in reduced SDS-PAGE. Proteinase inhibitory activities of α_2-M markedly decreased with incubation at 60 °C. The inhibitor showed optimum inhibitory activity at pH 10, indicating that the α_2-M was an alkaline proteinase inhibitor.

Various proteinase inhibitors from carp have been reported. Toyohara, Makinodan, Tanaka, and Ikeda (1983) reported that common carp (*Cyprinus carpio*) muscle contained both trypsin inhibitor and calpastatin. Yamada, Sakamoto, and Seki (1985) also found two calpastatin from carp skeletal muscle. They had molecular mass of 78 and 37 kDa based on SDS-PAGE and gel filtration, implying that each

calpastatin was a single polypeptide. In addition, cystatin was found in ovarian fluid of carp (Tsai, Chang, Huang, Chang, and Huang, 1996) and carp chorion (Chang, Weng, Li, and Huang, 1998). Hara and Ishihara (1987) reported that a serine proteinase inhibitor was purified from carp ordinary muscle. Isoelectric point (pI) was about 5.3 while it was stable over the pH range from 7.0-9.5, but was very unstable below pH 4.5. Trypsin, α-chymotrypsin, and elastase were strongly inhibited by the inhibitor. Molecular mass of this inhibitor was about 100 kDa by gel filtration and about 56 kDa by SDS-PAGE under reducing condition, suggesting that the inhibitor was a dimer of homologous subunits. In contrast, carp α_1-PI from perimeningeal fluid (Huang, Lee, Huang, and Chang, 1995), serum (Aranishi, 1999), seminal plasma (Wojtczak, Całka, Glogowski, and Ciereszko, 2007), and blood plasma (Mickowska, 2009) was considered to be a single polypeptide chain with molecular mass ranging from 54-62 kDa. Common carp sarcoplasmic protiens contained trypsin inhibitor with molecular mass of 35, 41, 44, 50 and 69 kDa (Siriangkanakun and Yongsawatdigul, 2012).

2.5.4 Proteinase inhibitor derived from plasma

Beef plasma protein (BPP) has been reported to contain two inhibitors including alpha2-macroglobulin (α_2-M) and kininogen. α_2-M composes of 4 identical subunits and is a nonspecific inhibitor (Chaudhuri, 1993), while kininogen is specific for cysteine proteinase (Rawlings and Barrett 1990). Wasson, Reppond, Babbitt, and French (1992) reported that no evidence of myosin heavy chain degradation was found when α_2-M was added to arrowtooth flounder surimi. BPP showed higher inhibitory activity on Pacific whiting autolysis than egg white (Morrissey, Wu, Lin, and An, 1993). Weerasinghe, Morrissey, Chung, and An (1996) reported that BPP

exhibited higher inhibition toward trypsin and papain than whey protein concentrate. Kang and Lanier (1999) found that gel strength of Pacific whiting surimi was improved with addition of 1% BPP. This was because BPP contained proteinase inhibitors and TGase. Benjakul, Visessanguan, Tueksuban, and Tanaka (2004) reported that BPP showed higher inhibition of lizardfish mince and washed mince autolysis, compared to egg white. In addition, egg white inhibited proteolysis in lizardfish sarcoplasmic fluid less effectively than beef plasma protein. However, addition of BPP is prohibited due to the occurrence of mad cow disease. Moreover, some BPP preparations resulted in off-flavors at concentrations higher than 1% (w/w) (Akazawa, Miyauchi, Sakurada, Wasson, and Reppond, 1993).

Pig plasma protein (PPP) contained a cysteine proteinase inhibitor with a molecular weingt of 55 kDa (Lee, Tzeng, and Jiang, 2000). The inhibitor considered to be L-kininogen, member of cystatin family, was responsible for endogenous cathepsins inhibition. Benjakul and Visessanguan (2000) reported that Pacific whiting proteinase, papain and trypsin were inhibited by PPP. Residual protein bands of PPP appeared with molecular weight of 60-63 kDa on trypsin and papain inhibitory activity staining indicating that they might be the active inhibitory components in PPP. In addition, Benjakul, Visessanguan, and Srivilai (2001) found that PPP displayed inhibitory activity towards trypsin, papain, muscle proteinase and viscera proteinase, suggesting that PPP was an effective inhibitor for cysteine and serine proteinases. Moreover, breaking force and deformation of bigeye snapper surimi increased when 0.5% PPP was added resulting from its proteinase inhibiotors. However, addition of PPP has been limited by Islamic dietary guideline (Halal food).

Addition of 0.5% chicken plasma protein (CPP) to bigeye snapper surimi increased breaking force and deformation due to its proteinase inhibitor (Rawdkuen, Benjakul, Visessanguan, and Lanier, 2004a). However, textural properties of surimi decreased at a level higher than 0.5% due to the dilution of myofibrillar protein or interference of myofibrillar protein cross-linking. Rawdkuen, Benjakul, Visessanguan, and Lanier (2004b) reported that CPP at a level of 2% (w/w) exhibited the highest inhibitory activity toward sarcoplasmic proteinases and autolysis of mince and washed mince from both bigeye snapper and lizardfish. Rawdkuen, Benjakul, Visessanguan, and Lanier (2007) reported that a cysteine proteinase inhibitor containing in CPP prevented the degradation of myosin heavy chain, tropomyosin and troponin-T in Pacific whiting surimi with incubation at 55 °C for 60 min as well as modori gel (55 °C for 30 min/90 °C for 20 min). Two proteinase inhibitors with molecular mass of 46 and 122 kDa found in CPP fraction were reported by Rawdkuen, Benjakul, Visessanguan, and Lanier (2006). The inhibitor with molecular mass of 46 kDa was identified as a papain-resistant protein while the 120-kDa protein was presumed to be a cysteine proteinase inhibitor. Addition of CPP enhanced textural properties of surimi but decreased in whiteness (Rawdkuen et al., 2004a).

2.5.5 Commercial food-grade proteinase inhibitors

Egg white, whey protein concentrate and potato powder are the additives most commonly used as commercial food-grade inhibitors in both surimi and surimi seafoods. Egg white (EW) contains several types of proteinase inhibitors including ovomucoid, ovoinhibitor, ovostatin, and cystatin. Ovomucoid (28 kDa) is specific for trypsin while ovoinhibitor (49 kDa) is a general serine proteinase inhibitor which can inhibit both trypsin and chymotrypsin (Kassell, 1970; Osuga and Feeney, 1977).

Ovostatin (780 kDa) has been reported that it is able to inhibit metallo proteinases. Structure and mechanism of this inhibitor is similar to α_2-M (Nagase, Brew, Harris, 1985). Cystatin (13.1 kDa) as a family of cysteine proteinase inhibitors has also been found in egg white (Stevens, 1991). Yongswatdigul and Piyadhammaviboon (2004) reported that addition of 1% EW showed higher autolytic inhibition in lizardfish surimi autolysis when compared to whey protein concentrate at the same concentration. Moreover, textural properties of lizardfish surimi was improved with addition of EW. Li, Lin, and Kim (2007) reported that EW was effective in preventing the degradation of myosin heavy chain in Alaska pollock surimi. Autolysis of Pacific whiting surimi was also inhibited by EW (Benjakul and Visessanguan, 2000).

Whey protein concentrate (WPC) is a by-product of cheese manufacturing. WPC with a molecular mass of approximately 101 kDa displayed inhibition toward trypsin and papain; however, it was more effective in inhibiting cysteine proteinase rather than serine proteinase (Weerasinghe et al., 1996). Rawdkuen and Benjakul (2008) reported that WPC was able to inhibit autolytic activity of tropical fish surimi including bigeye snapper, goatfish, threadfin bream and lizardfish. Moreover, textural properties of kamaboko gels of all surimi increased as added levels of WPC increased. Autolytic activity of Pacific whiting surimi was slightly inhibited with addition of 0.2% WPC (Akazawa et al., 1993). Weerasinghe et al. (1996) demonstrated that WPC at 3% or above was the most effective inhibition of autolytic proteinase activity from Pacific whiting surimi.

Potato powder (PP) contains serine and cysteine proteinase inhibitor (Weerasinghe et al. 1996; Rowan, Brzin, Buttle, and Barrett, 1990). Weerasinghe et al. (1996) found that a protein band with molecular weight of 68 kDa was detected on

the trypsin and papain inhibitory activity staining while high intensity of a protein band on trypsin inhibitory activity staining was observed at 31 kDa. Porter, Koury, and Kudo (1993) reported that inhibitors in PP showed effective inhibitory activity toward endoprotease activity in Pacific whiting and arrowtooth flounder muscle extracts. PP proved to be effective in stabilizing the gel-forming characteristics of Pacific whiting and arrowtooth flounder surimi at the 3-5% level. In addition, PP has been shown to be an inhibitor against autolysis of trout muscle (Kaiser and Belitz, 1973) and walleye Pollack (Reppond and Babbitt, 1993).

2.6 Mass spectrometry

Mass spectrometry (MS) is a powerful tool for the analysis of both large and small biomolecules such as proteins, peptides, amino acids, and glycoprotein. MS is a classical method for identification and characterization of interested proteins in solution or after electrophoresis. A typical mass spectrometer contains three components: ion source, mass analyzer, and detector. The first step in MS analysis, a sample which may be solid, liquid, or gas is loaded into the mass spectrometer and then vaporized and ionized by an ion source such as matrix assisted laser desorption/ionization and electrospray ionization. Then mass analyzers are used to separate the ions. The ions are separated according to their mass-to-charge ratio (m/z). The separated ions are then measured, and the results displayed on a chart. The use of MS has greatly aided proteomics because protein sequenceing is not simple and straightforward as DNA sequencing. MS has been used to analyze protein identification, complex protein mixtures, and N-glycosylation site.

2.6.1 Protein identification

Two techniques are widely used for protein identification by mass spectrometry: peptide mass fingerprinting (PMF) using matrix assisted laser desorption/ionization-time of flight (MALDI-T00OF) and partial amino acid sequencing using liquid chromatography with tandem mass spectrometry (LC-MS/MS). For protein identification, MS is replaced other classical techniques, for example, N-terminal sequencing based on Edmen degradation since its much more sensitive and can cope with protein mixture offering much higher sample throughput. Trypsin is the most commonly used for both PMF and partial amino acid sequencing because it is highly specific and cleaves peptide chains exclusively at the carboxyl side of the amino acids lysine or arginine that are commonly and widely distributed throughout most proteins (Olsen, Ong, and Mann, 2004). A small number of digested peptides usually yield sufficient information to permit protein identification by PMF and partial amino acid sequencing. The principles of both techniques are described below.

2.6.1.1 Peptide mass fingerprinting (PMF) using MALDI-TOF

MALDI is referred to as a soft ionization technique used in MS, allowing for analysis of large biomolecules that tend to fragment when conventional ionization methods are used (Karas and Hillenkamp, 1988). The ionization process of MALDI is triggered by a UV laser beam. A matrix is used to protect the sample from being destroyed by direct laser beam during ionization. The sample is mixed in a large quantity of matrix before allowing co-crystallized with a selected matrix. Hillenkamp, Karas, Beavis, and Chait (1991) reported that α-cyano-4-hydroxycinnamic acid was used a matrix for analysis of peptide and proteins smaller than 10,000 Da, while sinapinic acid was suitable for higher mass peptides. When the

probe is hit by a pulsed laser beam at high vacuum, partially vaporized matrix is desorbed from the surface and carries intact sample into gas phase. Protons are exchanged between analytes and matrix during expansion of the MALDI plume, resulting in formation of charged analytes (Karas, Gluckmann, and Schafer, 2000). The generated ions from MALDI are analyzed continuously by time-of-flight (TOF) mass analyzer. The velocity of the ions which are accelerated in equal energies depends on ions-mass-to-charge ratio (m/z) of the particle (heavier particles reach with lower speeds) (Weickhardt, Moritz, and Grotemeyer, 1996). This effective approach of protein identification is based on the accurate mass measurement of a group of peptides derived from a protein by sequence-specific proteolysis. The experimentally obtained masses are compared with the theoretical peptide masses in databases by means of mass search programs (Fabris et al., 1995). PMF technique is very quick and simple to acquire data while partial amino acid sequencing data acquisition takes several hours with several MS/MS spectra generated. However, the limitation of PMF still exist. PMF is not suitable for protein mixture analysis because it is unclear that all peptide masses observed are originally from the same protein species. In addition, the measured masses of protein modification are different from those without modifications so masses of modified proteins may be wrong (Poutanen, Salusjarvi, Rouhonen, Penttila, and Kalkkinen, 2001).

2.6.1.2 Partial amino acid sequencing using liquid chromatography with tandem mass spectrometry

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) technique is widely used in protein biochemistry and in proteomics for the identification and characterization of proteins. The digested peptides are submitted to liquid chromatography (LC) to separate components in a mixture. LC-MS/MS

shows higher sensitivity than PMF. This is because peptides were concentrated about 50-200 fold by LC before MS detection. An increase of peptide concentrations was translated into the increase of MS signal. Consequently, it can dramatically increase real sensitivity in identifying proteins. Polypeptides can be successfully transferred into gas phase by the ionization techniques including electrospray ionization (ESI) (Fenn, Mann, Meng, Wong, and Whitehouse, 1989) and MALDI (Karas and Hillenkamp, 1988). MS/MS is coupled with two mass analyzers. The mass analyzers are designed to select, collide, and separate ions which provide information of amino acid sequences. After the masses of mixture peptides are analyzed in the first mass analyzer, one of the interested peptides are further fragmented by collision-induced dissociation (CID). The precursor ion of the selected peptide passed into the collision cell where it undergoes low-energy collisions with inert gas such as argon and nitrogen. During collision, several bonds along the backbond are broken to produced smaller fragments. The resulting fragments are subsequently analyzed in the second mass analyzer in order to sequence the peptide. The fragments obtained are commonly designated as b- and y- ions, which denote fragmentation at amide bond with charge retention on the N- or C- terminus, respectively. Then a peptide sequence obtained can be used to identify a peptide in a protein database. Simpson (2003) reported that proteins in a complex mixture can be identified from CID spectrum of a single peptide and matches one or more tandem mass spectra to peptide sequences in the same protein. Therefore this method provides a high level of confidence in the identification process. Offengenden, Fentabil, and Wu (2011) identified three protein bands from hen egg white using LC-MS/MS. Protein bands with molecular mass of 350-400, 250, and 150 kDa corresponded to β-ovomucin, α-ovomucin, and ovostatin, respectively. LC-MS/MS was also used to identify tryptic peptides from low density lipoprotein

from hen egg yolk after separation on SDS-PAGE (Jolivet, Boulard, Beaumal, Chardot, and Anton 2006). Amino acid sequences of protein bands with molecular mass of 8 and 15 kDa corresponded to Apovitellenin I and a predicted protein similar to human apolipoprotein B-100 precursor was recovered in all the bands ranging from 55 to 190 kDa with apparent molecular masses of 55, 62, 73, 96, 98, 118, and 190 kDa. Proteins from rainbow trout was characterized by analysis of two-dimensional gel electrophoresis and identified by LC-MS/MS (Kjaersgard, Nørrelykke, Caron, and Jessen , 2006). The retrieved peptide sequences were used for protein identification by searching from the NCBI database. Several carbonylated proteins identified were nucleoside diphosphate, kinase, adenylate kinase, pyruvate kinase, actin, creatine kinase, tropomyosin, myosin light chains 1 and 2, and myosin heavy chain. Nano-high-performance liquid chromatography (HPLC)-MS/MS was used to generate profile of soluble proteins in Sauvignon Blanc wine (Kwon, 2004). Twenty proteins were identified including five proteins derived from the grape, 12 from yeast, two from bacteria, and one from fungi. Tolin, Pasini, Simonato, Mainente, and Arrigoni (2012) investigated allergenic proteins from 25 different wines using LC-MS/MS. Grape berry, wine yeast (*S. cerevisiae*), bacteria (*O. oeni*) and mold (*B. cinerea*) were generally found in every wine sample while 8 wines contained peptides belonging to egg and milk proteins, all of them known as potential allergens. In particular, ovalbumin, the major egg allergen, was detected in all 8 wines while 2 of them contained ovotransferrin. Moreover, milk allergens alpha- and beta-casein were identified in 2 wines.

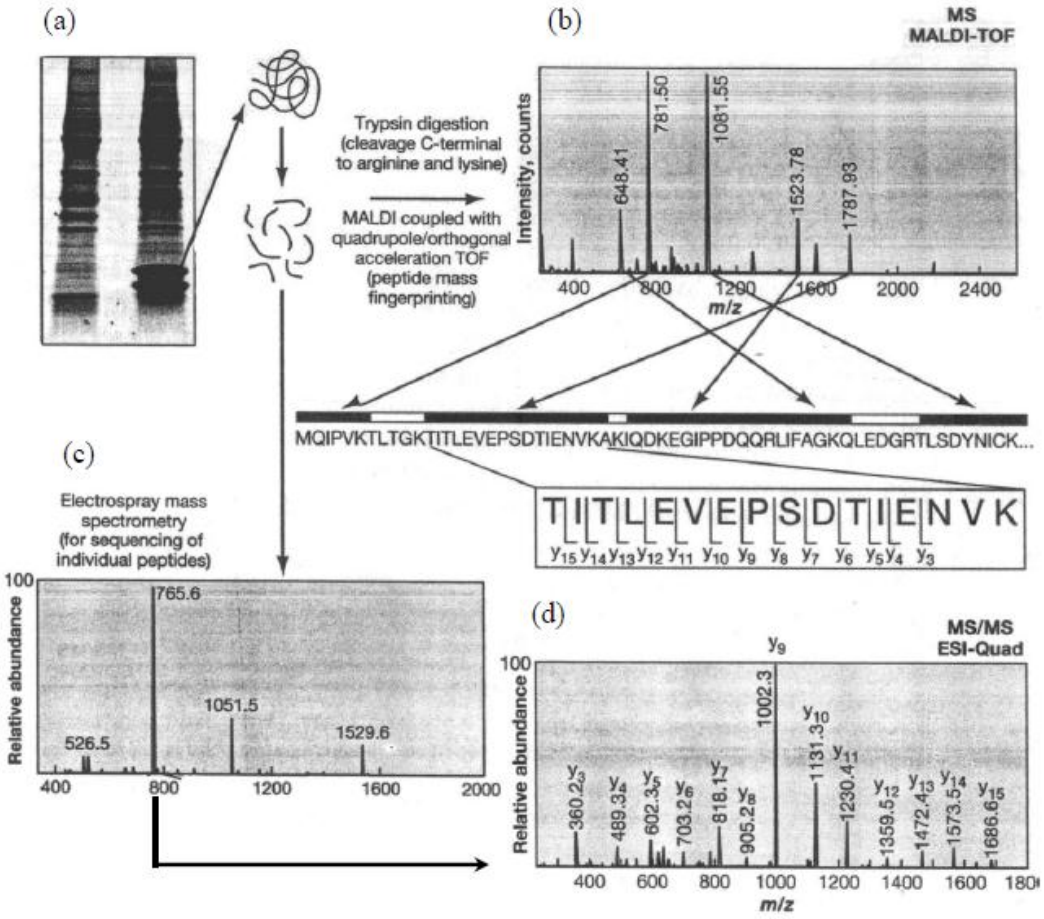

Figure 2.5 A strategy for mass spectrometric identification of proteins. (a) After SDS-PAGE gel, the interested protein bands are excised and digested in-gel with trypsin. (b) The resultant spectrum represents a peptide mass fingerprint (PMF) of a protein is derived from MALDI-TOF. This information permits the identification of the protein by matching of the experimentally obtained peptide masses against theoretically predicted peptides of proteins in publicly available database. Tryptic peptides derived from human ubiquitin (sequence below) are shown as an example. (c) Analysis of another aliquot of the peptide mixture using an electrospray ion-trap mass spectrometer, which usually coupled on-line to a RP-HPLC. Peptide masses at a given of time obtained from the first mass analyzer. One of these ions is selected to enter the collision cell and then the ion is fragmented. The fragmented ions

are separated in the second mass analyzer before being scanned out to the detector. (d) From the resultant MS/MS spectrum, amino acid sequence information can be derived using an algorithm that correlates the experimental spectrum with those in a database. In this example, the fragment ions in this spectrum correspond to amino acid residues TITLEVEPSDTIENVK. Protein can be identified from such peptide sequence information (Zugaro et al., 1998; Simpson, 2003).

2.6.2 Analysis of complex protein mixtures using gel-based liquid chromatography-tandem mass spectrometry

Gel-based liquid chromatography-tandem mass spectrometry (GeLC-MS/MS) has been proven to be a powerful and an efficient technique for analysis complexity of protein mixtures. In GeLC-MS/MS, proteins are separated by one-dimentional sodium dodecyl sulphate-PAGE (SDS-PAGE) then each gel lane is sliced into 5-20 slices prior to digestion. Digested peptides from each gel band are analyzed separately by LC-MS/MS (Paulo et al., 2010). Slicing the gel lane into smaller bands separates the protein mixtures into narrow molecular weight range, which increase the dynamic depth of the analysis. The separation at protein level can isolate some low abundant proteins from the high abundant ones. Although another proteomic technique, LC-MS/MS, can identify more than several peptides within one analysis and also enables the detection of proteins rarely found using 2D-PAGE based proteomics (Washburn, Wolters, and Yates, 2001), complexity of peptide mixtures limits the use of this method (Malmstrom, Lee, and Aebersold, 2007). This is because tryptic peptides from the high abundant proteins are detected across most of the fractions, making some low abundant ones ignored. In addition, GeLC-MS/MS can

evaluate protein profiles of complex biological samples. Therefore, this proteomic technique is suitable for analysis complexity of protein mixtures which are a limitation of other proteomic techniques (Rezaul, Wu, Mayya, Hwang, Han, 2005). Maksup, Roytrakul, and Supaibulwatana (2012) reported that 623 proteins with differing expression patterns under drought stress of three indica cultivars including IR 20, KDML105, and NSG19 were detected using GeLC-MS/MS and analyzed using Mascot software. Protein profiles of *Jatropha curcas* L. (physic nut) were evaluated using GeLC-MS/MS by Booranasrisak et al. (2013). This fruit was divided into eight developmental stages (stages I to VIII) based on their age and morphology. Significant changes were found in the relative abundance of 22 proteins during seed development, of which the expression levels for transcripts encoding for four of these proteins, acetyl CoA carboxylase, phosphoenolpyruvate carboxylase, mercaptopyruvate sulfurtransferase and 4-coumarate.

2.6.3 N-glycosylation site

N-linked glycans are attached to the protein backbone via an amide bond to asparagines residues. The N-linked glycans are located within a consensus sequence of Asn-Xxx-Ser/Thr, where Xxx can be any amino acids, except Pro. The glycan is able to be a high-mannose, hybrid, or complex type (Segu, Hussein, Novotny, and Mechred, 2010). Potential N-glycosylation sites can be predicted from the consensus sequence Asn-Xaa-Ser/Thr; however, it cannot be assumed that a site will actually be glycosylated. The combination of enzymatic method and LC-MS/MS is expected to be a powerful tool for the analysis of N-glycosylation sites.

Peptide-N-glycosidase F (PNGase F) is a common endoglycosidase that is widely used for deglycosylation of N-linked glycans. The enzyme PNGase F

cleaves the bond between the asparagine residue and N-acetylglucosamine of N-linked glycans groups. N-linked glycan is then removed from proteins while deamidating the originally glycosylated asparagines into aspartic acid (NH_2 group in asparagine changes into OH group at the glycosylation site) and the mass of deglycosylated peptides increases 0.984 Da (Offengenden, Fentabil, and Wu, 2011). For example, if asparagines (with molecular mass of 114.04 Da) is N-glycosylated, it is converted to aspartic acid (with molecular mass of 115.03 Da) after adding PNGase F. Therefore, the increase in mass of peptide after removal of the attached glycans enables the determination N- glycosylation sites using mass spectrometry. Offengenden et al. (2011) investigated N-glycosylation site of β-ovomucin from hen egg white. The sample was treated with PNGase F to remove N-linked carbohydrates from protein and submitted to LC-MS/MS. An increase in molecular mass by 1 Da was observed at N238 and N945, which corresponds to Asn, is 115 Da instead of 114 Da as a result of deglycosylation with PNGase F. Therefore, two N-glycosylation sites on β-ovomucin were observed at N238 and N 945. For α-ovomucin, 15 N-glycosylation sites exhibited glycosylated form while two N-glycosylation sites were not glycosylated. Based on LC-MS/MS, the mass difference, which corresponds to Asn is 114 Da which is not deamidated by PNGase F. Moreover, carp perimeningeal fluid α_1-PI (Huang et al., 1995) and serpin from rainbow trout seminal plasma (Mak et al., 2004) were found to contain two N-linked, while O-linked carbohydrate did not appear. Three N-linked glycosylation sites at N46, 83, and 247 were observed in human α_1-PI (Kolarich et al., 2006).

The measurement of exact mass differences between the native glycoproteins and the completely de-N-glycosylated proteins provides information for

the determination of the N-glycan content. N-linked glycan was commonly found in α_1-PI. Carbohydrate of inhibitor was removed by PNGase F. Wojtczak et al. (2007) reported that two isoforms (inhibitor A and inhibitor B) of α_1-PI from carp seminal plasma were glycoproteins. Molecular masses of inhibitor A decreased from 55.5 kDa to 48.5 kDa and from 54.0 to 47.5 kDa for inhibitor B after addition of PNGase F, suggesting that carbohydrate contents of inhibitor A and B were 12.6 and 12.1%, respectively based on SDS-PAGE. Bighead carp perimeningeal fluid α_1-PI (Huang et al., 1995) and rainbow trout seminal plasma α_1-PI (Mak, 2004) were reported to be glycoproteins with 15 and 16% carbohydrate contents, respectively. In addition, carbohydrate contents from several mammals α1–PI have also been reported. Carbohydrate contents from human α_1-PI (Carrell et al., 1982), rat α_1-PI (Chao, Chai, Chao, and Chao, 1990) and chinchilla α_1-PI (Diven, Vietmeier, Hempel, and Chambers, 1990) were 15, 13.2 and 15%. Suzuki et al. (2001) reported that the N-glycan contents of pigeon egg white after treatment with PNGase F were 3.5% in ovotransferrin; 17% in ovalbumins; and 31-37% in ovomucoid.

Glycosylations play an important role in folding of proteins. Gu et al. (1989) reported that stability of the ovomucoid molecule against tryptic hydrolysis and heat denaturation was contributed from its carbohydrate moiety. Residual inhibitory activity of glycosylated chicken cystatins was higher than that of unglycosylated form after incubation at 60-100 °C for 30 min (Tzeng and Jiang, 2004). Glycosylation of proteins increased stability and had a positive relationship with the length of the attached carbohydrate (Nakamura, Ogawa, and Nakai, 1998; Nakamura, Takasaki, Kobayashi, Kato, 1993). In addition, Wang, Eufemi, Turano, and Giartosio (1996) reported that carbohydrates might prevent unfolding of protein

molecules. Khan, Rasheedi, and Haq (2003) reported that activity of deglycosylated form of stem bromelain was lower than that of glycosylated form, suggesting that carbohydrate had effect on proteinase inhibitory activity of stem bromelain.

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CHAPTER III

EFFECT OF CONVENTIONAL WASHING AND ALKALI pH–SHIFT PROCESS ON AUTOLYSIS AND GELATION CHARACTERISTICS OF COMMON CARP AND GOATFISH

3.1 Abstract

Effect of conventional washing and alkali pH-shift process on autolysis and gel properties of freshwater fish (common carp, *Cyprinus carpio*) and marine fish (goatfish, *Upeneus* spp.) muscle proteins was investigated. Autolysis of both common carp and goatfish muscle was maximum at 65-70 °C. Washed mince of common carp (WM-C) showed higher autolytic activity than mince (M-C). However, washed mince of goatfish (WM-G) exhibited lower autolytic activity than mince (M-G). Endogenous proteolytic and transglutaminase activity were largely inactivated during alkali pH-shift process. Pepstatin A showed the highest inhibition toward autolysis at pH 4, suggesting that aspartic proteinase was predominant in both M-C and M-G. In addition, serine proteinases were mainly responsible for degradation of M-C at pH 7 and 10, while M-G contained serine- and metallo- proteinases at pH 7 and 9. The highest textural properties of common carp was found in gels prepared by the conventional 3 washing cycles, whereas goatfish gels made from alkali pH-shift process displayed the highest breaking force. Total sulfhydryl contents of common

carp and goatfish gels obtained from alkali-treated gels were the lowest, implying the formation of disulfide linkages. Setting did not improve gel properties of both species. Three-cycle washing improved whiteness of gels prepared from both species to a greater extent than the alkali pH-shift process.

3.2 Introduction

In conventional surimi production, washing is a process aiming at removing sarcoplasmic proteins and adipose fat. This process has been proven to improve gel-forming ability as it concentrates myofibrillar proteins, the major protein component for gelation. However, it inevitably decreases the yield of washed mince to only about 20-30% (Yongsawatdigul and Park, 2004). A pH-shift process has been introduced as an alternative to increase gel-forming ability of muscle proteins with higher protein recovery (Hultin and Kelleher, 1999). This process is refers to as either acid or alkali extraction followed by isoelectric precipitation and neutralization (Hultin and Kelleher, 1999). The distinct feature of this process is the retention of sarcoplasmic proteins, while removing membrane lipids. Several studies reported that textural properties of fish muscle proteins prepared by alkali pH-shift process was greater than those of conventional washing and acid pH-shift process (Kim, Park, and Choi, 2003; Kristinsson and Liang, 2006; Yongsawatdigul and Park, 2004).

Autolytic degradation is one of the most important factors affecting gelation of mince and washed mince. Proteolytic degradation of myofibrillar proteins, especially myosin, by endogenous proteinases has an adverse effect on gel-forming properties (An, Seymour, Wu, and Morrissey, 1994). Some proteinases, especially cathepsin L, cannot be completely removed by a typical washing process of Pacific whiting,

rendering an increase in proteolytic activity after washing (An, Weerasinghe, Seymour, and Morrissey, 1994). However, several studies reported that proteinase was inactivated during alkali pH-shift process (Kim et al., 2003; Kristinsson, Theodore, Demir, and Ingadottir, 2005). Information about autolytic activity of tropical fish, particularly common carp and goatfish, as affected by protein recovery processes, namely 3-washing cycles and alkali pH-shift process, has not been thoroughly investigated. Understanding such effects would lead to a more efficient means to utilize these species.

Endogeous trasglutsaminase (TGase) is considered as a transferase catalyzing the formation of covalent non-disulfide cross-linking between γ-carboxyamide groups of glutamine and ε-amino groups of lysine (Folk, 1980). Catalytic reaction of TGase is known to enhance setting process (Yongsawatdigul, Worratao, and Park, 2002). Setting is a process where surimi paste was pre-incubated at a certain temperature below 40 °C before cooking to form gel at 90 °C (Lanier, 2000). Tadpitchayangkoon and Yongsawatdigul (2009) reported that textural properties of alkali pH-shift treated striped catfish (*Pangasius hypophthalmus*) which was subjected to setting process was higher than those prepared from a direct heating method. However, setting phenomenon of tropical fish species prepared from alkali pH-shift process has not been clearly demonstrated.

Common carp (*Cyprinus carpio*) is an economically important aquacultured freshwater fish with the annual world production around 3.7 million metric tons in 2011 (FAO, 2013). They are not widely consumed because of pinbones, leading to its low value. Goatfish (*Upeneus* spp.) is also one of important tropical marine fish used for surimi production in Southeast Asia. However, gel-forming ability of goatfish

surimi is lower than other tropical counterparts because of its high endogenous proteolytic activity (Rawdkuen and Benjakul, 2008). Gelation prepared by alkali pH-shift process of common carp (freshwater) and goatfish (marine fish) has not been thoroughly investigated, particularly in relation to endogenous proteinase and TGase activities. Understanding the nature of each species under different processes (washing versus pH-shift) would lead to more efficient utilization of fishery resource to obtain the optimal gel quality. Therefore, objectives of this study were to compare the effect of conventional washing and alkali pH-shift process on autolysis and gel-forming ability of common carp (*Cyprinus carpio*) and goatfish (*Upeneus* spp.).

3.3 Materials and methods

3.3.1 Chemicals

Phenylmethanesulfonyl fluoride (PMSF), N-tosyl-L-lysine chloromethyl ketone (TLCK), N-tosyl-phenylalanine chloromethyl ketone (TPCK), trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64), soybean trypsin inhibitor (STI), pepstatin A, iodoacetic acid (IAA), monodansylcadaverine (MDC), and *N*, *N*-dimethylated casein (DMC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethylenediaminetetraacetic disodium salt (EDTA), bovine serum albumin (BSA) and L-tyrosine were purchased from Fluka (Buchs, Switzerland). Trichloroacetic acid (TCA) was purchased from BDH Chemicals Ltd (Poole, UK). Dithiothreitol (DTT) was obtained from Bio Basic Inc. (Markham, Ontario, Canada). Reagents used for gel electrophoresis were purchased from Promega (Madison, WI, USA). All other reagents and chemicals were of analytical grade.

3.3.2 Fish

Common carp (*Cyprinus carpio*) with the size of 2-2.5 kg were purchased from the freshwater fish wholesale market, Nakhon Ratchasima, and transported to Suranaree University of Technology laboratory in polystyrene foam boxes filled with ice within 30 min. Fish were approximately less than 6 h postharvest. Fish were manually eviscerated and deskinned immediately upon arrival. Fish fillets were ground using a meat grinder (Model 8-22, Marblehead, Ohio, USA) with a screen size of 1.5-mm perforation plate. Ground samples were vacuum-packed in a plastic bag, kept at -20 °C, and used within 3 months. Frozen goatfish (*Upeneus* spp.) mince was obtained from Andaman Surimi Industry, Samutsakorn province. Frozen surimi was vacuum-packed kept at -20 °C and used within 3 months.

3.3.3 Sample preparation

Mince of common carp (M-C) and goatfish (M-G) were thawed under running tap water (25 °C) for 20 min. For preparation of washed mince, mince was homogenized with distilled water (4 °C) at a mince/water ratio of 1:3 (w/v) and continuously stirred for 5 min. The homogenate was filtered with three layers of cheesecloth. The washing cycle was repeated twice by distilled water and the third washing cycle was carried out using 0.3% NaCl. Subsequently, the homogenate was centrifuged at 5000×g (Sorvall Legend MACH 1.6/R, Thermo Electron LED GmbH, Lengensellbold ,Germany) for 10 min at 4°C. The precipitate was referred to as washed mince of common carp (WM-C) and goatfish (WM-G).

The alkali pH-shift process was carried out according to Hultin and Kelleher (2000). Fish mince was homogenized with cold distilled water at ratio 1:9 (w/v). The pH of homogenate was adjusted to pH 11 using 2 N NaOH. The

homogenate was centrifuged at 10000×g at 4 ℃ for 20 min. The soluble proteins were collected and then the pH of homogenate was adjusted to the isoelectric point using 2 N HCl with slow stirring. The precipitate was filtered with a layer of cheesecloth and then the sample was dewatered by centrifugation at 6000×g at 4 °C for 20 min. The final pH was adjusted to pH 7 using 2 N NaOH. The samples were referred to as fish protein isolate of common carp (FPI-C) and goatfish (FPI-G).

3.3.4 Autolytic activity assay

Autolytic activity was determined using the method of Yongsawatdigul and Piyadhammaviboon (2004). Three grams of all samples were incubated at different temperatures (0, 40, 50, 55, 60, 65, 70, 75, and 80 °C) for 1 h. Autolytic reaction was stopped by addition of 27 mL of cold 5% trichloroacetic acid (TCA) solution. The mixture was homogenized for 1 min. Then, the homogenate was incubated at 4 °C for 15 min, and centrifuged at 10000×g (Sorvall Legend Micro 17R, Thermo Electron LED GmbH, Lengensellbold ,Germany) for 15 min at 4 °C. Blanks were kept on ice and treated in the same manner as the samples. Supernatants were analyzed for TCA-soluble oligopeptide peptides content by Lowry method (Lowry et al, 1951) using L-tyrosine as a standard. To determine total soluble protein, three grams of sample were mixed with 27 mL of 5% hot sodium dodecylsulfate (SDS) solution at a ratio of (w/v). Autolytic activity was expressed as nmol of tyrosine/mg protein/h.

Optimum pH for autolytic activity of all samples was also measured. Three grams of samples were added to 9 mL of different buffers including 0.2 M McIlvaines' buffer (0.2 M sodium phosphate, 0.1 M sodium citrate) for pH 2.0-7.0, 0.1 M Tris-HCl buffer for pH 8-9, and 0.1 M glycine-NaOH for pH 10-11. The

mixture was homogenized for 1 min. All samples were incubated at 65 °C, except for M-C samples which were incubated at 70 °C for 1 h. The reaction was stopped by addition of 18 mL of cold 7.5% TCA. The mixture was homogenized, centrifuged and determined for TCA-soluble oligopeptide content as described above.

3.3.5 Proteinase inhibitors

Three grams of M-C and M-G were homogenized with 9 mL of different buffers. The homogenate was mixed with 1 mL of proteinase inhibitor solution to obtain the final concentration of 0.01 mM SBTI, 100 μM TLCK, 100 μM TPCK, 1 mM PMSF, 10 mM EDTA, 10 μM pepstatin A, 10 μM E-64, or 1 mM IAA. M-C and M-G samples were incubated for 1 h at 70 °C (at pH 4, 7, and 10) and 65 °C (pH 4, 7 and 9), which were the optimum conditions found from the autolytic assay for each species, respectively. The reaction was stopped by addition of 18 mL of cold 7.5% TCA solution. The mixture was homogenized, centrifuged and determined oligopeptide content as previously described. The sample without any inhibitors was used as a control. Blanks for both control and sample were mixed with inhibitors and kept on ice, and TCA was immediately added. The degree of inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{(TC - TC_b) - (TI - TI_b)}{(TC - TC_b)} x100$$

where, TC = oligopeptide content of control (without inhibitor) incubated at the optimum condition , TC_b = oligopeptide content of the control (without inhibitor) incubated at 0 °C, TI = oligopeptide content of sample (with inhibitor) incubated at the optimal condition , TI_b = oligopeptide content of sample (with inhibitor) incubated at 0 °C.

3.3.6 TGase activity

TGase activity of all samples was determined by the method of Takagi, Saito, Kikuchi, and Inada (1986). The samples were homogenized in four volumes of extraction buffer (10 mM NaCl, 5 mM EDTA, 20 mM Tris–HCl, pH 8.0). Homogenates were centrifuged at 16000×g for 20 min at 4 °C. The supernatants were filtered through glass wool and used as a crude extract. The assay mixture was contained 1.0 mg/mL DMC, 15 μM MDC, 5 mM $CaCl_2$, 3 mM DTT, 50 mM Tris–HCl (pH 8.0), and 100 μL of crude enzyme. After incubation at 37 °C for 10 min, EDTA solution was added to a final concentration of 20 mM to stop the reaction. The fluorescence intensity was measured with excitation and emission wavelengths of 350 and 480 nm, respectively, using a Shimadzu spectrofluorometer. Enhancing factor was determined to be 1.16 in this study. One unit of TGase activity was defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of MDC into DMC per 1 min.

3.3.7 Gel preparation

Gels of all samples (M-C, M-G, WM-C, and WM-G) were prepared to contain 2% NaCl and 80% moisture content. Gels from FPI-C and FPI-G were also prepared in the absence of NaCl. The samples were chopped in a Stephan vacuum cutter (UM5, Stephan Machinery Co., Columbus, OH, USA) for 6 min and final chopping temperature was <12 °C. The pastes were stuffed into 22-cm-diameter cellulose casing. Subsequently, samples were pre-incubated at 25 °C for 2 h, 40 °C for 30 min, or 65°C for 30 min and followed by 90 °C for 30 min. Samples without pre-incubation were also prepared by heating at 90 °C for 30 min. Then, gels were cooled in iced water for 20 min and stored overnight in a refrigerator.

3.3.8 Total sulfhydryl content

Total SH groups of pastes and gels treated at various treatments were determined according to Monahan, German, and Kinsella (1995). Samples (1 g) were homogenized in a solubilizing buffer (0.2 M Tris-HCl, 2% SDS, 10 mM EDTA, 8 M urea, pH 7.0). The homogenates were heated at 100 °C for 5 min and centrifuged at 10000×g for 15 min. One mL of the supernatant was added 0.01 mL Ellman's reagent (10 mM 5,5'-dinitrobis [2-nitrobenzoic acid]). The mixtures were incubated at 40 °C for 25 min. The absorbance measured at 412 nm was used to calculate total SH content using the extinction coefficient of 13600 $M^{-1}cm^{-1}$. Protein content was determined using Lowry method with BSA as a standard (Lowry, Rosebrough, Farr, and Randall, 1951).

3.3.9 Texture and color analysis

The chilled gels were left at room temperature for 2 h. Textural properties of gels were measured using a Texture Analyzer (TA-XT2 Stable Micro System, Surrey, U.K.) equipped with a spherical probe (5-mm dia.). Gel samples were cut into pieces of 2.5 cm length. Penetration test was performed at a probe speed of 1 mm/s. Breaking force (g) and deformation distance (mm) were recorded.

Color values (L*, a*, b*) of samples were measured using a colorimeter (Minolta, Tokyo, Japan). Whiteness of gels was calculated using the equation L* - 3b* (Park, Yongsawadigul, and Lin, 1994).

3.3.10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of surimi gels were evaluated using SDS-PAGE (Laemmli, 1970). Three g of samples were solubilized in 18 mL of 5% hot SDS

solution. Then, the homogenate was heated at 90 °C for 30 min and centrifuged at 10000×g for 10 min at room temperature. The supernatant was mixed with the treatment buffer at 1:1 ratio and boiled for 3 min. Protein sample (25 µg) were loaded on polyacrylamide gel (4% stacking gel and 10% running gel). Gels were run at a constant voltage setting of 120 V. Gels were stained with 0.125% Coomassie Brilliant Blue R-250 for 1 h and destained in a solution containing 25% ethanol and 10% acetic acid.

3.3.11 Statistical analyses

All experiments were performed in duplicate. Data were analyzed for the degree of variation and significance of difference using an analysis of variance (ANOVA) and differences among treatment means were evaluated by Duncan's multiple range test (DMRT). The statistical analysis was performed using SPSS Statistic Program Version 14.0 (SPSS Inc, Chicago, IL, USA). Significance of differences was defined at $P < 0.05$.

3.4 Results and discussion

3.4.1 Autolytic activity

Proteolytic activity of M-C was the highest at 70 °C, while optimum temperature of both WM-C and FPI-C were observed at 65 °C (Figure 3.1a). This result implied that active proteinase(s) exhibiting optimal activity at 70 °C might be removed during conventional washing and inactivated during alkali pH-shift process. All goatfish samples exhibited the maximum activity at 65 °C (Figure 3.1b). Washing and the pH-shift process markedly decreased autolytic activity of goatfish. Predominant proteinases responsible for autolytic degradation of goatfish are likely to

be in the sarcoplasmic fraction, which were readily removed by washing and inactivated by alkali pH-shift process.

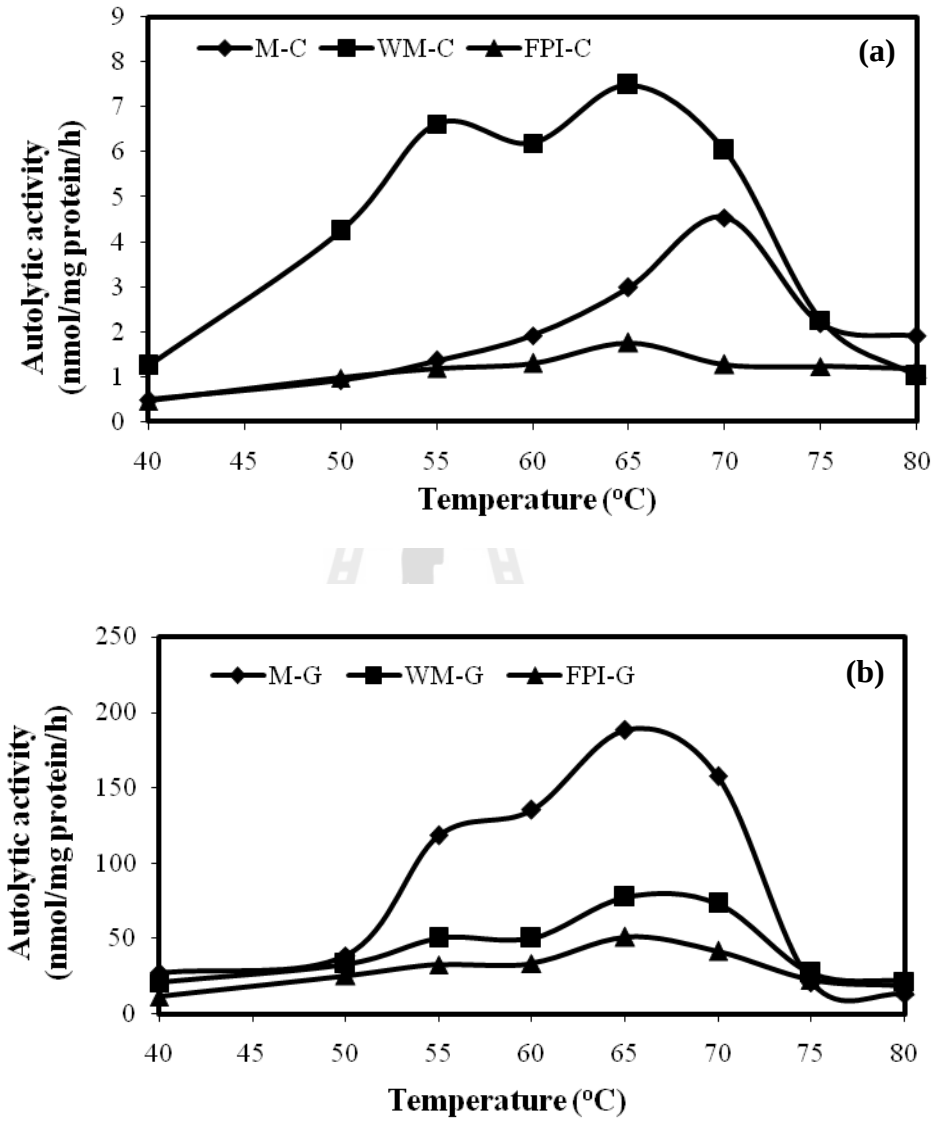

Figure 3.1 Effect of temperature on autolytic activity of common carp (a) and goatfish (b) mince subjected to 3-cycle washing and alkali pH-shift process. M-C, mince of common carp; WM-C, washed mince of common carp; FPI-C, fish protein isolate of common carp; M-G, mince of goatfish; WM-G, washed mince of goatfish; FPI-G, fish protein isolate of goatfish.

Tropical species including lizardfish, tilapia and small scale mud carp also exhibited the maximum activity around 65-70 °C (Yongsawatdigul, Park, Virulhakul, and Viratchakul, 2000; Yongsawadigul and Piyadhammaviboon, 2004; Yongsawadigul, Piyadhammaviboon, and Singchan, 2006). Relatively high activity at 70 °C appeared to be a distinct autolytic characteristic of tropical fish. WM-C showed higher autolytic activity than M-C (Figure 3.1a). This result suggested that proteinases in common carp were tightly bound to myofibril and were not removed by washing. Osatomi, Sasai, Cao, Hara, and Ishihara (1997) purified a myofibril-bound proteinase from common carp muscle. Myofibril-bound proteinases were also found in crucian carp (Kinoshita, Toyohara, and Shimizu, 1990). In contrast, a marked decrease of proteolytic activity of goatfish was observed in WM-G (Figure 3.1b), indicating the nature of sarcoplasmic proteinases. An et al. (1994) found that cathepsin B and H from Pacific whiting were largely removed during conventional washing. TCA-soluble oligopeptide contents of FPI-C and FPI-G were lowest in the respective species, implying that endogenous proteinases were inactivated during alkali pH-shift process. Tadpitchayangkoon and Yongsawatdigul (2009) reported that alkaline extraction of striped catfish (*Pangasius hypophthalmus*) resulted in inactivation of endogenous proteinase activity.

For pH profiles of M-C and WM-C, major activity was found at pH 4 and 10 (Figure 3.2a), indicating the involvement of acid and alkaline proteinases. Autolytic degradation of M-G optimally occurred at pH 7, and 9, and much smaller peak at pH 4 (Figure 3.2b). Neutral and alkaline proteinases appeared to be more predominant in goatfish muscle than acid counterparts.

After 3-cycle washing, proteinases of M-G were largely removed as judged by a decrease of autolytic activity. The pH-shift process drastically reduced proteolytic activities of both species. Kim et al. (2003) also reported a significant reduction of cathepsin L- and B-like activity in Pacific whiting mince after alkali pH-shift process. Therefore, alkali pH-shift process could be an effective means to minimize proteolytic activity in goatfish. It should be note that proteolytic activity of common carp was much less than that of goatfish at all temperature and pH values studied. Thus, proteolysis of common carp during processing would be less problematic.

3.4.2 Effect of proteinase inhibitors

The effect of various proteinase inhibitors was investigated at the optimum acidic (pH 4), neutral (pH 7) and alkaline (pH 10 for M-C and pH 9 for M-G) condition. Although autolytic activity of M-C at pH 7 was relatively low, it was selected for the study since this is a typical pH of fish mince products. Pepstatin A inhibited the autolytic activity of M-C and M-G at pH 4 by 60% and 30%, respectively (Table 3.1). E-64 and iodoacetic acid were able to inhibit proteolytic activity of M-G at pH 4 by about 40%, but not in M-C. E-64 and iodoacetic acid are specific inhibitors for cysteine proteinases. These results indicated that aspartic proteinases were mainly responsible for proteolysis of M-C while both aspartic- and cysteine- proteinases were responsible for the degradation of M-G at pH 4. Proteinases in M-C were strongly inhibited by TLCK, STI, and PMSF at pH 7 and 10. TLCK and STI inhibit trypsin-like proteinases, while PMSF is an irreversible serine proteinase inhibitor.

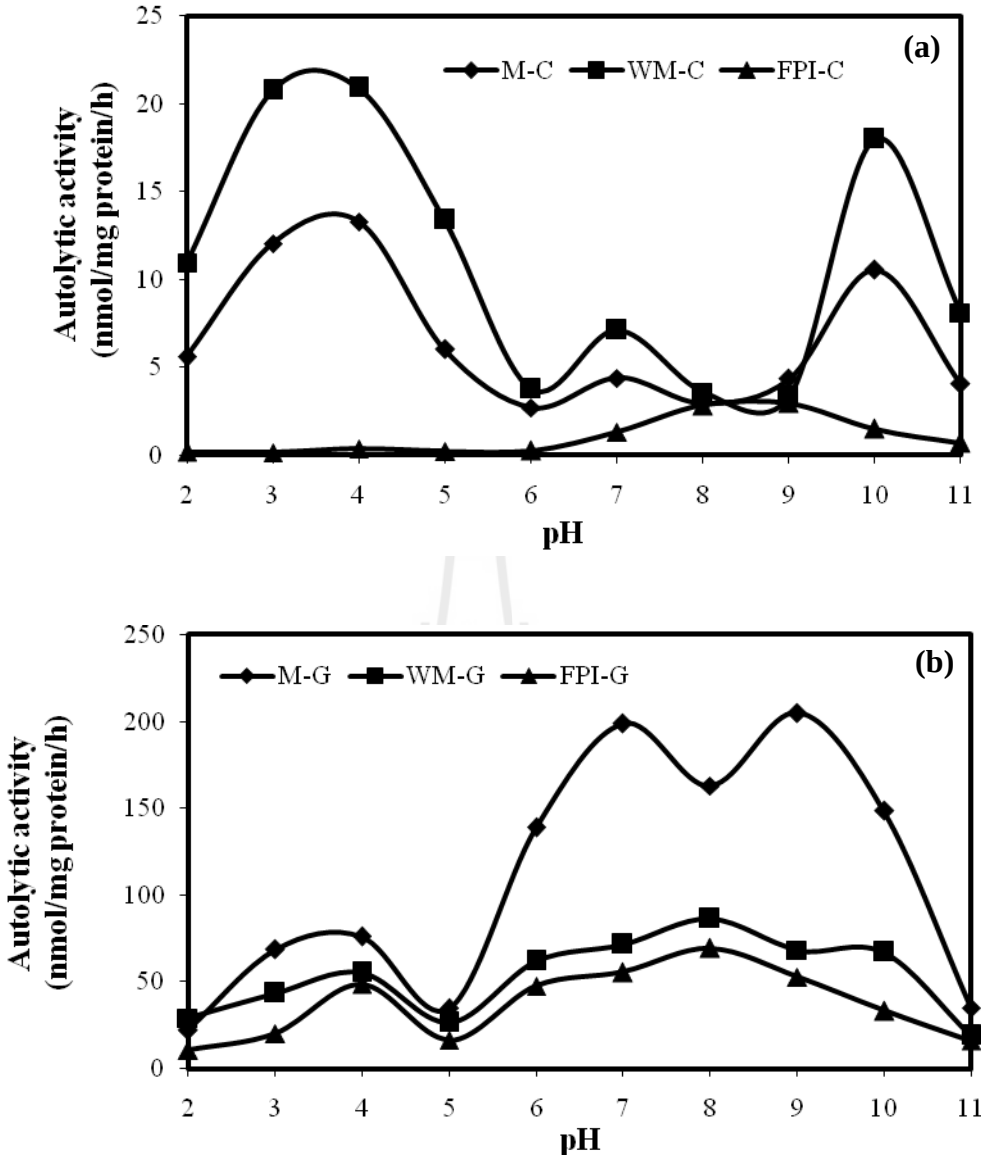

Figure 3.2 Effect of pH on autolytic activity of common carp (a) and goatfish (b) mince subjected to 3-cycle washing and alkali pH-shift process. Abbreviations are the same as figure 3.1.

In M-G, inhibitory effects of TLCK, SPI and PMSF were relatively high (50-70%) at pH 7 and 10. Moreover, the inhibitory effect of EDTA, a metallo proteinase inhibitor, was also observed. Yongsawatdigul et al. (2000) reported that

proteolytic activity of tilapia surimi was mainly inhibited by STI and leupeptin, suggesting that proteinase(s) involved was serine type. STI strongly inhibited proteolysis of washed lizardfish mince, while leupeptin and E-64 had little inhibition effect, suggesting that both serine and cysteine proteinase(s) were responsible for myosin heavy chain degradation of washed lizardfish mince (Suwansakornkul, Itoh, Hara, and Obatake, 1993). At pH 7 and 10, trypsin-like proteinases were responsible for autolytic degradation of M-C, whereas serine- and metallo- proteinases were involved in proteolysis of M-G.

3.4.3 TGase activity

TGase activity of washed mince from both species was lower than that of respective minces ($P < 0.05$, Table 3.2). Tissue TGase is present in cytosol and is removed when tissue is disintegrated and washed with water. Yongswatdigul and Piyadhammaviboon (2007) reported that TGase was easily removed during washing. TGase activity of FPI-C and FPI-G was lowest ($P < 0.05$), indicating that the enzyme was largely inactivated during alkali pH-shift process. It has been known that endogenous TGase is responsible for the formation of covalent non-disulfide cross-linking between glutamine and lysine, resulting in an increase in gel strength during setting (Joseph et al., 1994). Therefore, it is unlikely that endogenous TGase would significantly contribute to textural properties of alkali-treated gels of these species.

Table 3.1 Effect of various inhibitors on autolytic activity of mince from common carp and goatfish.

Inhibitor	Degree inhibitition (%)					
	M-C			M-G		
	pH 4	pH 7	pH 10	pH 4	pH 7	pH 9
Control	0	0	0	0	0	0
TPCK	1.26 ± 0.04	18.94 ± 3.79	22.59 ± 3.61	8.89 ± 0.94	8.32 ± 1.73	10.93 ± 1.58
TLCK	5.57 ± 0.038	79.45 ± 8.93	68.02 ± 4.58	0	57.82 ± 3.92	54.05 ± 2.95
E-64	12.22 ± 1.62	21.49 ± 2.96	19.85 ± 1.95	43.27 ± 1.97	11.39 ± 6.43	17.78 ± 3.82
SPI	0	81.49 ± 1.98	65.93 ± 3.04	3.32 ± 0.83	65.66 ± 2.84	66.91 ± 5.61
IAA	12.51 ± 2.84	18.64 ± 2.84	16.34 ± 2.87	37.7 ± 4.62	9.16 ± 0.97	5.16 ± 4.73
Pepstatin A	59.76 ± 4.92	24.16 ± 4.97	14.63 ± 1.63	32.04 ± 5.81	19.79 ± 1.28	4.51 ± 2.18
PMSF	2.27 ± 0.85	63.85 ± 3.59	71.28 ± 5.94	1.93 ± 0.05	59.74 ± 2.51	57.12 ± 2.64
EDTA	4.34 ± 1.09	1.37 ± 0.05	1.98 ± 4.38	10.67 ± 2.96	35.02 ± 7.16	30.09 ± 2.96

Note: M-C, mince of common carp; M-G, mince of goatfish.

Table 3.2 TGase activity of common carp and goatfish muscle proteins subjected to washing and alkali pH-shift process.

Samples	TGase activity (unit/mg protein)	
	Common carp	Goatfish
Mince	0.176 ± 0.031^{a}	0.258 ± 0.011^{a}
Washed mince	0.114 ± 0.029^{b}	0.164 ± 0.029^{b}
Fish protein isolate	0.027 ± 0.005^{c}	0.035 ± 0.002^{c}

Mean $\pm$ SD values with different letters in column are different ($P < 0.05$).

3.4.4 Total sulfhydryl content

Total sulfhydryl (SH) content of pastes varied with treatments in both species (Figure 3.3). The total SH content of mince was comparable to that of washed mince ($P > 0.05$). The lowest total SH content of paste were found in fish protein isolate of both species ($P < 0.05$). Similar result was reported in alkali-treated rockfish (Yongsawatdigul and Park, 2004) and striped catfish (Tadpitchayangkoon and Yongsawatdigul, 2009). This result implied that alkali pH-shift process induced oxidation of sulfhydryl groups of both species. Park (2009) reported that conventional washing process avoids any possible denaturation while pH-shift process induces chemical denaturation. Kristinsson, and Hultin (2003) reported that cod myosin had more exposed reactive SH groups when the myosin was treated by alkali treatment, presumably promoting myosin head-to-head aggregation. This result suggested that alkaline treatment induced oxidation of sulhydryl groups, resulting in the formation of disulfide bonds. Total SH content of all gels markedly decreased when subjected to any studied heating conditions as compared to their respective pastes. This was probably because heating induced exposure of SH groups, resulting in more sulfhydryl

group interchanges. These results implied that fish protein isolate of both species contained more intermolecular disulfide bonds than mince and washed mince.

3.4.5 Textural properties

M-C and M-G gels exhibited the lowest breaking force at all pre-incubation temperature studied ($P < 0.05$, Figure 3.4). Three-cycle washing improved textural properties of both species ($P < 0.05$). This was probably because of a removal of sarcoplasmic proteins. Moreover, endogenous proteinases in goatfish were removed during washing (Figure 3.1b and 3.2b), leading to a decrease of proteolysis. Although myofibril-bound proteinases were found in WM-C (Figure 3.1a and 3.2a), their low activity did not severely disintegrate gel texture (Figure 3.4a and 3.4c).

Both breaking force and deformation distance of M-C and WM-C gels were improved when pre-incubated at 25 and 40 °C, with 40 °C-pre-incubation being the best condition for gel improvement ($P < 0.05$). Textural properties of tropical fish species, such as threadfin bream, bigeye snapper, and small scale mud carp were also improved with pre-incubation at 40 °C (Yongsawatdigul et al, 2002; Benjakul and Visessanguan, 2003; Yongswatdigul et al., 2006). However, optimum condition for setting of M-G and WM-G gels was found at low setting temperature of 25 °C ($P < 0.05$). The textural improvement under setting condition of both common carp and goatfish could also be partly attributed to endogenous TGase activity, resulting in the formation of a stronger gel. In addition, pre-incubation at low temperature could induce unfolding and exposure of buried hydrophobic and sulfhydryl groups of actomyosin, leading to hydrophobic interactions and/or disulfide linkages (Hemung and Yongsawatdigul, 2005; Yongsawatdigul and Sinsuwan, 2007). However, textural properties of both M-G and WM-G gels did not enhance at 40 °C. The degree of

proteolysis might appear to be more predominant than the extent of cross-linked formation at 40 °C as goatfish exhibited high endogenous proteolytic activity.

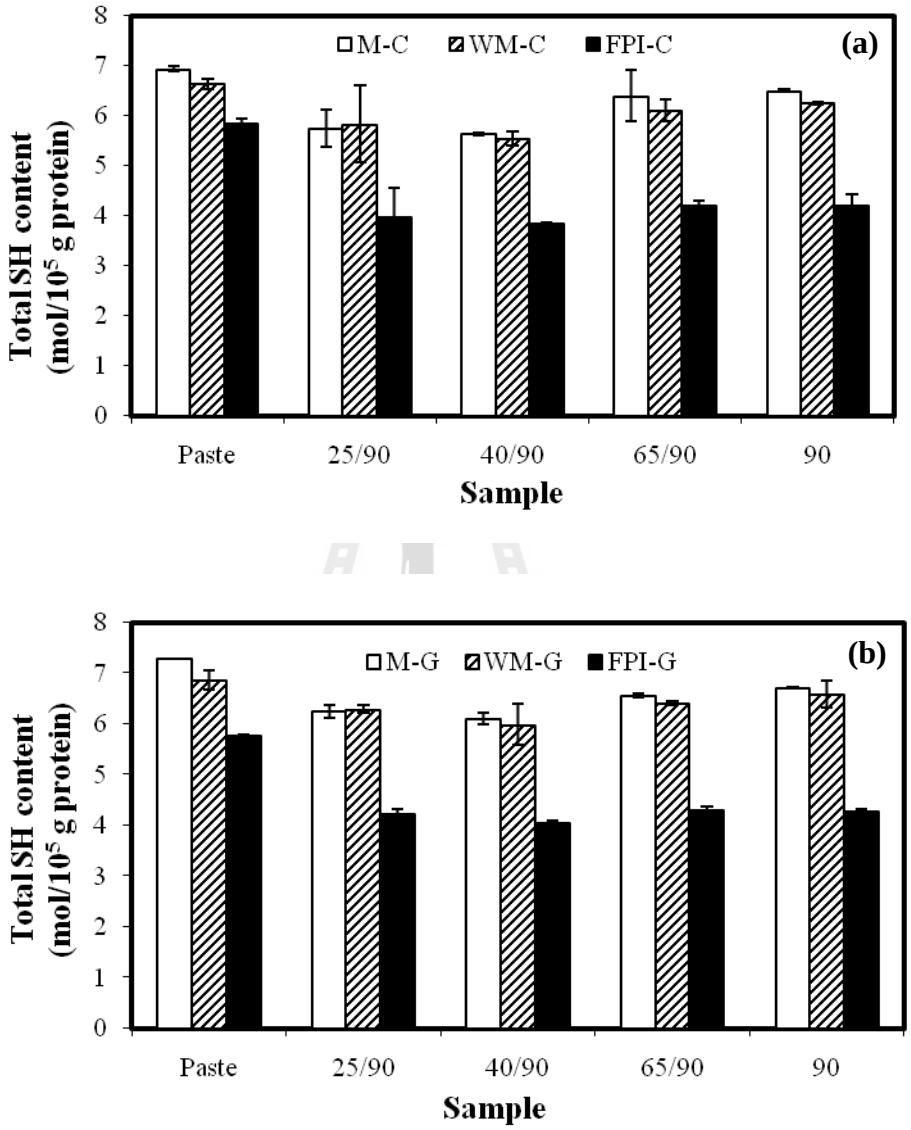

Figure 3.3 Total SH content of paste and gels of common carp (a) and goatfish (b) prepared from washing and an alkali-pH shift process and subjected to various heating regimes. Abbreviations are the same as figure 3.1.

Gels from common carp and goatfish showed the lowest textural properties when pre-incubated at 65 °C. This was obviously resulted from the presence of endogenous proteinases. A drastic decrease of both breaking force and deformation distance was apparently observed in M-G gels, corresponding to high activity of both serine- and metallo- proteinases (Table 3.1).

FPI-G gels exhibited greater breaking force than WM-G gels at all heating conditions ($P < 0.05$). A decrease of proteolytic activity and a decrease of total SH content appeared to positively contribute to an enhancement of breaking force for FPI-G gels. Kim et al. (2003) reported that the best gelation was obtained when Pacific whiting fish proteins were treated at pH 11 under nonsetting condition. This was due to inactivation of cathepsin L-like enzymes in Pacific whiting at pH 11. However, Pacific whiting gel prepared by acid solubilization process exhibited lower breaking force than conventional washing process due to residual activity of cathepsin L-like enzymes (Choi and Park, 2002). Alkali pH-shift process appeared to be more effective in reducing endogenous proteolytic activity of muscle proteins.

At 25 and 40 °C-preincubation, FPI-C gels showed lower breaking force than WM-C gels ($P < 0.05$). A possible explanation is that FPI-C sample contained limited endogenous TGase activity (Table 3.2), impeding the extent of cross-linkings. Although alkali pH-shift process can minimize proteolytic activity in common carp muscle proteins (Figure 3.1a and 3.2a), breaking force value of FPI-C gel was comparable to that of WM-C gels pre-incubated at 65 °C ($P > 0.05$). Since endogenous proteinase activity was relatively low in common carp muscle, the proteinase inactivation by an alkali pH-shift treatment did not significantly improve its gel texture.

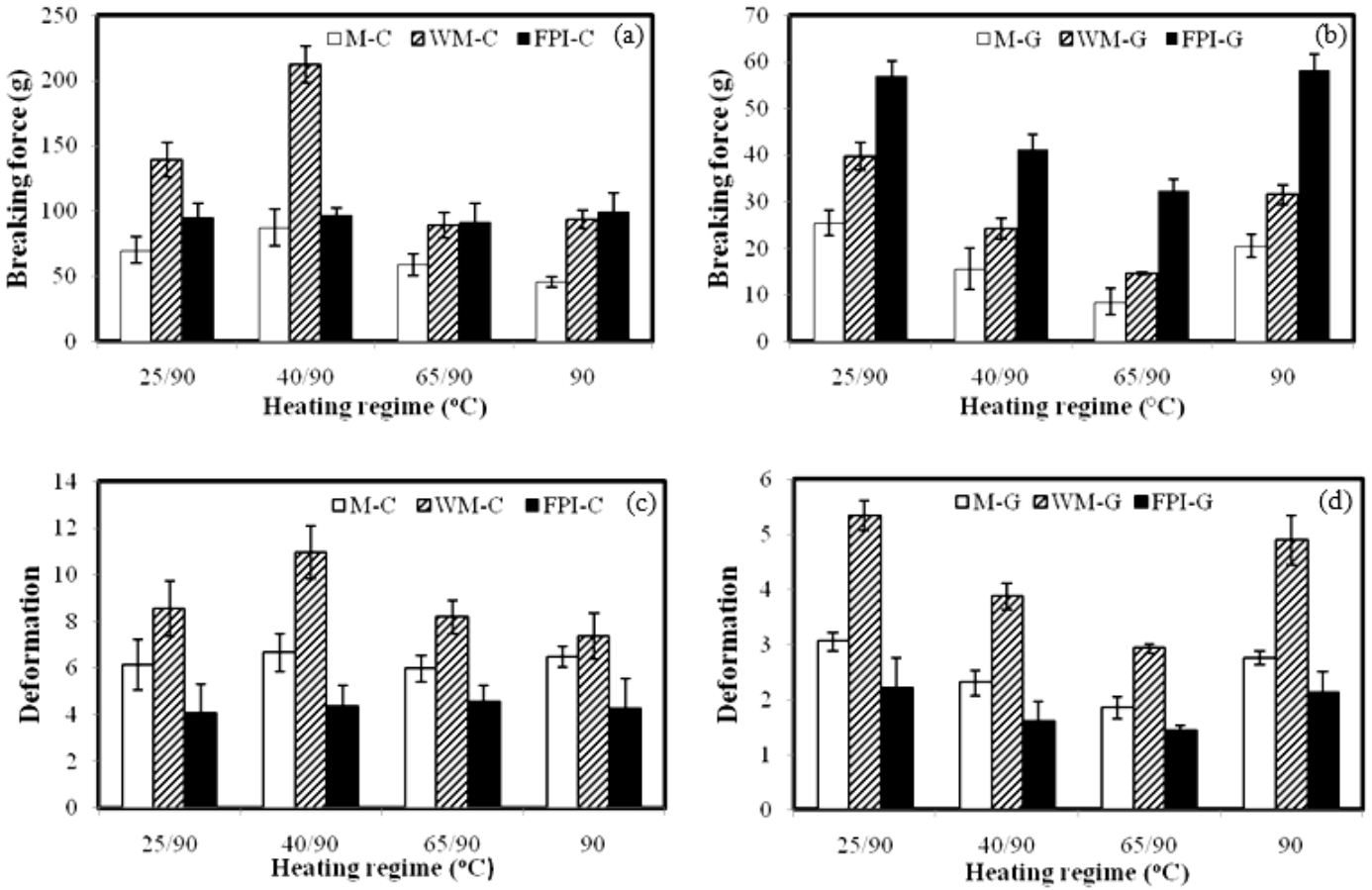

Figure 3.4 Textural properties of gels of common carp (a, c) and goatfish (b, d) from various treatments and heating regimes. Abbreviations are the same as figure 3.1.

In addition, conformational changes induced by alkali pH-shift treatment of some freshwater fish and tropical species might result in inferior gel network formation. Tilapia, sardine and, mackerel prepared by alkali pH-shift process also showed lower breaking force and deformation than did that from conventional method (Rawdkuen, Sai-Ut, Khamsorn, Chaijan, and Benjakul, 2009; Chaijan, Benjakul, Visessanguan, and Faustman, 2006).

Breaking force value of FPI-G gels pre-incubated at low temperature (25 °C) was comparable to that cooked at 90 °C ($P > 0.05$). In addition, breaking force of FPI-C gels pre-incubated at low (25 °C) and high (40 °C) temperature setting was the same as that at 90 °C ($P > 0.05$). Endogenous TGase activities in FPI-C and FPI-G samples were largely inactivated during alkali pH-shift process (Table 3.2), yielding minimal setting effect. Kim and Park (2008) found that setting did not promote textural properties of alkali-extracted fish protein isolate of Alaska pollock. Alkali pH-shift process resulted in the lowest deformation values in both common carp and goatfish ($P < 0.05$). Kristinsson and Hultin (2003) reported an increase in surface hydrophobicity of cod myosin when treated in alkaline pH, indicating the role of hydrophobic interactions in gel formation of FPI. Alkali pH-shift process might induce aggregation but reduce protein-water binding, leading to high breaking force but low deformation. From this result, setting did not enhance gel quality of both species prepared by alkali pH-shift treatment. In general, common carp gels showed higher textural properties than goatfish gels. Therefore, common carp could be a potential alternative raw material for surimi-based products.

3.4.6 SDS-PAGE patterns

Degradation of myosin heavy chain (MHC) and actin was not found in M-C gels at all heating regimes. Although myofibrillar-bound proteinases were not removed during conventional washing process (Figure 3.1a and 3.2a), no obvious degradation of MHC and actin in WM-C gels was observed. This result confirmed that proteolysis in common carp was insignificant. In addition, it did not appear that alkali pH-shift process induced degradation of FPI-C. Therefore, low textural properties of FPI-C gels might result from inactivation of TGase activity (Table 3.2) and conformational changes induced by alkali pH-shift treatment of common cap muscle protein. Severe degradation of MHC and actin of M-G gels occurred at all heating regimes applied, especially at 65 °C (Figure 3.5). In addition, troponin and tropomyosin also disappeared when pre-incubated at 65 °C. Various proteinases including serine- and metallo- proteinases were responsible for degradation of M-G at pH 7.0 (Table 3.1). After conventional washing, higher intensity of actin and troponin was observed in WM-G gels, indicating a decrease in proteolysis. However, loss of tropomyosin was still observed in WM-G gels at 65 °C. Yongsawatdigul and Piyadhammaviboon (2004) reported that both MHC and tropomyosin of lizardfish surimi were preferentially hydrolyzed at 65 °C. Intensity of MHC band of FPI-G gels was much higher than that of WM-G gels when pre-incubated at 25 and 40 °C. Changes of actin intensity were subtle in FPI-G gels. This indicated less proteolysis after alkali pH-shift treatment, corresponding to its high breaking force (Figure 3.4b). Interestingly, a band with molecular mass of 37 kDa, which was presumed to be troponin, disappeared in the paste and gels of FPI-G.

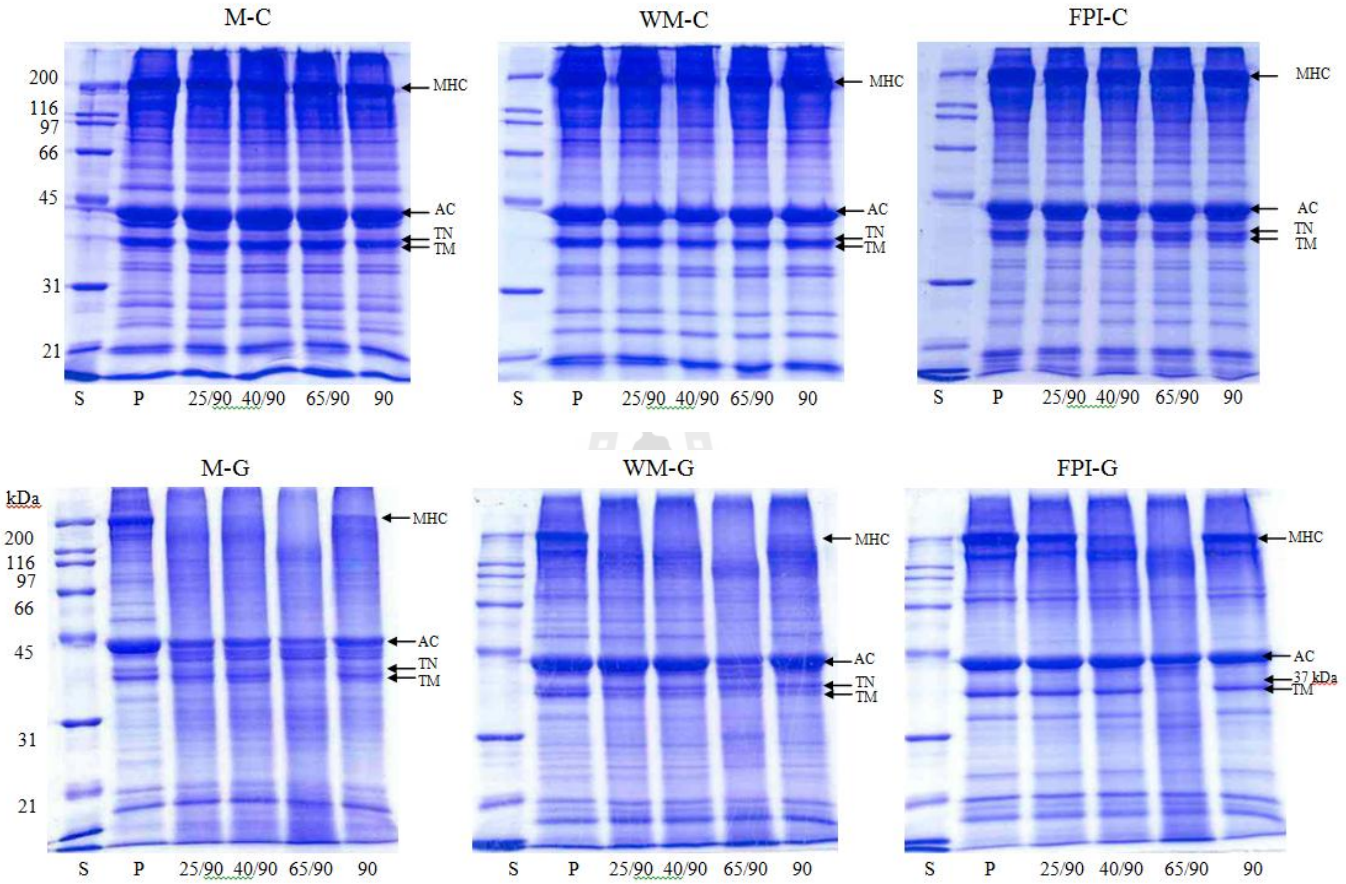

Figure 3.5 SDS-PAGE pattern of pastes and gels prepared using different conditions and subjected to various heating regimes. S, standard markers; P, paste; MHC, myosin heavy chain; AC, actin; TN, troponin; TM, tropomyosin. Abbreviations are the same as figure 3.1.

Alkali pH-shift process might induce interactions of troponin with other proteins. Alternatively, troponin might undergo degradation under alkali pH-shift treatment. Yongsawatdigul and Park (2004) reported that proteins with molecular mass of 42 and 120 kDa interacted with other proteins through disulfide linkages during gelation of alkali fish protein isolate. Disappearance of MHC at 65 °C of FPI-G indicated the presence of residual endogenous proteolytic activity. This result confirmed that alkali pH-shift process minimized proteolytic activity in goatfish muscle proteins, resulting in higher textural properties. Less MHC degradation in all treatments of common carp resulted in higher gel texture as compared to goatfish.

3.4.7 Color

Since heating regimes had no effect on whiteness of gels, only whiteness value of sample pre-incubated at 40 °C are shown in Figure 3.6. Whiteness of gels from both common carp and goatfish displayed similar trends. The whiteness of washed mince gels was higher than that of mince and alkali-treated gels ($P < 0.05$). Both mince and alkali-treated gels contained myoglobin and hemoglobin, resulting in higher a* value than washed mince. L* value of washed mince gels was also the highest due to a removal of heme proteins. Alkali-produced protein isolates from mackerel (Chaijan, Benjakul, Visessanguan, and Faustman, 2006), rockfish (Yongsawatdigul and Park, 2004), menhaden (Perez-Mateos and Lanier, 2006), and Atlantic croaker (Kristinsson and Liang, 2006) also showed higher whiteness values than that of conventionally-washed gels.

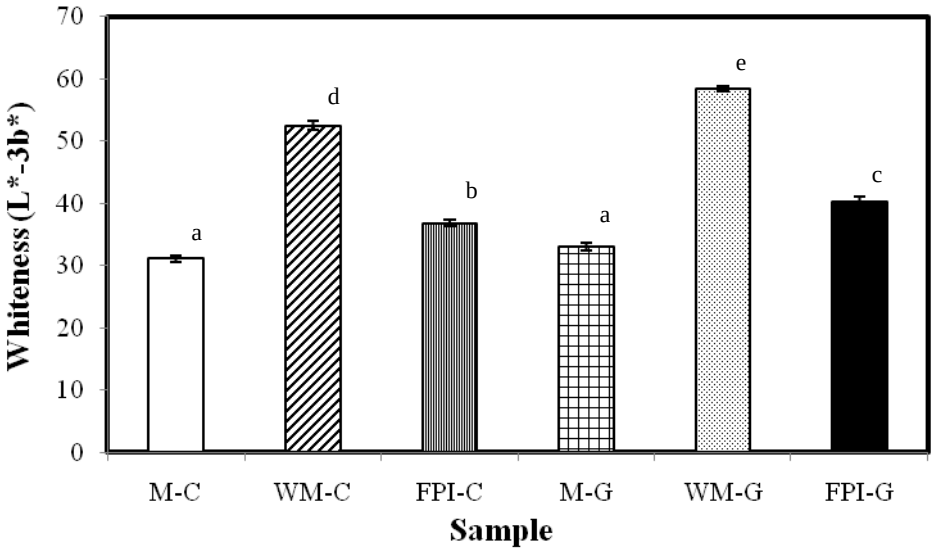

Figure 3.6 Whiteness of gels prepared from various treatments and pre-incubated at 40 °C. Abbreviations are the same as figure 3.1. Different letters with the same species are significantly different ($P < 0.05$).

3.5 Conclusions

Alkali pH-shift process can effectively reduce autolytic activity of common carp and goatfish. In addition, TGase activity of both species markedly decreased by alkali pH-shift process. Common carp exhibited much lower proteolytic activity than goatfish. At pH 7, typical pH of fish mince products, major proteinases in common carp mince was serine proteinases while that of goatfish mince was serine- and metallo-proteinases. Breaking force of goatfish gels was greatly improved by alkali pH-shift process while a 3-cycle washing process tremendously enhanced textural properties of common carp gels. Setting did not significantly increase gel qualities of both species prepared by alkali pH-shift process.

3.6 References

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CHAPTER IV

TRYPSIN INHIBITOR ACTIVITY AND GEL-ENHANCING EFFECT OF SARCOPLASMIC PROTEINS FROM COMMON CARP

4.1 Abstract

The objective of this study was to investigate the effect of common carp (*Cyprinus carpio*) sarcoplasmic proteins (SP) on proteinase inhibitory activity and gel improving ability of threadfin bream (*Nemipterus* spp.) surimi. SP displayed 89 and 54% inhibitory activity toward trypsin at 40 and 65 °C, respectively. Protein bands with molecular mass of 35, 41, 47, 52, and 69 kDa appeared on trypsin inhibitory activity staining under non-reducing condition when incubated at 40 °C, while two protein bands with molecular mass of 35 and 54 kDa were observed at 65 °C. Trypsin inhibitory activity of SP showed good thermal stability in the temperature range of 35-60 °C. In addition, it still retained 88% of its inhibitory activity up to 60 min when incubated at 40°C. Addition of 0.18 g protein of SP/100 g increased breaking force and deformation of threadfin bream surimi gel. However, when SP was added in combination with various $CaCl_2$ concentrations, it did not improve the textural properties. Myosin heavy chain of threadfin bream surimi was greater retained when SP was added, coinciding with a decrease in trichloroacetic acid-oligopeptide content. These results indicated that SP from common carp showed

inhibitory activity towards trypsin and endogenous proteinases of threadfin bream surimi. In addition, SP from common carp could be a potential protein additive for improving textural properties of fish protein gel.

4.2 Introduction

Endogenous proteinases have been well known to cause an adverse effect on the gel-forming ability of fish muscle proteins. One of the major proteinases responsible for textural degradation of fish muscle protein is classified as a trypsin-like serine proteinase. Several studies have reported on trypsin-like proteinases in fish muscle proteins. Martone, Busconi, Folco, and Sanchez (1991) reported that hake skeletal muscle contained trypsin-like proteinases which degraded major contractile muscle tissue and myofibrils. Choi, Cho, and Lanier (1999) found that trypsin-like proteinase was the most predominant proteinase in Atlantic menhaden muscle and caused poor textural properties. It has also been reported that serine proteinases are prevalent in tropical fish surimi including lizardfish (Cao, Osatomi, Hara, and Ishihara, 2001; Yongswatdigul and Piyadhammaviboon, 2004), threadfin bream (Kinoshita, Toyohara, and Shimizu, 1990), and croaker (Ohkubo, Osatomi, Hara, Ishihara, and Aranishi, 2005). To alleviate the problems of textural degradation caused by endogenous proteinases some commercial food-grade proteinase inhibitors including egg white powder and whey protein concentrate have been used.

Sarcoplasmic proteins (SPs) are proteins extracted from muscle tissue by water or low ionic strength solution. SPs consist of several enzymes involved in muscle metabolism with glyceraldehydes 3-phosphate dehydrogenase (GAPDH) being the most abundant species (Jafarpour and Gorczyca, 2012). SPs are known to interfere

with gelation of myofibrillar proteins as: proteinases in SPs induce myofibrillar proteins degradation; and some enzymes in SPs, namely GAPDH, bind with fish myofibrils, limiting proper myofibrillar cross-linking (Jafarpour and Gorczyca, 2012). For this reason, SPs are typically removed during surimi production by washing. Recently, several studies have revealed that SPs positively contribute to the gelation of myofibrillar proteins. Morioka and Shimizu (1993) found that gels prepared from SPs with molecular mass of 94, 40, and 26 kDa displayed high gel strength. Jafarpour and Gorczyca (2009) reported that textural properties of threadfin bream surimi with added freeze-dried SPs powder from common carp (*Cyprinus carpio*) were improved. Thus far, it has not been well established why SPs exhibit gel-enhancing effect. Some studies have shown that endogenous transglutaminase (TGase) contained in SPs could be a major gel-promoting factor (Yongsawatdigul and Piyadhammaviboon, 2007). In addition, endogenous proteinase inhibitor(s) could also contribute to textural improvement of fish protein gels. Piyadhammaviboon and Yongsawatdigul (2010) reported that SPs from threadfin bream (*Nemipterus* spp.) exhibited inhibitory activity toward trypsin. A better understanding on the gel-enhancing role of SPs would lead to more effective strategies for recovery and utilization of SP as a functional ingredient. The objectives of this study were to investigate proteinase inhibitory activity of SP extracted from common carp (*C. carpio*). Moreover, the gel enhancing effect of common carp SP on the gel-forming ability of threadfin bream (*Nemipterus* spp.) surimi was elucidated.

4.3 Materials and methods

4.3.1 Chemicals

Casein was purchased from Sigma Chemical Co. (St Louis, MO, USA). Bovine serum albumin (BSA), trypsin from hog pancreas and L-tyrosine were purchased from Fluka (Buchs, Switzerland). Trichloroacetic acid (TCA) was purchased from BDH Chemicals Ltd (Poole, UK). Egg white power (EW) was purchased from Igrecha (Seichessur Le Loir, France).Whey protein concentrate (WPC) (PSMD 505) was obtained from Arla Food Ingredient (Tokyo, Japan). Soy protein isolate (SPI) was purchased from Solae (SURO EX45 IP, Solae c/o DuPont China Holding Co., Ltd., Shanghai, China). Reagents used for gel electrophoresis were purchased from Promega (Madison, WI, USA). Other chemicals and reagents used were of analytical grade.

4.3.2 Fish

Common carp (*Cyprinuscarpio*) with the size of 2-2.5 kg were purchased from the freshwater fish wholesale market, Nakhon Ratchasima, Thailand. Fish were transported to Suranaree University of Technology laboratory in polystyrene foam boxes filled with ice within 30 min. Fish were manually eviscerated and deskinned immediately upon arrival. Fish fillets were ground using a meat grinder (Model 8-22, Marblehead, Ohio, USA) with a screen size of 1.5-mm perforation plate. Ground samples were vacuum-packed in a plastic bag and kept at -20 °C. Frozen threadfin bream (*Nemipterus* spp.) surimi was obtained from Andaman Surimi Industry, Samutsakorn province. The frozen surimi was vacuum-packed kept at -20 °C and used within 6 months.

4.3.3 Preparation of sarcoplasmic proteins

Frozen common carp mince was thawed under running tap water (25°C) for about 20 min. The samples were homogenized using an IKA homogenizer (IKA Works Asia, Bhd, Malaysia) with distilled water (4°C) at a mince to water ratio of 1:3 (w/w) and continuously stirred for 5 min. The homogenate was filtered through three layers of cheesecloth. Subsequently, the sample was centrifuged at 10000xg) Sorvall Legend MACH 1.6/R, Thermo Electron LED GmbH, Lengensellbold ,Germany) for 20 min at 4°C. The supernatant was kept in a refrigerator (4 °C) overnight, leading to the formation of the floating fat on the surface. The crystallized fat was manually skimmed off and the remaining supernatant was referred to as sarcoplasmic protein (SP). Protein contents of SP were approximately 11 $\pm$ 2 mg/mL.

4.3.4 Proteinase inhibitory activity assay

Trypsin inhibitory activity of EW, WPC, SPI and SP was assayed using casein as a substrate according to the modified method of An, Weerasinghe, Seymour, and Morrissey (1994). The reaction mixture (500 µL) consisted of 0.1 mM Tris-HCl (pH 8), 100 µL of trypsin solution (0.4 mg/mL) and 2.2 mg protein of EW, WPC, SPI or SP. After pre-incubation at either 40 or 65 °C for 10 min, 200 µl of 1% (w/v) casein solution in 0.1 mM Tris-HCl, pH 8 was added and the reaction was further incubated at the respective temperatures for 1 h. The reaction was stopped by adding 400 µL of cold 50% trichloroacetic acid (TCA). Blanks were carried out in the same manner, except the substrate solution was added after addition of 50% TCA. The mixture was centrifuged at 10000xg (Sorvall Legend Micro 17R, Thermo Electron LED GmbH, Lengensellbold ,Germany) for 10 min at 4 °C. TCA-soluble oligopeptide content in the supernatant was analyzed by Lowry method (Lowry,

Rosebrough, Farr, and Randall, 1951) using tyrosine as a standard. Degree of inhibition (%) was calculated as follows:

$$\text{Inhibition} = \frac{(C_0 - C_b) - (I_0 - I_b)}{(C_0 - C_b)} \text{x}100$$

Where C_0 is TCA-soluble oligopeptide content of control without protein additives (EW, WPC, SPI or SP). C_b is TCA-soluble oligopeptide content of control blank, I_0 is TCA-soluble oligopeptide content of sample with protein additive, and I_b is TCA-soluble oligopeptide content of sample blank.

4.3.5 Inhibitory activity staining

Inhibitory activity staining was conducted under non-reducing conditions according to the method of Garcia-Carreno, Dimes, and Haard (1993) with slight modifications. Inhibitory activity staining of EW, WPC, SPI and SP against trypsin was compared. The samples were separated in 4 % stacking gel and 10 % running gel in the presence of 0.12% casein. Several proteins were mixed with the treatment buffer in the absence of β-mercaptoethanol. Thirty micrograms of each protein were loaded. Gels were run at a constant voltage setting of 120 V on ice. After electrophoresis, gels were washed with 2.5% Triton X-100 for 15 min to remove SDS and then washed with deionized water. The gels were incubated with 50 mL of trypsin solution (0.4 mg/mL) at either 40 or 65 °C for 1 h and then rinsed with deionized water twice. Gels were stained in 0.125% Coomassie Brilliant Blue R-250 and destained in a solution containing 25% ethanol and 10% acetic acid. Proteins exhibiting trypsin inhibitory activity appeared as blue bands on a clear background.

4.3.6 Thermal stability

The effect of temperature on trypsin inhibitory activity of SP was investigated by pre-incubating SP at various temperatures of 35, 40, 45, 50, 55, 60, 65 and 70 °C for 10 min. Subsequently, samples were immediately cooled in iced water for 10 min. The residual trypsin inhibitory activity was determined at 40 °C for 60 min using casein as a substrate as described above. Remaining inhibitory activity was calculated by taking the activity of SP without incubation as 100%.

Thermal stability at 40 °C was also conducted by incubating SP at 40 °C for 60 min. Residual trypsin inhibitory activity was determined at various time intervals. Remaining inhibitory activity was calculated by taking inhibitory activity of sample without incubation as 100%.

4.3.7 Effect of SP on surimi gelation

Threadfin bream surimi pastes were prepared to obtain final moisture content of 80% and 2% NaCl, with SP addition of 0.18 g protein/100 g total weight and various $CaCl_2$ concentrations (0, 0.1, 0.3, and 0.5%). Surimi pastes were prepared using a mortar and pestle. The pastes were filled into a microplate with a diameter of 5 mm and depth of 10 mm. The filled microplates were then vacuum-packed and pre-incubated at 25 °C for 2 h, 40 °C for 30 min, or 65 °C for 30 min prior to heating at 90 °C for 30 min. Samples directly heated at 90 °C for 30 min was used as the control. Subsequently, samples were cooled in iced water for 20 min and stored overnight in a refrigerator (4 °C). The chilled gels were equilibrated at room temperature for 2 h before texture measurement using a Texture Analyzer (TA-XT2 Stable Micro System, Surrey, U.K.). Breaking force (g) and deformation (mm) were determined using a 2-mm cylindrical probe. Penetration test was achieved at a test

speed of 1 mm/s. For each treatment, mean values of breaking force (g) and deformation (mm) were obtained from at least eight measurements.

TCA-soluble oligopeptide contents of surimi samples were determined according to Yongsawatdigul and Piyadhammaviboon (2004). Two grams of surimi samples were added 18 mL of cold 5% trichloroacetic acid (TCA) solution. The mixture was homogenized for 1 min using an IKA homogenizer (IKA Works Asia, Bhd, Malaysia). The homogenate was centrifuged at 10000xg (Sorvall Legend Micro 17R, Thermo Electron LED GmbH, Lengensellbold ,Germany) for 10 min at 4°C. The supernatant was analyzed for TCA-soluble oligopeptide peptides content by Lowry method (Lowry et al, 1951) using L-tyrosine as a standard. TCA-soluble oligopeptide content was expressed as nmol of tyrosine/g sample.

4.3.8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of surimi gels were evaluated using SDS-PAGE (Laemmli, 1970). Samples were solubilized in 5% SDS solution according to the method of Yongsawatdigul and Park (2004). Thirty micrograms of proteins were loaded on polyacrylamide gel (4% stacking gel and 10% running gel). Gels were run at a constant voltage setting of 120 V. Gels were stained with 0.125% Coomassie Brilliant Blue R-250 and destained in a solution containing 25% ethanol and 10% acetic acid.

4.3.9 Statistical analyses

All experiments were performed in duplicate. Data were analyzed for the degree of variation and significance of difference using an analysis of variance (ANOVA) and differences among treatment means were evaluated by Duncan's

multiple range test (DMRT). The statistical analysis was performed using SPSS Statistic Program Version 14.0 (SPSS Inc, Chicago, IL, USA). Significance of differences was defined at $P < 0.05$.

4.4 Results and discussion

4.4.1 Inhibitory activity of common carp SP

On the basis of these preliminary studies, trypsin was strongly inhibited by common carp SP, whereas α-chymotrypsin and papain were slightly inhibited. Therefore, trypsin was selected as a target enzyme. Activity of trypsin at either 40 or 65 °C was comparable ($P > 0.05$). EW showed the highest trypsin inhibitory activity of 94% and 78% at 40 and 65 °C, respectively ($P < 0.05$, Figure 4.1). WPC inhibited trypsin to a lesser extent than EW, but to a similar degree with SPI at both temperatures ($P > 0.05$). EW is known to be a highly specific serine proteinase inhibitor, whereas WPC was a more effective inhibitor for cysteine proteinases than serine proteinases (Weerasinghe, Morrissey, Chung, and An, 1996). Typically, trypsin inhibitors remained in SPI with lower amounts than in raw soybean (Baker and Rackis, 1986). Inhibitory activity of SP was lower than that of EW at both temperatures, but was comparable to that of WPC and SPI at 40 °C. For all studied inhibitors, inhibition ability was higher at 40 °C than at 65 °C. A decrease of inhibitory activity at high temperature implied that inhibitors might undergo thermal denaturation during thermal treatment. Several endogenous proteinase inhibitors have been reported from various parts of fish. Nagashima, Takeda, Ohta, Shimakura, and Shiomi (2004) reported that skin mucus of pufferfish, *Takifugu pardalis*, contained 2 trypsin inhibitors with molecular mass of 57 and 47 kDa. Li, Lin, and Kim (2008)

purified a cysteine proteinase inhibitor from chum salmon (*Oncorhynchus keta*) plasma, which presumably was a glycoprotein and classified as a kininogen. A cysteine proteinase inhibitor in glassfish (*Liparis tanakai*) eggs was classified as a member of the family I cystatins (Ustadi, Kim, and Kim, 2005). These results demonstrated that SP from common carp contained trypsin inhibitors.

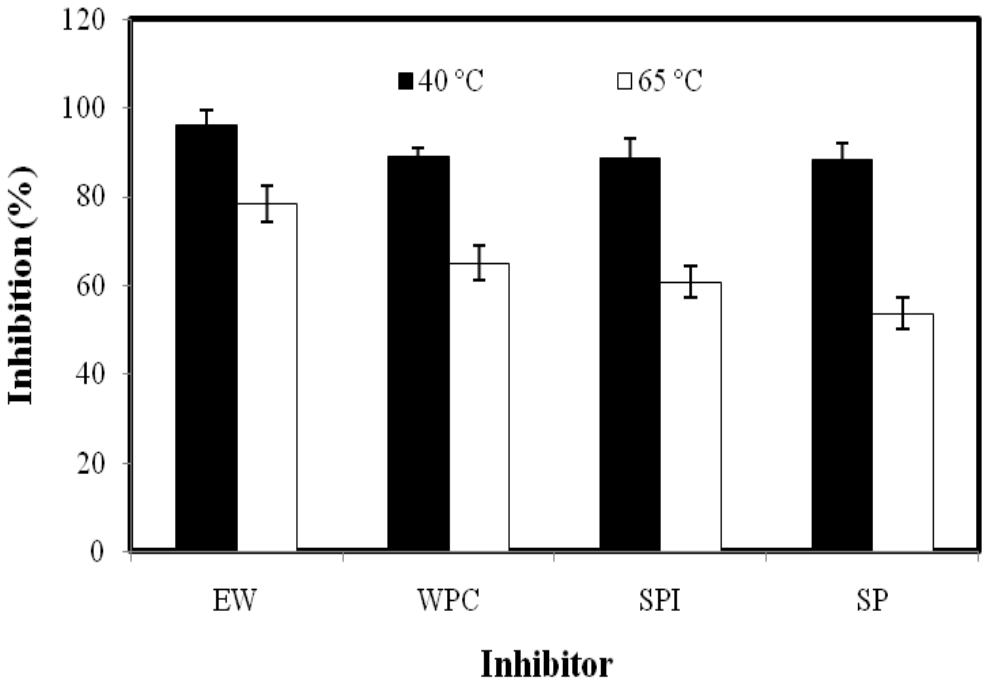

Figure 4.1 Trypsin inhibitory activity of various proteins at 40 and 65 °C. EW, egg white powder; WPC, whey protein concentrate; SPI, soy protein isolate; SP, sarcoplasmic protein.

4.4.2 Inhibitory activity staining

SP contained various proteins with molecular mass ranging from 23 to 150 kDa under nonreducing condition (Figure 4.2a). SP exhibited an effective trypsin inhibition at both 40 and 65 °C as judged by retention of blue bands on inhibitory

activity staining (Figure 4.2b and c). At 40 °C, trypsin inhibitors with molecular mass of 35, 41, 47, 52, and 69 kDa were evident (Figure 4.2b). In contrast, only 2 predominant protein bands with molecular mass of 35 and 54 kDa appeared at 65 °C (Figure 4.2c). The decrease in trypsin inhibition at 65 °C observed in Figure 4.1 was probably caused by thermal denaturation of potential inhibitors with molecular mass of 41, 44, and 50 kDa. These results implied that inhibition of SP at 40 and 65 °C was the action of different proteins. A trypsin inhibitor purified from lizardfish (*Saurida wanieso*) showed molecular mass of 50 kDa (Cao et al., 2001). Choi, Park, and Kim (2002) reported that molecular mass of the purified trypsin inhibitor from egg of skipjack tuna (*Katsuwonus pelamis*) was 78 kDa as estimated by gel filtration and 39 kDa by SDS-PAGE.

At 40 °C, EW, WPC, and SPI showed inhibitory activity against trypsin. Weerasinghe et al. (1996) reported that purified ovomucoid showed molecular mass of 65.6 kDa under nonreducing condition. A 60-kDa protein corresponding to ovomucoid was evident after being subjected to trypsin hydrolysis at 40 and 65 °C (Figure 3.2b). However, intensity of ovomucoid at 65 °C was lower than that at 40 °C. This was probably due to thermal denaturation as the onset temperature of ovomucoid was reported to be 66.37 °C (Julia et al., 2007). Major whey protein components are α-lactalbumin, β-lactoglobulin, and bovine serum albumin with molecular mass around 14, 18, and 66 kDa, respectively (Gezimati, Creamer, and Singh, 1997). Two protein bands of WPC appeared with molecular mass of approximately 148 and 48 kDa on trypsin inhibitory activity staining. It is postulated that the protein band with molecular mass of about 48 kDa might be bovine serum albumin. Bovine serum albumin did not directly inhibit trypsin, although it appeared

on trypsin inhibitory activity staining in earlier work (Piyadhammaviboon and Yongsawatdigul 2010). This was probably because bovine serum albumin contains a rigid structure with 17 disulfide bonds and is less susceptible to trypsin hydrolysis. Moreover, Weerasinghe et al. (1996) found that bovine serum albumin did not interact with active sites of trypsin and was not hydrolyzed by proteinases.

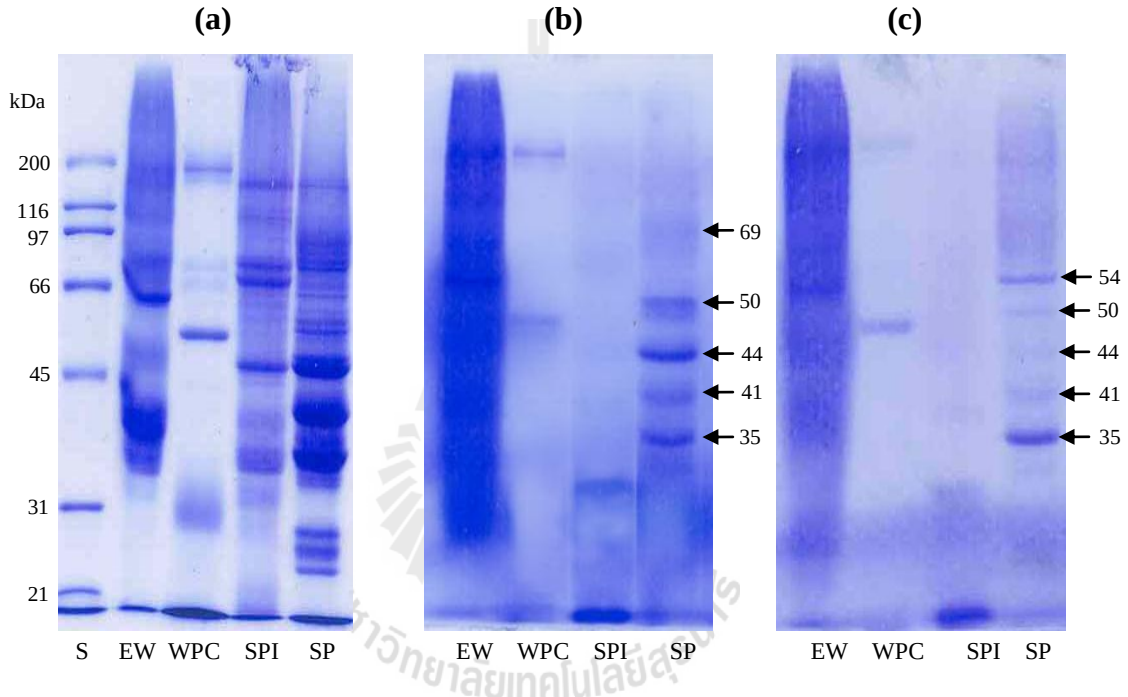

Figure 4.2 SDS-PAGE patterns on 10% polyacrylamide gel stained with Coomassie blue (a) and trypsin inhibitory activity at 40 °C (b) and 65 °C (c) under non-reducing condition. S, standard markers; EW, egg white power; WPC, whey protein concentrate; SPI, soy protein isolate; SP, sarcoplasmic proteins.

The protein band with molecular mass of 148 kDa might be a trypsin inhibitor in WPC. Weerasinghe et al. (1996) reported that WPC with a molecular

mass of approximately 101 kDa displayed inhibition toward papain and trypsin. Retention of protein bands with molecular mass of about 33 kDa appeared in SPI (Figure 4.2c). Baker and Rackis (1986) reported that SPI might contain trypsin inhibitor activity as high as 40% of that found in raw soybeans. A 33-kDa protein was presumed to be a Kunitz inhibitor whose molecular mass was reported to be more than 20 kDa under nonreducing condition by Garcia-Carreno, Toro, Diaz-Lopez, Hernandez-Cortez, and Ezquerra (1996).

4.4.3 Thermal stability

Trypsin inhibitory activity of SP increased with temperature up to 60 °C and markedly decreased at higher temperatures (Figure 4.3a). SPs were likely to undergo thermal denaturation at temperature greater than 60 °C. This result explains a reduced inhibitory activity toward trypsin at 65 °C (Figure 4.1). Proteinase inhibitor from chum salmon (*O. keta*) egg showed low thermal stability at 20 to 40 °C (Kim, Ustadi, and Kim, 2006). The remaining cysteine inhibitory activity from Atlantic salmon (*Salmo salar* L.) skin was observed after incubation at 80 °C (Ylonen et al., 1999). Ustadi, Kim, and Kim (2005) reported that remaining inhibitory activity of glassfish (*L. tanakai*) egg inhibitor toward papain at 65 and 80 °C was 60.8% and 40.1%, respectively.

As the gel-setting process for tropical surimi is typically conducted at a relatively high temperature of 40 °C to enhance gelation, thermal stability of trypsin inhibitory activity of common carp SP was investigated. Trypsin inhibitory activity retained approximately 88% when incubated at 40 °C for 60 min (Figure 3.3b). This result indicated a relatively high thermal stability of common carp SP at 40 °C.

Cysteine proteinase inhibitor from chum salmon (*O. keta*) plasma retained 70% of its inhibitory activity after being pre-incubated at 50 °C for 30 min (Li et al., 2008).

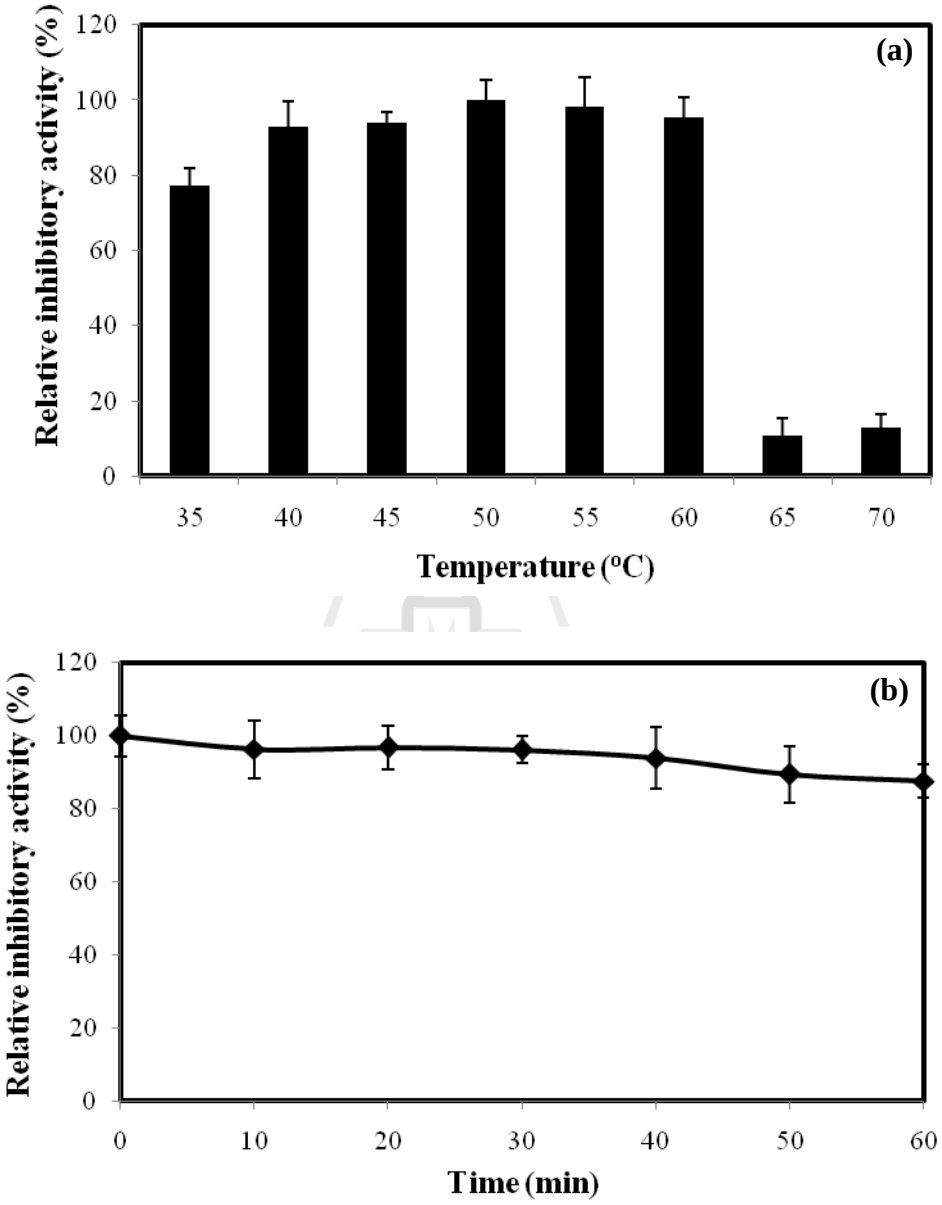

Figure 4.3 Effect of temperature on trypsin inhibitory activity of SP (a), and stability of trypsin inhibitory activity of SP at 40 °C (b).

4.4.4 Inhibition of proteolysis

Gel-enhancing effect of Ca^{2+} varies with surimi species and concentration of Ca^{2+} (Park, 2005). In the absence of SP and Ca^{2+}, threadfin bream surimi pre-incubated at 25 and 40 °C showed lower TCA-soluble oligopeptide contents than at 65 °C ($P < 0.05$, Figure 4.4). Endogenous proteinases in threadfin bream were heat-activated serine proteinases (Toyohara and Shimizu, 1988; Kinoshita, Toyohara, and Shimizu, 1990). In a direct heating process at 90 °C, low TCA-soluble oligopeptide contents were observed due to thermal inactivation of endogenous proteinases. When SP was added, about 40% autolytic inhibition was observed at both temperatures regardless of Ca^{2+} addition (Figure 4.4).

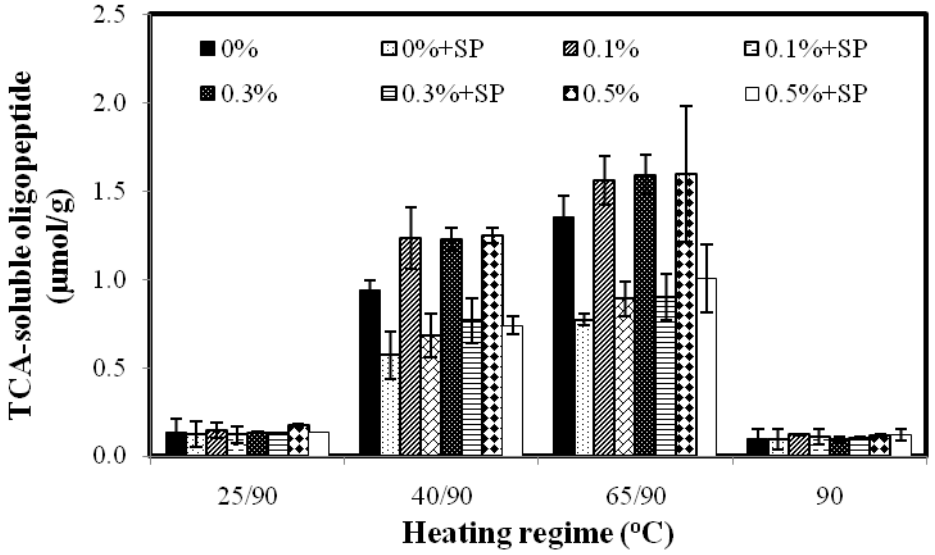

Figure 4.4 TCA-soluble oligopeptide contents of threadfin bream surimi mixed with common carp sarcoplasmic proteins (0.18 g protein/100 g total weight) and subjected to various heating conditions. SP, sarcoplasmic protein; numbers indicate addition level of $CaCl_2$.

Addition of 0.1% to 0.5% Ca^{2+} appeared to increase proteolysis of threadfin bream surimi. It is likely that endogenous proteinases of threadfin bream are activated by $Ca^{2+.}$ Similar results were also observed with goatfish surimi (Benjakul, Yarnpakdee, Visessanguan, and Phatcharat, 2010). The results suggest that common carp SP can inhibit proteolytic activity in threadfin bream surimi at both 40 and 65 °C.

4.4.5 Effect of SP on gel properties of surimi

In the absence of SP and Ca^{2+} textural properties of threadfin bream surimi gels were highest when pre-incubated at 40 °C for 30 min ($P < 0.05$, Figure 4.5). Textural improvement with pre-incubation at 40 °C could also be partly attributed to endogenous transglutaminase (TGase) activity (Yongsawatdigul, Worratao, and Park, 2002). Textural properties of surimi gel were lowest when pre-incubated at 65 °C ($P < 0.05$), corresponding to its high TCA-soluble oligopeptide content (Figure 4.4). Addition of 0.1% Ca^{2+} in the absence of SP under preincubation at 40 °C resulted in improvement of breaking force (Figure 4.5a) despite an increased TCA-soluble oligopetide content (Figure 4.4). However, addition of 0.1% Ca^{2+} did not further improve textural properties of threadfin bream surimi gels treated under other heating regimes. Fish endogenous TGase is a Ca^{2+}- dependent enzyme and shows the highest catalytic activity at 50 °C for threadfin bream liver (Hemung and Yongsawatdigul, 2008) and 37 to 50 °C for tilapia (Worratao and Yongsawatdigul, 2005).

Addition of 0.1% Ca^{2+} probably activated endogenous TGase activity at 40 °C. Protein cross-linking mediated by TGase and degradation of myofibrillar proteins induced by Ca^{2+}-activated proteinases were likely to occur simultaneously during pre-incubation at 40 °C, but overall textural properties were governed by the

action of endogenous TGase as the extent of proteolysis at 40 °C was limited. Moreover, Ca^{2+} directly induced the unfolding of both myosin and actin, promoting hydrophobic interactions and disulfide linkages, which would occur to a greater extent at 40 °C than at 25 °C (Hemung and Yongsawatdigul, 2005). Textural properties of surimi gels did not improve at higher Ca^{2+} addition of 0.3 and 0.5% under all heating regimes applied. Besides the action of Ca^{2+}-activated proteinases, high concentrations of Ca^{2+} might interfere with excessive conformational changes and impede a proper gel network formation of myosin.

When SP was added to surimi alone, breaking force of gels pre-incubated at 40 and 65 °C increased approximately to 58.8% and 104.9%, whereas deformation increased to around 18.6% and 36.2%, respectively. These results were in agreement with the low TCA-soluble oligopeptide content (Figure 4.4), confirming the inhibitory activity of common carp SP toward threadfin bream proteolysis. Yongsawatdigul and Piyadhammaviboon (2007) reported that SP from tilapia (*Oreochromis niloticus*) enhanced the textural quality of lizardfish (*Saurida* spp.) surimi by the action of endogenous TGase. The common carp SP used in this study exhibited TGase activity of 41.6 units/mL, which was lower than that reported in tilapia by Yongsawatdigul and Piyadhammaviboon (2007). Therefore, TGase contributed to the gel-enhancing effect of common carp SP to a lesser extent. Addition of SP had no enhancing effect on gels pre-incubated at 25 °C and gels heated directly at 90 °C. The latter 2 conditions did not allow endogenous proteinases to be fully active as seen by relatively low TCA soluble oligopeptide content (Figure 4.4). This also implied that the gel-forming ability of SP itself was unlikely to be the main reason for its gel-enhancing effect.

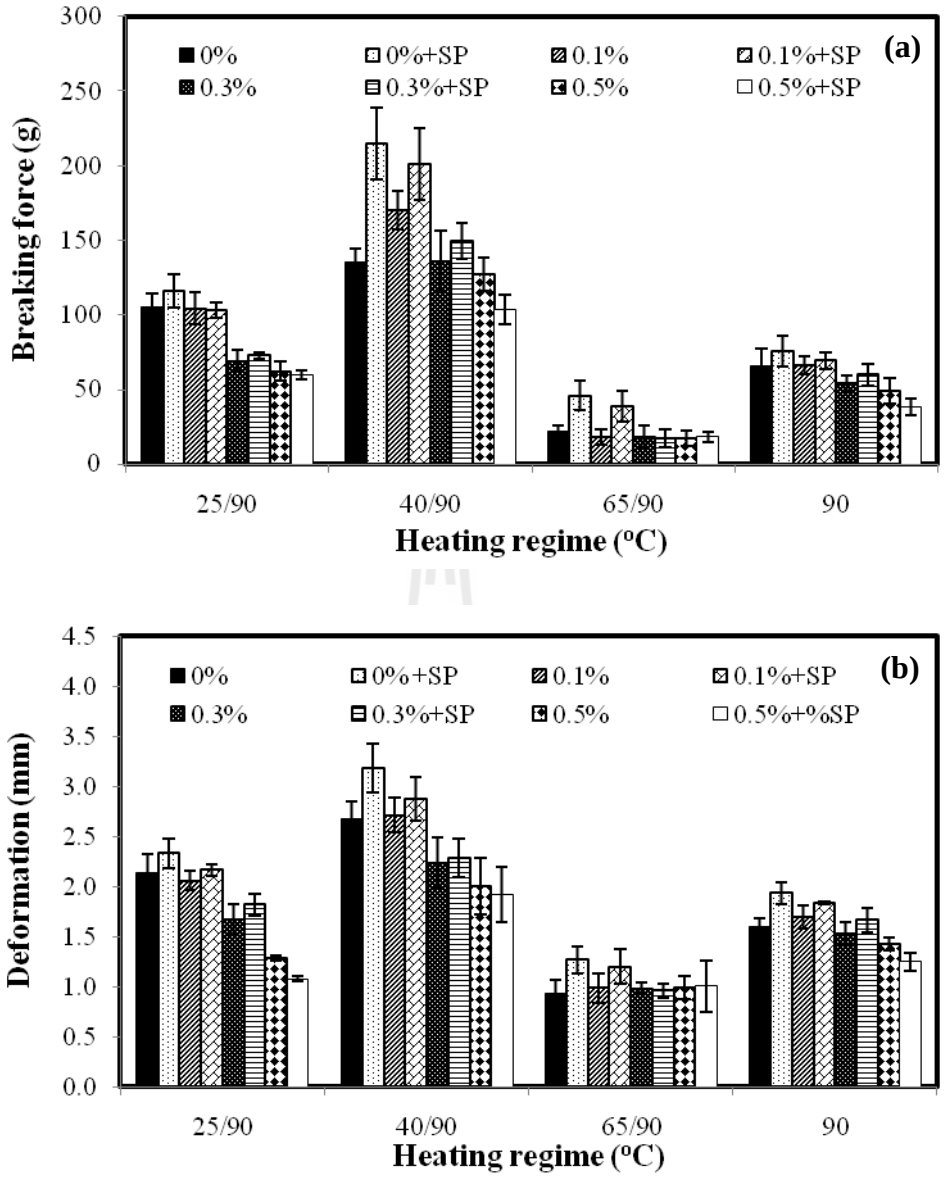

Figure 4.5 Breaking force (a) and deformation (b) of threadfin bream surimi mixed with common carp sarcoplasmic proteins (0.18 g protein/100 g total weight) and subjected to various heating conditions. SP, sarcoplasmic protein; numbers indicate addition level of $CaCl_2$.

At 40 °C-pre-incubation, the extent of gel improvement by addition of SP at 0.18 g protein/100 g alone was greater than the addition of 0.1% Ca^{2+} (Figure

4.5). The combined addition of SP and 0.1% Ca^{2+} showed lower textural properties than SP alone ($P < 0.05$, Figure 4.5). The lack of synergism between SP and Ca^{2+} implied that contribution of protein cross-linking catalyzed by endogenous TGase is less important than the integrity of muscle protein resulting from inhibition of endogenous proteolysis. These results indicate that addition of common carp SP at 0.18 g protein/100 g alone was sufficient for the textural properties of threadfin bream surimi when pre-incubated at either 40 or 65 °C.

4.4.6 SDS-PAGE patterns

Severe degradation of myosin heavy chain (MHC) occurred when threadfin bream surimi was pre-incubated at 40 and 65 °C (Figure 4.6). Loss of actin and tropomyosin was also observed at 65 °C (Figure 4.6a). Degradation of myofibrillar proteins along with high TCA-soluble oligopeptide and low textural properties of gels pre-incubated at 65 °C clearly illustrated the evidence of proteolysis caused by endogenous proteinases at this temperature. When SP was added, retention of MHC, actin, and tropomyosin was observed. This was concomitant with lower TCA-soluble oligopeptide content and improved textural properties of gels. These results confirm that SP exhibited inhibitory activity against proteolysis of major myofibrillar proteins of threadfin bream surimi both in the absence and presence of 0.1% Ca^{2+}. Significant loss of myofibrillar proteins was also observed in samples pre-incubated at 40 °C, but with slightly different patterns to those pre-incubated at 65 °C (Figure 4.6b). Only degradation of MHC occurred, but not actin and tropomyosin. Loss of MHC in this condition could have arisen for 2 reasons. First, MHC was hydrolyzed by endogenous proteinases, which occurred to a lesser extent than at 65 °C. Second, a decrease was due to a crosslinking reaction catalyzed by endogenous

TGase. However, the cross-linked proteins were not distinctively observed, confirming a minimal cross-linking reaction mediated by endogenous TGase. Addition of SP proved to reduce the loss of MHC caused by proteolysis. Due to such an inhibitory effect, a significant increase of textural properties was obtained when pre-incubated at 40 °C. The setting phenomenon has been known to improve textural properties of fish protein. Tropical fish require a higher setting temperature around 40 °C as their muscle proteins exhibit higher thermal stability (Lanier, Carvajal, and Yongsawatdigul, 2005). The efficacy of setting would be hampered by the presence of endogenous proteinases. Addition of a proteinase inhibitor would be a practical means to reduce proteolysis and attain the possible maximum gel strength induced by setting. Common carp SP offers several advantages compared to other commercial protein inhibitors. It is derived from fish, allowing compatibility with fish protein gel products. In addition, an effective concentration to reduce proteolysis is as low as 0.18%, whereas effective concentration of food-grade proteinase inhibitors ranges from 1% to 4% (Weerasinghe et al., 1996).

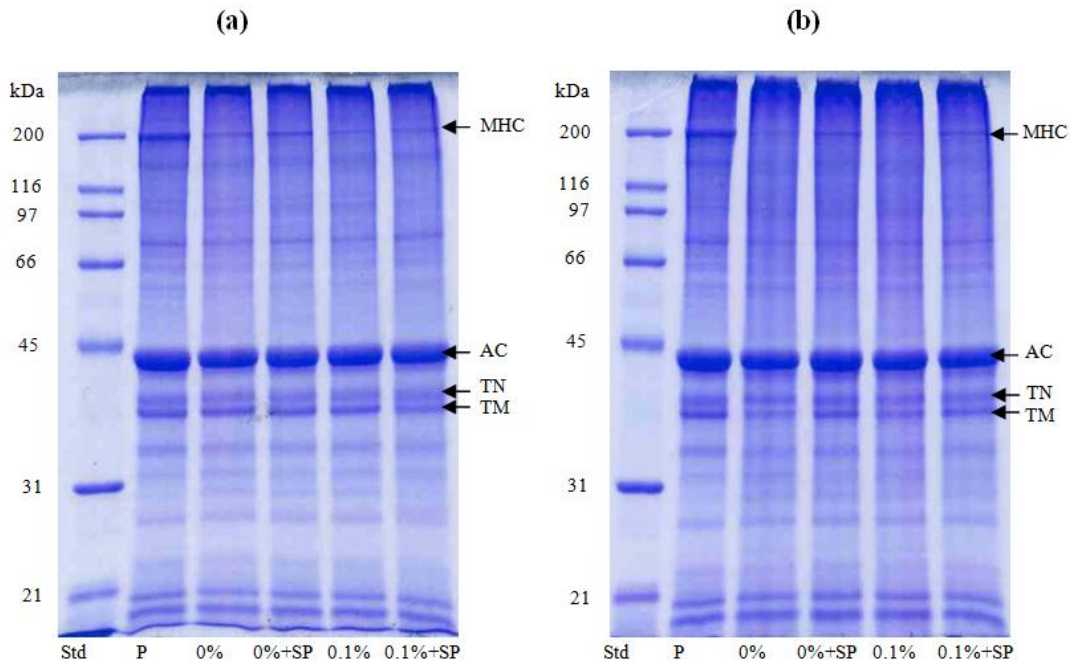

Figure 4.6 SDSGE (10% polyacrylamide) pattern of threadfin bream surimi with added sarcoplasmic protein (0.18 g protein/100 g total weight) and pre-incubated at 65 °C (a) and 40 °C (b) for 30 min, followed by heating at 90 °C for 10 min. Std, standard markers; P, raw paste; SP, sarcoplasmic protein; MHC, myosin heavy chain; AC, actin; TN, troponin; TM, tropomyosin; numbers indicate addition level of $CaCl_2$.

4.5 Conclusions

SP from common carp exhibited inhibitory activity against trypsin. Inhibitory activity of SP toward trypsin was lower than that of EW, but was comparable to that of WPC and SPI at 40 °C. Trypsin inhibitor activity staining of nonreducing SDS-PAGE revealed 5 protein bands with molecular mass of 35, 41, 44, 50, and 69 kDa with incubation at 40 °C. However, only 2 proteins were found with molecular mass

of 35 and 54 kDa when incubated at 65 °C. SP alone effectively reduced proteolysis of threadfin bream surimi and increased its textural properties to a greater extent than 0.1% Ca^{2+}. Common carp SP might be a potential alternative food-grade inhibitor applied to enhance textural properties of surimi.

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CHAPTER V

PURIFICATION AND IDENTIFICATION OF PROTEINASE INHIBITOR FROM COMMON CARP SARCOPLASMIC PROTEINS

5.1 Abstract

Proteinase inhibitor from common carp (*Cyprinus carpio*) sarcoplasmic proteins was purified using heat treatment, ammonium sulfate precipitation, anion exchange, affinity chromatography and gel filtration with a yield of 2.83% and purification fold of 129.23. The purified common carp proteinase inhibitor showed molecular mass of 47 (inhibitor I) and 52 (inhibitor II) kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing condition. The inhibitor I and II were considered to be alpha-1-proteinase inhibitor (α1-PI) based on LC-MS/MS. Isoelectric point (pI) of common carp inhibitor I and II were 5.3 and 5.4. The inhibitor I and II were glycoproteins and molecular mass after peptide-N-glycosidase F (PNGase F) treatment was 38 and 45 kDa, respectively with N-glycosylation sites of both inhibitors were determined to be at N214 and N226. The common carp α1-PI was stable up to 60 °C. A decrease of inhibitory activity was found with addition of 10-100 mM $CaCl_2$. Trypsin was strongly inhibited while α-chymotrypsin and papain were slightly inhibited by the common carp α1-PI. Moreover, common carp α1-PI effectively reduced autolytic degradation of bigeye snapper surimi by 0.025%.

5.2 Introduction

Sarcoplasmic protein is defined as a group of muscle proteins that exist in the fluids within and between muscle fibers and are soluble in water or low ionic strength solution (Park, 2009). Fish muscle protein is composed of 20-40% sarcoplasmic proteins, depending on species (Okada, 1999). Sarcoplasmic proteins contain many enzymes involved in muscle metabolism. Major sarcoplasmic proteins of fish were glycolytic enzymes, including phosphorylase, lactate dehydrogenase, enolase, creatine kinase, aldolase, and glyceraldehyde phosphate dehydrogenase (Scopes, 1970). In addition, proteinases can be prevalently found in sarcoplasmic fraction To maintain homeostasis of fish muscle proteins, the regulation of proteolytic activity in tissues by proteinase inhibitors is a prerequisite (Wojtczak, Całka, Glogowski, and Ciereszko, 2007).

Proteinase inhibitors found in sarcoplasmic protein fraction of threadfin bream and common carp were able to inhibit serine proteinase, especially trypsin (Piyadhammaviboon and Yongsawatdigul 2010; Siriangkanakun and Yongsawatdigul, 2012). The proteinase inhibitors that exhibit selective interaction of serine proteinases are referred to as serpins. The main serpin is alpha-1-proteinase inhibitor (α1-PI) that is found in blood plasma, seminal plasma, serum of mammalian and fish species (Mickowska, 2009; Wojtczak et al., 2007; Aranishi, 1999). Several proteinase inhibitors were found in fish muscle proteins. Cao et al., (2000) reported that white croaker (*Argyrosomus argentatus*) skeletal muscle contained a serine proteinase inhibitor with molecular mass of 55 kDa, which was identified to be phosphoglucose isomerase. A serine proteinase with molecular mass of 65 kDa was purified from white croaker (*Micropogon opercularis*) skeletal muscle (Sangorrín, Folco, Martone,

and Sánchez, 2001). Muscular proteinase inhibitors could have an important role in controlling proteolytic activity of fish muscle.

Common carp (*Cyprinus carpio*) is an economically important aquacultured fish around the world. Global aquaculture production for common carp in 2011 was estimated to be 3.7 million metric tons (FAO, 2013). Proteolysis of common carp muscle is relatively lower than other species. This could be due to lower endogenous proteolytic activity. In addition, natural proteinase inhibitor(s) could contribute to the limited proteolysis. Proteinase inhibitor from common carp muscle has not yet been identified and characterized. Recovery of a proteinase inhibitor from fish sarcoplasmic proteins could be a means to fully utilize fishery resource as a large amount of sarcoplasmic proteins is largely removed in surimi processing. To achieve efficient utilization of fish muscular proteinase inhibitor, its biochemical characteristics must be fully realized. Therefore, the objectives of this study were to purify and identify proteinase inhibitor from common carp (*Cyprinus carpio*) sarcoplasmic proteins and investigate the efficacy of the proteinase inhibitor in inhibiting the autolysis of bigeye snapper surimi.

5.3 Materials and methods

5.3.1 Chemicals

Casein was purchased from Sigma Chemical Co. (St Louis, MO, USA). Bovine serum albumin (BSA), trypsin from hog pancreas and L-tyrosine were purchased from Fluka (Buchs, Switzerland). Boc-Asp(oBzl)-ProArg-AMC was purchased from Bachem A.G. (Bubendorf, Switzerland). Diethylaminoethyl (DEAE)-Sephacel, Sephacryl S-300 and concanavalin A (Con A)-Sepharose were purchased

from GE Healthcare *(*Uppsala, Sweden*).* Peptide -*N*-Glycosidase F (PNGase F) was purchased from New England BioLabs (Hitchin, Hertfordshire, UK). Pepsin, thermolysin and α-chymotrypsin were purchased from Sigma Chemical Co. Papain was obtained from Enzybel International, S. A. (Liege, Belgium). Reagents used for gel electrophoresis were purchased from Promega (Madison, WI, USA). Other chemicals and reagents used were of analytical grade.

5.3.2 Preparation of sarcoplasmic proteins

Fresh common carp (*Cyprinus carpio*) were purchased from the freshwater fish wholesale market, Nakhon Ratchasima, Thailand. Fish were transported to Suranaree University of Technology laboratory in polystyrene foam boxes filled with ice within 30 min. Fish were approximately 2-2.5 kg each. Fish were degutted, deskinned, and filleted manually upon arrival. Fish fillets were ground using a meat grinder (Model 8-22, Marblehead, Ohio, USA) with a 1.5-mm perforation plate. The ground meat was homogenized with 3 volumes of 50 mM Tris-HCl (pH 7.5) using an IKA homogenizer (IKA Works Asia, Bhd, Malaysia) and continuously stirred for 5 min. The homogenate was filtered through three layers of cheesecloth, then centrifuged at 10000×g (Sorvall Legend MACH 1.6/R, Thermo Electron LED GmbH, Lengensellbold, Germany) for 20 min at 4°C. The supernatant was kept in a refrigerator (4 °C) overnight. The floated lipid layer was removed and the supernatant was referred to as sarcoplasmic protein. Protein contents of sarcoplasmic protein were approximately 11±2 mg/mL.

5.3.3 Purification

Sarcoplasmic proteins were heated at 60 ºC for 5 min, cooled in iced water immediately for 10 min, and centrifuged at 10000×g for 20 min at 4 °C.

Supernatant was collected and fractionated by 50-70% ammonium sulfate. Precipitated proteins were collected by centrifugation at 10000×g for 20 min at 4 °C. The precipitates were dissolved in 50 mM Tris-HCl, pH 7.5 and subsequently dialyzed overnight against 50 mM Tris–HCl, pH 7.5 using SnakeSkinTM pleated dialysis tubing with 10 kDa molecular weight cutoff (MWCO) (Pierce Chemical Co., Rockford, IL, USA). The dialysates were applied to a DEAE-Sephacel column equilibrated with 50 mM Tris–HCl, pH 7.5 and eluted using a linear gradient of 0-1.0 M NaCl, 50 mM Tris–HCl, pH 7.5. Five-ml fractions were collected at a flow rate of 1 mL/min. Fractions exerting trypsin inhibitory activity were pooled and concentrated using 10-kDa MWCO ultrafiltration membrane (Amicon, Billerica, MA, USA). The retentate was dialyzed against 50 mM Tris-HCl, pH 7.5 for 24 h and loaded onto a Con-A Sepharose column equilibrated with 50 mM Tris-HCl, pH 7.5 and then eluted with 0-0.5 M methyl-α-D-glucopyranoside in 50 mM Tris–HCl, pH 7.5. Fractions of 3 mL were collected at a flow rate of 0.3 mL/min. The pooled fractions containing trypsin inhibitory activity were concentrated using 10-kDa MWCO ultrafiltration membrane. The concentrated sample was applied to Sephacryl S-300 column equilibrated with 50 mM Tris–HCl, pH 7.5 and then eluted with the same buffer at a flow rate of 0.5 mL/min. Fractions containing trypsin inhibitory activity were pooled and used for characterization. Protein content was measured by Lowry method (Lowry, Rosebrough, Farr, and Randall, 1951) using bovine serum albumin as a standard.

5.3.4 Inhibitory activity assay

Trypsin inhibitory activity was determined according to the method of Barrett and Kirschke (1981) and Ishida, Sugiyama, Sato, and Nagayama (1995) with

slight modifications. The reaction mixture containing 0.7 mL of 50 mM Tris–HCl (pH 8), 0.1 mL of purified proteinase inhibitor (10 µg/mL) and 0.1 mL trypsin solution (0.01 mg/mL) was added and pre-incubated at 37 °C for 5 min. After pre-incubation, 0.1 mL of 10 µM of Boc-Asp(oBzl)-Pro-Arg-AMC was added and the reaction was further incubated at 37 °C for 10 min. The reaction was terminated by addition of 1.5 mL of stopping solution (methanol:n-butanol: deionized water = 35:30:35, v/v/v). Fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) was measured with a spectrofluorophotometer RF-1501 (Shimadzu Co., Kyoto, Japan) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. One unit of trypsin inhibitory activity was defined as inhibition of one unit of trypsin activity, whereas one unit of trypsin activity was defined as one nmol of AMC releases/min/mL.

5.3.5 Inhibitory activity staining

Inhibitory activity staining was conducted under a nonreducing condition according to the method of Garcia-Carreno, Dimes, and Haard (1993) with slight modifications. The samples were separated in 4% stacking gel and 10% running gel in the presence of 0.12% casein. Proteins were mixed with the treatment buffer in the absence of β-mercaptoethanol. Then, the samples were loaded onto the gel without boiling. The gel was run at a constant voltage setting of 120 V on ice. After electrophoresis, the gel was washed with 2.5% Triton X-100 for 15 min to remove SDS and washed with deionized water. The gel was incubated with 50 mL trypsin solution (0.4 mg/mL) at 37 °C for 1 h and then rinsed with deionized water twice. The gel was stained in 0.125% Coomassie Brilliant Blue R-250 and destained in a solution

containing 25% ethanol and 10% acetic acid. Trypsin inhibitors were indicated by blue bands on a clear background.

5.3.6 Protein identification by LC-MS/MS

5.3.6.1 Sample preparation

Purified trypsin inhibitor was separated on a 4-12% Bis-Tris Novex mini-gel (Invitrogen, Carlsbad, CA). Gel was stained by Coomasie brilliant blue R-250. The protein bands with molecular mass of 47 (inhibitor I) and 52 (inhibitor II) kDa, indicating trypsin inhibitory activity were excised. The gel pieces were washed with 25 mM NH_4HCO_3 followed by acetonitrile. The gels were reduced with 10 mM DTT at 60°C followed by alkylation with 50 mM iodoacetamide at room temperature. Subsequently, the samples were digested with trypsin (Promega, Madison, WI, USA) at 37°C for 4 h. and the supernatant was quenched with formic acid and analyzed directly without further processing.

5.3.6.2 Mass spectrometry

The gel digests were analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Orbitrap Velos Pro. Peptides were loaded on a trapping column and eluted over a 75 μm analytical column at 350 nL/min; both columns were packed with Jupiter Proteo resin (Phenomenex, CA, USA). The mass spectrometer was operated in data-dependent mode, with MS performed in the Orbitrap at 60,000 full width at half maximum resolution and MS/MS performed in the linear trap quadrupole. The fifteen most abundant ions were selected for MS/MS. Data were searched using a local copy of Mascot with the following parameters: enzyme (trypsin); database (SwissProt all kingdoms); fixed modification (carbamidomethyl); variable modifications (oxidation, acetyl,

deamidation, pyro-glu); mass values (monoisotopic); peptide mass tolerance (10 ppm); fragment mass tolerance (0.8 Da); max missed cleavages (2). Mascot DAT files were parsed into the Scaffold software for validation, filtering and to create a nonredundant list per sample. Data were filtered using a minimum protein value of 99%, a minimum peptide value of 50% (Prophet scores) and requiring at least two unique peptides per protein.

5.3.7 Isoelectric point determination

The isoelectric point (pI) of the purified proteinase inhibitors was determined using PhastSystem Electrophoresis System with PhastGel isoelectric focusing (IEF) 3-9 (GE Healthcare, Uppsala, Sweden). One microliter of the purified trypsin inhibitor was applied to the PhastGel 3/9 IEF gels as described by the manufacturer. The broad range IEF mix 3.6 - 9.3 (Sigma Aldrich) was used. Protein bands were visualized by silver staining in the development unit of the PhastSystem, following the instruction given by GE Healthcare for IEF analysis. Calibration proteins were trypsinogen (9.3), lectin (8.2, 8.6, 8.8), myoglobin (6.8, 7.2), carbonic anhydrase isozyme I (6.6), carbonic anhydrase isozyme II (5.9), β-lactoglobulin A (5.1), trypsin inhibitor (4.0), and amyloglucosidase (3.6).

5.3.8 Enzymatic deglycosylation

Inhibitor I and II were treated with peptide -*N*-glycosidase F (PNGase F) (New England Biolabs, Hitchin, Hertfordshire, UK) according to manufacturer's instructions. PNGase F-treated and untreated samples were separated on a 4-12% Bis-Tris Novex mini-gel (Invitrogen, Carlsbad, CA). The gel was stained with Coomassie Brilliant Blue R-250. Protein bands were excised. Each protein band was reduced,

alkylated and digested as described in section 5.3.6.1. Digested peptides were analyzed by nano LC/MS/MS as described earlier (section 5.3.6.2).

5.3.9 Thermal stability

Thermal stability of purified proteinase inhibitor was determined by pre-incubating the purified proteinase inhibitor for 10 min at different temperatures ranging from 30 to 90 °C. Subsequently, samples were immediately cooled down in iced water for 10 min. The residual trypsin inhibitory activity was determined at 37 °C for 10 min as described above. Remaining inhibitory activity was expressed as the relative inhibitory activity, compared with that of the untreated sample.

5.3.10 Effect of $CaCl_2$ on trypsin inhibitory activity

Purified proteinase inhibitor (10 µg/mL) was incubated in 50 mM Tris-HCl (pH 7.5) containing various $CaCl_2$ concentrations (0-100 mM) at 40 °C for 30 min. The residual inhibitory activity against trypsin was then determined as described above. Remaining trypsin inhibitory activity was calculated by taking the inhibitory activity of purified proteinase inhibitor without $CaCl_2$ as 100%.

5.3.11 Inhibitory activity towards various proteinases

The purified trypsin inhibitor was examined for inhibitory activity against trypsin, α-chymotrypsin, thermolysin, papain and pepsin. All proteinases except pepsin were determined using azocasein as a substrate while hemoglobin was used for pepsin. Purified proteinase inhibitor (100 µg/mL) was mixed with 0.6 mL of 50 mM Tris-HCl (pH 7.5) for trypsin, chymotrypsin and thermolysin, 2 mM DTT in 50 mM Tris-HCl (pH 7.5) for papain, and 50 mM glycine-HCl buffer (pH 2.0) for pepsin. All samples were pre-incubated for 5 min prior to the addition of 0.2 mL of 1% substrate (azocasein or hemoglobin). After 1-h incubation, 0.5 mL of cold 50%

TCA was added and then the precipitate was removed by centrifugation at 10000xg for 10 min. Supernatants were added to 0.1 mL 10 N NaOH and the absorbance measured at 450 nm. For pepsin, TCA-soluble oligopeptide content in the supernatant was analyzed by Lowry method (Lowry et al., 1951) using tyrosine as a standard. Control samples were carried out in the same manner, except the substrate solution was added after addition of 50% TCA.

5.3.12 Inhibition of autolysis

Degradation of muscle proteins in bigeye snapper surimi was evaluated using SDS-PAGE (Laemmli, 1970). Frozen bigeye snapper surimi without addition of egg white powder and other protein additives were obtained from Andaman Surimi Industry (Samutsakorn, Thailand). Surimi blocks were kept at -20 ºC. Bigeye snapper surimi was mixed with purified proteinase inhibitor at a level of 0.25, 0.5 and 1 mg protein/100 g total weight and incubated at either 40 or 65 °C for 1 h. The reaction was stopped by adding 18 mL of 5% hot sodium dodecylsulfate (SDS) solution, homogenized (IKA Works Asia, Bhd, Malaysia), and heated at 90 °C for 30 min. Then, samples were centrifuged at 10000×g (Centrifuge 5415D, Eppendorf, Hamburg, Germany) for 10 min at room temperature. The supernatant was mixed with the treatment buffer containing 100 mM ß-mercaptoethanol in a 1:1 volume ratio and boiled for 5 min. Thirty micrograms of proteins were loaded on polyacrylamide gel (4% stacking gel and 10% running gel) and running at a constant voltage setting of 120 V. Gels were stained in 0.125% Coomassie Brilliant Blue R-250 and destained in a solution containing 25% ethanol and 10% acetic acid.

5.3.13 Statistical analyses

All experiments were conducted in duplicate. Data were analyzed for degree of variation and significance of difference using an analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was applied to determine differences ($P < 0.05$) between treatment means. The statistical analysis was performed using SPSS Statistic Program Version 17.0 (SPSS Inc., Chicago, Ill., U.S.A.).

5.4 Results and discussion

5.4.1 Purification

The purification of common carp proteinase inhibitor is summarized in Table 5.1. After heat treatment and ammonium sulfate precipitation, purity increased by 7.01 folds. Trypsin inhibitor was found in the bound fraction of DEAE-Sepharose (Figure 5.1a). Other proteins were largely removed by this technique, resulting in a 23-fold increase (Table 5.1). The trypsin inhibitor showed strong affinity to Con A-Sepharose, showing a glycoprotein characteristic. This step effectively captured the trypsin inhibitor as evidenced by extremely high purity of 107 folds. Gel filtration with Sephacryl S-300 removed some larger proteins, resulting in 129-fold increase with 2.8% yield. Aranishi (1999) isolated a trypsin inhibitor from carp serum with a purity of 17-fold and 3% recovery, while a purity of 73.4-fold and a yield of 20.4% were obtained from a serine protein inhibitor from rainbow trout seminal plasma (Mak et al., 2004).

5.4.2 Molecular mass and inhibitory activity staining

Crude sarcoplasmic proteins contained various proteins with their molecular mass ranging from 23 to 150 kDa (Figure 5.2a). Fish sarcoplasmic proteins comprise of several types of proteins including enzymes and heme proteins (Lanier, Carvajal, and Yongsawatdigul, 2005). After various purification steps, 2 protein bands with molecular mass of 47 (inhibitor I) and 52 (inhibitor II) kDa estimated by SDS-PAGE under nonreducing condition were observed (Figure 5.2a). The same result was obtained under reducing condition (data not shown), suggesting that they are not subunits linked by disulfide linkages. After inhibitory activity staining, two inhibitory zones corresponding to the molecular mass of 47 and 52 kDa were also detected on the gel under nonreducing condition (Figure 5.2b).

Table 5.1 Purification table of purified proteinase inhibitor from common carp sarcoplasmic proteins.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purity (fold)	Yield (%)
Crude extract	2056.41	1380.21	0.67	1	100
Heat treatment	916.85	1037.26	1.13	1.69	75.15
$(NH_4)_2SO_4$ precipitation	182.36	858.29	4.71	7.01	62.19
DEAE-Sephacel	10.37	159.25	15.36	22.89	11.54
Con A-Sepharose	1.29	92.69	72.08	107.39	6.72
Sephacryl S-300	0.45	39.03	86.73	129.23	2.83

These proteins were likely to be a trypsin inhibitor. Aranishi (1999) reported that trypsin inhibitor from carp serum was a single polypeptide with molecular mass of 55 kDa based on SDS-PAGE under reducing and nonreducing condition. Mak et al. (2004) reported that molecular mass of purified serine proteinase inhibitor from rainbow trout seminal plasma was about 56 kDa and it was also a single polypeptide chain. Trypsin inhibitor from common carp blood plasma was a single polypeptide chain with molecular weight of 54 kDa (Mickowska, 2009).

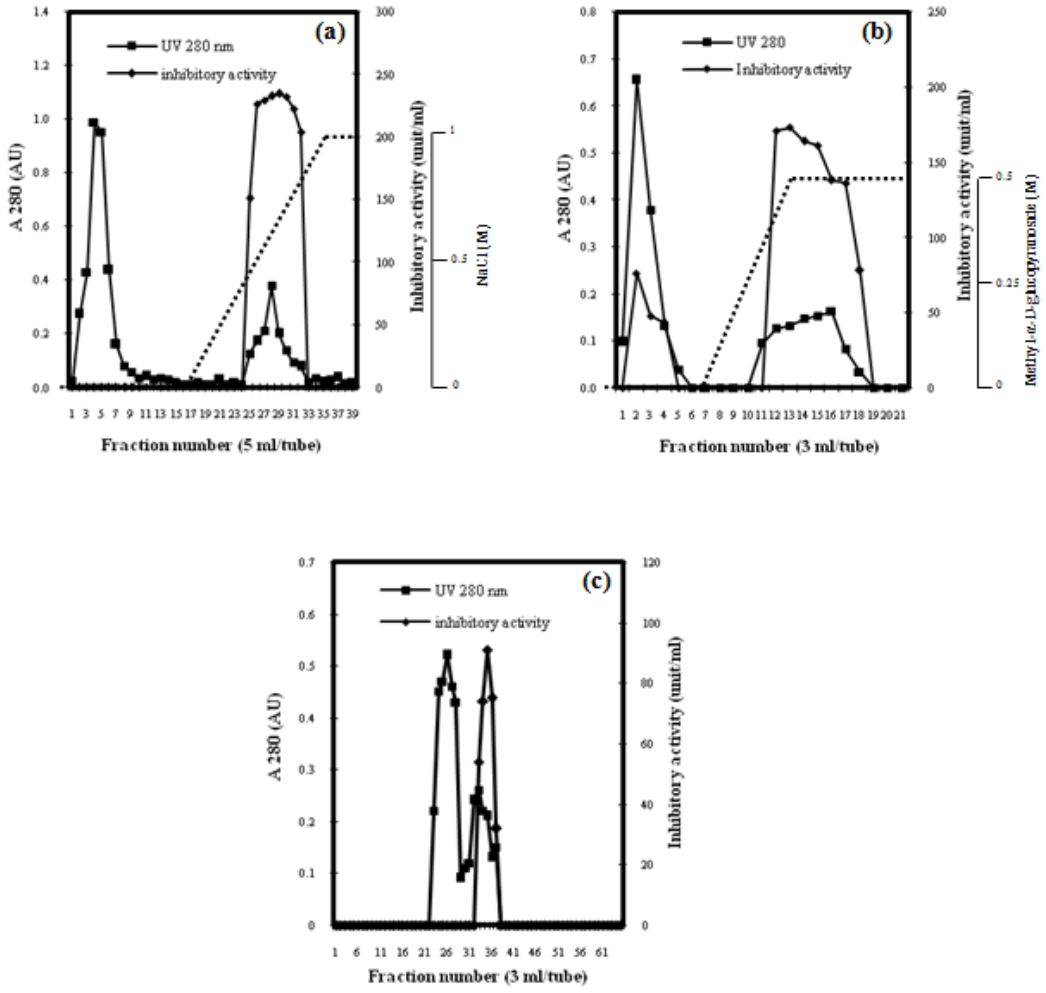

Figure 5.1 Chromatograms of common carp trypsin inhibitor obtained from DEAE-Sephacel (a), Con A-Sepharose (b) and Sephacryl S-300 gel filtration (c).

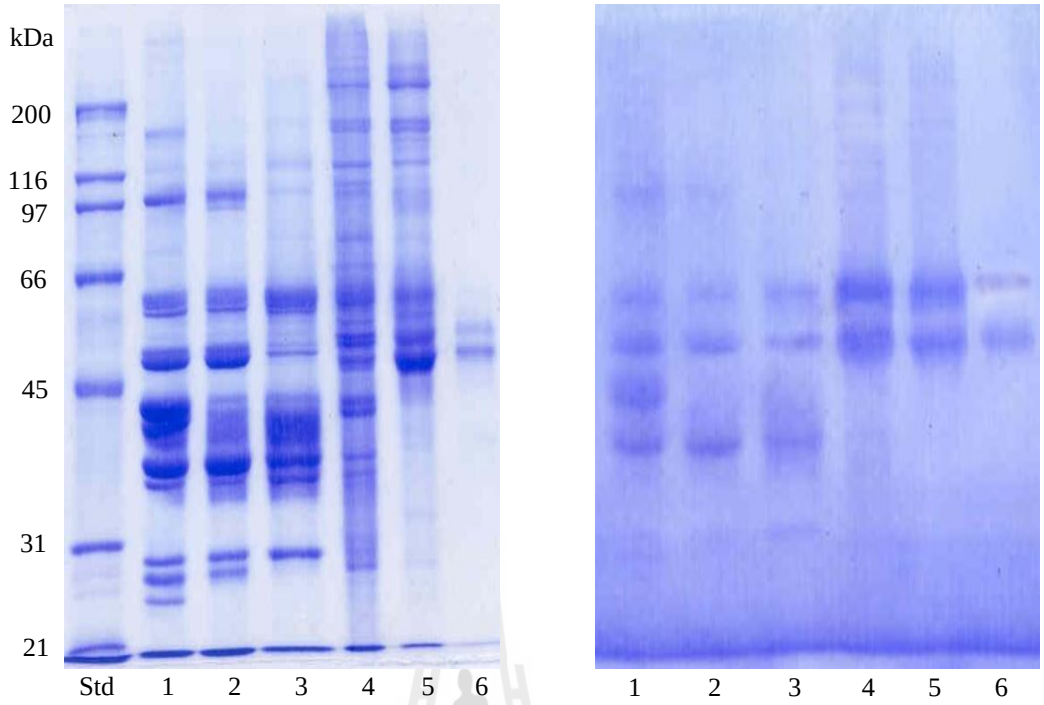

Figure 5.2 Protein patterns at various purification steps of proteinase inhibitor stained by Coomassie blue (a) and trypsin inhibitory activity staining (b). Std, Standard markers; lane 1, crude; lane 2, heat treatment; lane 3, 50-70% $(NH_4)_2SO_4$ precipitation; lane 4, DEAE Sephacel; lane 5, Con A-Sepharose; lane 6, Sephacryl S-300 under nonreducing condition.

5.4.3 Protein identification

Based on LC-MS/MS, amino acid sequences derived from two proteins were matched with those of alpha-1-antitrypsin homolog from common carp (Table 5.2). Alpha-1-antitrypsin, also known as alpha-1-proteinase inhibitor (α1-PI), is a member of serine proteinase inhibitor family (serpins) which inhibits serine proteinases. Two isoforms of α1-PI were found in common carp seminal plasma, guinea pig plasma, and equine (Wojtczak et al., 2007; Suzuki, Yoshida, Ichimiya, Yamamoto, and Sinohara, 1990; Pellegrini, Zweifel, and Fellenberg, 1985). Common

carp α1-PI might have two isoforms with molecular mass of 47 and 52 kDa. In mammals, α1-PI is synthesized by the liver and released into the blood and reaches tissues to protect them against excessive proteolysis. Serpins in fish muscle prevent self-destructive proteolysis to maintain homeostasis. α1-PI has been purified from serum and plasma of various mammals and fish (Patterson, 1991; Abrams, Kimbel, and Weinbaum, 1978; Koj, Hatton, Wong, and Regoeczi, 1987; Martorana et al., 1993; Catanese and Kress, 1993; Berninger and Mathis, 1976; Aranishi, 1999; Wojtczak, et al., 2007; Mickowska, 2009). This is the first report of α1-PI purified from skeletal muscle.

Table 5.2 Significant hits of two purified proteinase inhibitors from Mascot search.

Sample	Accession number	Protein	Species	Score	Sequence coverage
Inhibitor I (47 kDa)	A1AT_CYPCA	Alpha-1-antitrypsin homolog	*Cyprinus carpio* (Common carp)	3691	53%
Inhibitor II (52 kDa)	A1AT_CYPCA	Alpha-1-antitrypsin homolog	*Cyprinus carpio* (Common carp)	5963	57%

The pI of common carp α1-PI from sarcoplasmic protein were about 5.3 and 5.4 (Figure 5.3). The pI of carp blood plasma α1-PI and carp seminal plasma α1-PI was determined to be 5.5 and 5.1, respectively (Mickowska, 2009; Wojtczak et al., 2007). Lower values of mammalian α1-PI have been reported to be 4.2-4.9 (Patterson, 1991; Mattes, Matthiessen, Tureck, and Schwarz, 2001). Fetz et al. (2004) found that

four bands of α1-PI from cat (*Felis catus*) with pI between 4.3-4.5 while the observed pI values of canine α1-PI was 4.6-4.7 (Melgarejo, Williams, and Griffith, 1996).

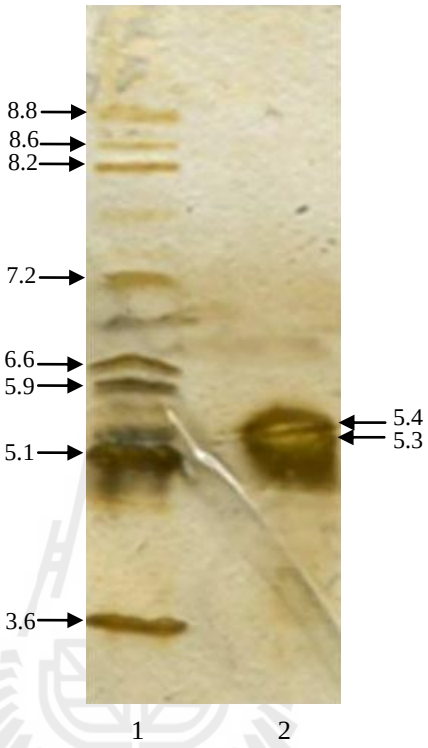

Figure 5.3 pI of purified proteinase inhibitor from common carp. 1, standard pI; 2, purified proteinase inhibitor.

5.4.4 Identification of N-glycosylation sites

Inhibitor I and II might be glycoproteins as they bound to Con A-Sepharose. To prove this, cleavage of all N-linked oligosaccharide was performed using PNGase F. The molecular mass of deglycosylated proteins decreased from 47 to 42 kDa for inhibitor I and from 52 to 47 kDa for inhibitor II (Figure 5.4), suggesting that these inhibitors are glycoproteins. Based on mass differences, inhibitor I and inhibitor II contained 11 and 10 % of N-glycan content, respectively. Serpins have been reported to be glycoproteins (Roberts, Mathialagan, Duffy, and Smith, 1995).

Carbohydrate content of serpins was in the range of 10 to 20% (Carrell et at., 1982). α1-PI from fish contained carbohydrate contents 12-16%, while those from mammalians, including human, rat, and chinchilla possessed 13.2-15% carbohydrate (Huang, Chen, Chen, Huang, and Chang, 1995; Mak, 2004; Wojtczak et al., 2007; Carrell et al., 1982; Chao, Chai, Chao, and Chao, 1990; Diven, Vietmeier, Hempel, and Chambers, 1990).

N-glycans are covalently linked to asparagines that are located within a consensus sequence of -N-X-S/T-, where X can be any amino acids, except P. Thus, a potential N-glycosylation site can be predicted from the consensus sequence -N-X-S/T-. To identify the exact N-glycosylation sites of two inhibitors, both PNGase F and LC-MS/MS were used. PNGase F was added to remove N-linked carbohydrates from proteins. PNGase F cleaves the bond between the asparagine residue and N-linked carbohydrates with the conversion of the asparagines to aspartic acid via deamidation, resulting in an increase in molecular mass by 1 Da (Khoshnoodi, Hill, Tryqqvason, Hudson, and Friedman, 2007). The mass difference was exploited to identify N-glycosylation sites using LC-MS/MS.

Based on this principle, an increase in molecular mass by 1 Da was observed at N214 and N226 of both inhibitor I and II after PNGase F treatment from MS/MS ion chromatogram (Table 5.3). A representative MS/MS data of one of the N-glycosylation sites containing a deamidated asparagine, N214, is presented in Figure 5.5. The mass difference between y11 and y10 of a peptide (^{206}YDIYQDPVN^{214}QTTVMMVPYK^{224}), which corresponds to Asn, was 115 Da instead of 114 Da (Figure 5.5). An increase in molecular mass by 1 Da resulted from NH_2 group

in asparagine changed into OH group in aspartic acid. Based on these results, each common carp inhibitor has two N-glycosylation sites at N214 and N226.

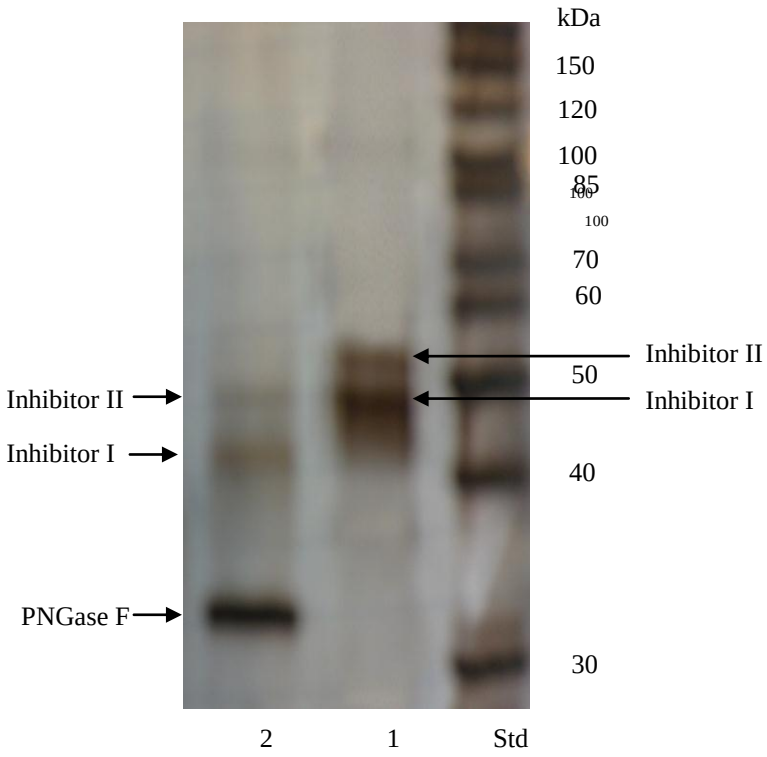

Figure 5.4 Molecular mass pattern of inhibitor I and II (lane 1) and after PNGase F treatment (lane 2). Std, Standard markers.

α1-PI of common carp seminal plasma had two N-linked glycosylation sites (Wojtczak et al., 2007). Two N-linked carbohydrate site chains were also found in carp perimeningeal fluid α1-PI while O-linked carbohydrate did not appear in the presence of O-glycosidase treatment (Huang et al., 1995). Moreover, Mak et al. (2004) revealed that serpin from rainbow trout seminal plasma which was similar to α1-PI contained two N-linked but not O-linked carbohydrate side chains. Human α1-PI exhibits three N-linked glycosylation sites at N46, 83, and 247 (Kolarich et al., 2006). O-linked carbohydrate has never been reported in fish α1-PI. Kininogen, a cysteine

proteinase inhibitor, from Atlantic salmon (*Salmo salar* L.) skin was found to have both N-and O-glycosylated form (Ylönen et al, 1999).

Table 5.3 Glycosylation sites of inhibitor I and II obtained from LC-MS/MS data of tryptic peptides by MASCOT database.

Inhibitor	Site	Peptide position	Mass	m/z Obs	Sequence
Inhibitor I	214	206-224	2305.06	1153.54^{2+}	YDIYQDPVNQTTVMMVPYK
	226	225-238	1525.68	763.85^{2+}	GNTSMMIIFPDDGK
Inhibitor II	214	206-224	2337.05[a]	780.02^{3+}	YDIYQDPVNQTTVM*M*VPYK
	226	225-238	1525.68	763.85^{2+}	GNTSMMIIFPDDGK

[a] Calculated mass (deamidated Asn) with modified methionine

*Oxidation of methionine.

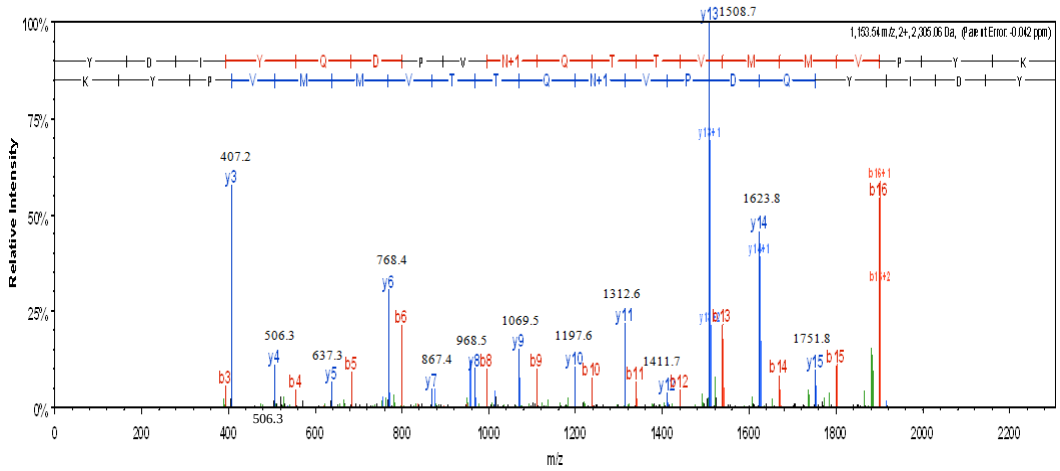

Figure 5.5 MS/MS ion chromatogram of inhibitor I peptide (^{206}YDIYQDPVNQTTVMMVPYK^{224}) with deglycosylated asparagines at N214.

Carbohydrate moiety had no influence on the proteinase inhibitory activity of α1-PI (Guzdek, Potempa, Dubin, and Travis, 1990). However, several studies revealed that glycosylation of proteins increases stability and has a positive relationship with the length of the attached carbohydrates (Nakamura, Ogawa, and Nakai, 1998; Nakamura, Takasaki, Kobayashi, and Kato, 1993). Wang, Eufemi, Turano, and Giartosio (1996) reported that carbohydrates which were attached to proteins might prevent unfolding of protein molecules.

5.4.5 Thermal stability

Common carp α1-PI purified from sarcoplasmic protein retained 100% activity up to 60 °C (Figure 5.6). High activity (80%) remained at 70 °C. The carp serum α1-PI showed maximum thermal stability at 50 °C and lost stability above 60 °C (Aranishi, 1999). The difference could be arisen from different incubation between the study of Aranishi (30 min) and this study (10 min). Purified trypsin inhibitor from white croaker (*Micropogon opercularis*) skeletal muscle was stable at temperature below 60 °C (Sangorrín et al., 2001). Purified trypsin inhibitor from the egg of skipjack tuna showed thermal stability at temperature below 40 ºC (Choi, Park, and Kim, 2002).

Aranishi (1999) reported that α1-PI from carp serum exhibited high thermal stability due to its compact tertiary structure. It could be speculated that glycosylation of the common carp α1-PI might contribute to its thermal stability. The carbohydrate moiety of ovomucoid contributes to the stability of ovomucoid against tryptic hydrolysis and thermal denaturation (Gu et al., 1989). Residual inhibitory

activity of glycosylated chicken cystatins was higher than that of deglycosylated form at 60-100 °C for 30 min (Tzeng and Jiang, 2004).

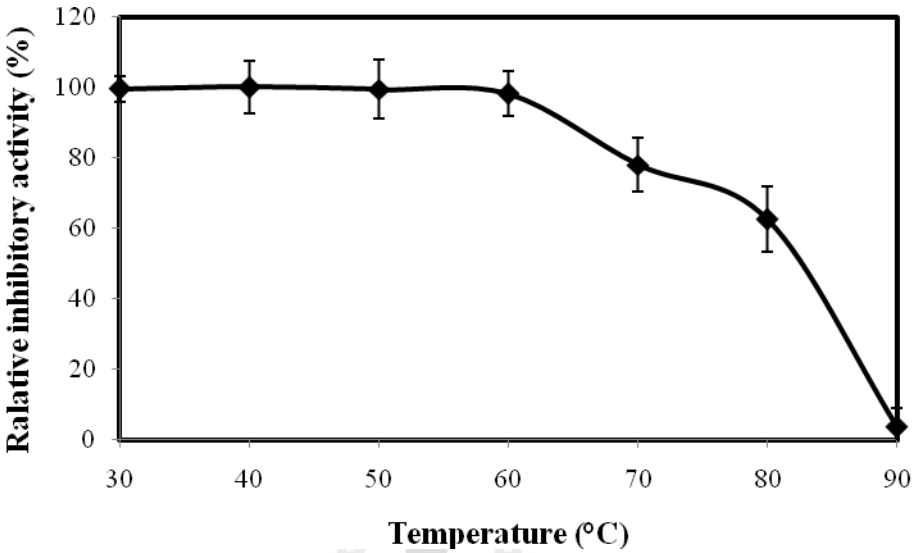

Figure 5.6 Effect of temperature on stability of purified common carp α1-PI.

5.4.6 Effect of $CaCl_2$ on trypsin inhibitory activity

Addtion of Ca^{2+} has been reported to improve textural properties of surimi depending on surimi species and concentration of Ca^{2+} (Park, 2005). Inhibitory activity of α1-PI decreased to 80% at 10 mM Ca^{2+} and remained at that residual activity when Ca^{2+} concentration increased up to 100 mM (Figure 5.7). Contrarily, trypsin inhibitory activity from egg of skipjack tuna increased with addition of a final Ca^{2+} concentration of 10 mM (Choi et al., 2002). Ovostatin, trypsin inhibitor, from sea urchin eggs required a low concentration of Ca^{2+} for activity (Yamada and Aketa, 1988). This result implied that Ca^{2+} might induce conformational change of the common carp α1-PI, leading to a decrease of α1-PI inhibitory activity.

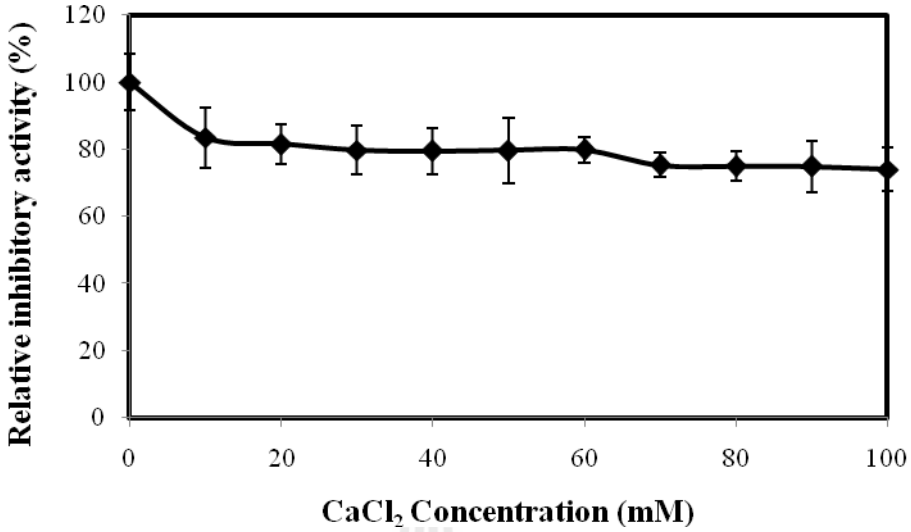

Figure 5.7 Effect of $CaCl_2$ on proteinase inhibitory activity of the common carp α1-PI at 40 °C.

Ca^{2+} is also known as a destabilizing salt in the Hofmeister series and promotes ‘‘salting in’’ of protein (Baldwin, 1996). Ca^{2+} ion decreases the free energy required to transfer the nonpolar amino acids into water and thus reduces intramolecular hydrophobic interactions, resulting in an increased protein unfolding (von Hippel and Wong, 1965). Binding of Ca^{2+} to β-lactoglobulin induced partial unfolding, leading to increased hydrophobicity (Jeyarajah and Allen, 1994). Ca^{2+} also induced the unfolding of myosin and actin (Hemung and Yongsawatdigul, 2005). Since pI of the common carp inhibitor I and II was approximately 5.3-5.4 (Figure 5.3), they were negatively charged at pH 7.5. This would likely lead to formation of Ca^{2+} bridges among proteins, rendering a decrease in inhibitory activity. In addition, Siriangkanakun and Yongsawatdigul (2012) reported that the combined addition of common carp crude sarcoplasmic protein in conjunction with $CaCl_2$ at 10-50 mM to threadfin bream surimi showed lower textural properties than crude sarcoplasmic

protein alone. This was probably due to Ca^{2+}-induced loss of inhibitory activity of α1-PI contained in the sarcoplasmic protein.

5.4.7 Inhibitory activity of common carp α1-PI

The common carp α1-PI completely inhibited trypsin, while thermolysin and pepsin were not inhibited (Table 5.4). α-Chymotrypsin and papain were slightly inhibited. Serpins including α1-PI have been reported to covalently bind to the target enzyme. The serpin interaction with their target proteinase is determined by the P1-P1′ bond. The target proteinase cleaves the P1-P1′ bond located within a reactive center loop structure of the inhibitor, 30-40 amino acids from the C-terminus, and forms an ester bond between its active serine residue (serine hydroxyl of proteinase) and P1 (the carboxyl group of the serpin reactive site). This reaction causes irreversible distortion of active site of the target proteinase (Patterson, 1991; Bousquet, Duranton, Mely, and Bieth, 2003).

α1-PI from chinchilla, mouse, and monkey inhibited trypsin, while α1-PI from carp serum, horse, pig, and wallaby inhibited both trypsin and chymotrypsin (Diven et al., 1990; Lamontagne, Gauldie, and Koj, 1981; Berninger, and Mathis, 1976; Aranishi, 1999; Patterson, Bell, and Shaw, 1991b; Gahne and Juneja, 1986; Patterson, Bell, and Shaw, 1991a). α1-PI from rat and bighead carp (*Aristichthys nobilis*) perimeningeal fluid strongly reduced activity of trypsin, chymotrypsin and elastase (Chao et al., 1990; Huang et al., 1995). Wojtczak et al. (2007) reported that α1-PI from fish seminal plasma differed in species-specific manner in their abilities to recognize and bind individual serine proteinases.

Table 5.4 Inhibitory specificity of the common carp α1-PI.

Proteinase	**Inhibition (%)**
Serine proteinase	
Trypsin	100 ± 1.89
α-Chymotrypsin	20.71 ± 1.52
Metallo proteinase	
Thermolysin	0
Cysteine proteinase	
Papain	8.93 ± 1.32
Aspartic proteinase	
Pepsin	0

5.4.8 Effect of common carp α1-AT on autolysis

In the absence of α1-PI, bigeye snapper surimi underwent severe textural degradation at 65 °C as observed from disappearance of myosin heavy chain (MHC) and tropomyosin (TM) (Figure 5.8). In addition, protein degradation also occured at 40 °C, which would negatively affected textural properties of gels subjected to setting. Intensity of MHC and TM increased with the levels of α1-PI addition, especially at 65 °C preincubation. This result demonstrated that the common carp α1-PI can be used to reduce proteolysis of bigeye snapper surimi. Addition of crude common carp sarcoplasmic proteins to threadfin bream surimi also improved both breaking force and deformation and reduced proteolysis of threadfin bream surimi (Siriangkanakun and Yongsawatdigul, 2012). It should be noted that an effective concentration to reduce proteolysis is only 0.025%, suggesting its strong inhibition towards

endogenous proteinases of bigeye snapper proteinases. Serine proteinases were responsible for myosin heavy chain degradation of washed bigeye snapper mince (Benjakul, Leelapongwattana, and Visessanguan, 2003). Therefore, this could be a promising alternative food grade inhibitor for controlling proteolysis in proteinase-laden surimi.

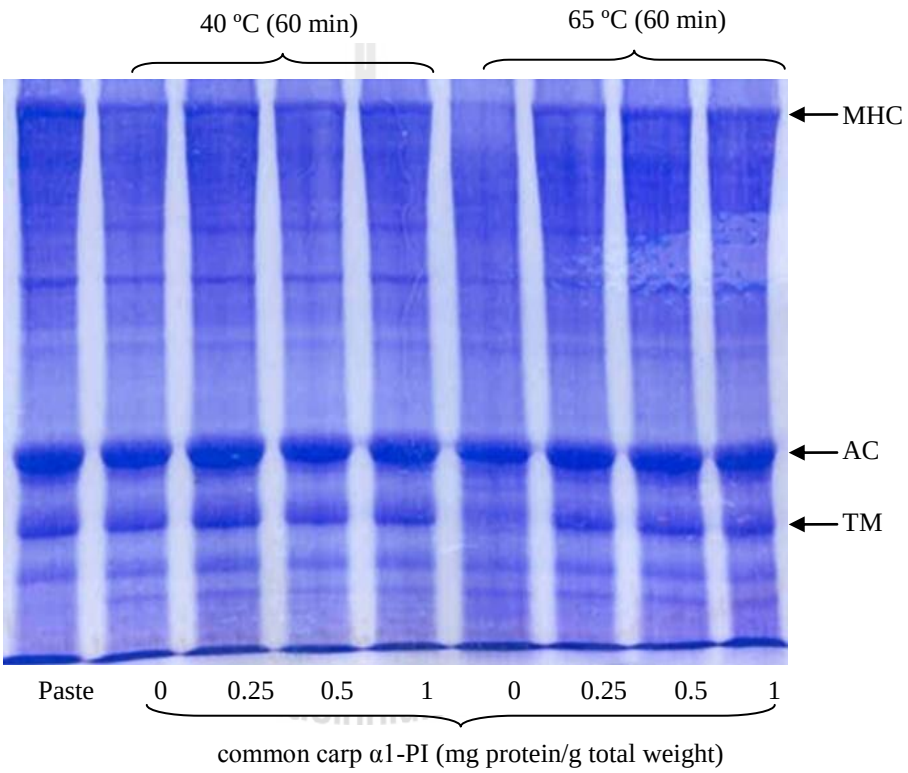

Figure 5.8 Autolytic pattern of bigeye snapper surimi at incubated at 40 and 65. Numbers indicate addition level of the common carp α1-PI (mg protein/g total weight); MHC, myosin heavy chain; AC, actin; TM, tropomyosin.

5.5 Conclusions

Two proteinase inhibitors with molecular mass of 47 and 52 kDa were purified from common carp sarcoplasmic protein**.** They are identified to be common carp α1-

PI based on LC-MS/MS. The pI of common carp sarcoplasmic protein α1-PI were 5.3 and 5.4. They are glycoprotein and have two N-glycosylation sites at both N214 and N226. Inhibitory activity of α1-PI decreased at temperature above 60 °C. $CaCl_2$ concentration up to 100 mM decreased inhibitory activity by 20%. The inhibitors exhibited remarkable antitrypsin activity. Moreover, they were able to reduce proteolysis of bigeye snapper surimi. Therefore, the common carp α1-PI could be a potential proteinase inhibitor to prevent tissue softening of fish muscle.

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CHAPTER VI

IDENTIFICATION OF TRYPSIN INHIBITOR BY GELC-MS/MS IN SARCOPLASMIC PROTEINS OF THREE TROPICAL FISH AND THEIR INHIBITORY PROPERTIES

6.1 Abstract

Sarcoplasmic proteins (SP) from three fish species were fractionated by 50-70% ammonium sulfate precipitation. Lyophilized fractionated SP of threadfin bream (TB-SP), bigeye snapper (BS-SP) and yellow croaker (YC-SP) showed 80-92% trypsin inhibitory activity. The molecular mass on trypsin inhibitory activity staining gel electrophoresis of all 3 species included 32, 33, 37, 45, 48 and 50 kDa. TB-SP and YC-SP also showed a band at 95 kDa. Alpha-1-proteinase inhibitor with molecular mass ranging from 45 to 50 kDa was identified by GeLC-MS/MS in YC-SP. Trypsin inhibitory activity of fractionated SP showed good stability at 30-60 °C and up to 0.2 M NaCl. TB-SP and YC-SP effectively inhibited autolytic degradation of threadfin bream washed mince. Addition of 0.5 and 1% TB-SP in conjunction with setting at 37 and 65 °C improved textural properties of threadfin bream surimi. Therefore, TB-SP could be a promising protein ingredient for enhancing surimi gel texture.

6.2 Introduction

Endogenous proteinases in fish muscle inhibit proper gel network development, resulting in poor gel texture. Trypsin-like proteinase is a major enzyme responsible for degradation of myofibrillar protein and gel-weakening of various tropical fish. Choi, Cho, and Lanier (1999) found that trypsin-like proteinase was responsible for textural degradation of Atlantic menhaden muscle. Cao, Osatomi, Hara, and Ishihara (2000) found trypsin-like serine myofibril-bound proteinase in lizardfish (*Sauridawanieso*) surimi with maximum activity at 55-60 °C. Food grade proteinase inhibitors including egg white powder and whey protein concentrate have been applied as a means to control proteolysis of fish muscle protein (Li, Lin, and Kim, 2007; Yongswatdigul and Piyadhammaviboon, 2004).

During surimi processing, sarcoplasmic proteins are removed by washing process and discarded in the waste stream because they are believed to interfere with gelation of myofibrillar proteins. However, addition of fish sarcoplasmic protein has been reported to increase surimi gelation (Kim, Yongsawatdigul, Park, and Thawornchinsombut, 2005; Jafarpour and Gorczyca, 2009). Gel-enhancing effect of fish sarcoplasmic protein has been attributed from various factors, including residual endogenous transglutaminase (Yongsawatdigul and Piyadhammaviboon, 2007) and the presence of protein(s) with gel-forming ability (Morioka, Kurashima, and Shimizu, 1992). In addition, endogenous proteinase inhibitor in sarcoplasmic protein has been reported to be a key factor improving myofibrillar protein gelation (Siriangkanakun and Yongsawatdigul, 2012). Therefore, sarcoplasmic protein might be an alternative ingredient for controlling proteolysis of proteinase-laden muscle proteins. However, muscular proteinase inhibitors might vary among fish species.

Endogenous muscular proteinase inhibitors have been reported in freshwater fish, common carp and crucian carp (Siriangkanakun and Yongsawatdigul, 2012; Sun et al., 2009). Information on muscular proteinase inhibitor of marine fish, which are economically important to seafood industry, is still limited, particularly threadfin bream (*Nemipterus* spp.), bigeye snapper (*Priacanthus* spp.) and yellow croaker (*Larimichthys* spp.). These species are important tropical marine fish used for surimi production.

Gel-based liquid chromatography-tandem mass spectrometry (GeLC-MS/MS) has been proven to be a powerful and an efficient approach for proteomic analysis of protein mixtures (Rezaul, Wu, Mayya, Hwang, and Han, 2005). Proteins are pre-fractionated by one-dimentional sodium dodecyl sulfate-PAGE (SDS-PAGE) then the entire protein bands are excised and further subdivided into smaller sections before trypsin digestion and LC- MS/MS. Slicing the gel lane into smaller bands separates the protein mixtures into narrow molecular mass range so some low abundant proteins will be separated from the high abundant ones. Shotgun proteomics can identify several proteins within one analysis but proteins showed low abundant are ignored. Sarcoplasmic proteins consist of several types of proteins and enzymes involved in muscle metabolism. Hence, GeLC-MS/MS could be applied to identify sarcoplasmic proteins of interest.

Objective of this study was to investigate the proteinase inhibitory activity of sarcoplasmic proteins extracted from 3 tropical marine species, threadfin bream, bigeye snapper, and yellow croaker. Moreover, profiles from sarcoplasmic proteins were evaluated using GeLC-MS/MS technique.

6.3 Materials and methods

6.3.1 Chemicals

Chymotrypsin, papain, trypsin, phosphorylase b, glyceraldehydes-3-phosphate dehydrogenase, creatine kinase, and ovomucoid were purchased from Sigma Chemical Co. (St Louis, MO, USA). Egg white powder (EW) was obtained from Igrecha (Seichessur Le Loir, France). Reagents used for gel electrophoresis were purchased from Promega (Madison, WI, USA). All other reagents and chemicals were of analytical grade.

6.3.2 Sarcoplasmic protein and washed mince

Frozen threadfin bream (*Nemipterus* spp), bigeye snapper (*Priacanthus* spp.) and yellow croaker (*Larimichthys* spp.) were used. Fish were thawed under running tap water (25°C) for 20 min. Subsequently, fish were beheaded, deskinned and eviscerated manually. Both ordinary and dark muscles were collected. Fish mince was homogenized with cold water at a ratio of 1:3 (w/v) using a homogenizer for 5 min. The homogenate was filtered through three layers of cheesecloth and then centrifuged at 10000×g (Sorvall RC-5C Plus, Dupont, Wilmington, Del., USA) for 20 min at 4 °C. The supernatant was kept in a 4 °C cold room overnight and fat layer in the supernatant was removed manually. The supernatant was referred to as sarcoplasmic protein. The sarcoplasmic protein content of threadfin bream, bigeye snapper and yellow croaker was approximately 11.6 ± 2, 12.8 ± 2 and 12.8 ± 2 mg/mL, respectively.

Washed threadfin bream mince was prepared by 3-cycle washing at a ratio of fish to mince of 1:3. The third washing cycle was carried out using 0.3% NaCl solution. Water removal at each washing cycle was achieved by centrifugation at

10000×g for 15 min at 4 °C. Frozen threadfin bream (*Nemipterus* spp.) surimi without addition of protein additives were obtained from Andaman Surimi Industry (Samutsakorn, Thailand). The sample was cut into 1-kg blocks, vacuum-packed, and kept at -20 °C.

6.3.3 Preparation of lyophilized sarcoplasmic protein powder

Ammonium sulfate fractionation of sarcoplasmic proteins was carried out at 50-70% to precipitate proteins with the highest inhibitory activity. The precipitates were collected and dissolved in small amount of cold distilled water for complete solubilization and dialyzed overnight against cold distilled water at 4 °C using SnakeSkinTM pleated dialysis tubing with 10-kDa molecular weight cut off (MWCO) (Pierce Chemical Co., Rockford, IL, USA). The dialysate was lyophilized and kept at -20 °C throughout the study. The lyophilized fractioned sarcoplasmic proteins from threadfin bream, bigeye snapper, and yellow croaker were referred to as TB-SP, BS-SP and YC-SP, respectively.

6.3.4 Proteinase inhibitory activity assay

Proteinase inhibitory activity of TB-SP, YC-SP and BS-SP was determined against trypsin, α-chymotrypsin and papain using casein as a substrate according to the modified method of An, Weerasinghe, Seymour, and Morrissey (1994). Lyophilized samples were dissolved in deionized water to obtain protein content of 11 mg/mL. For trypsin and α-chymotrypsin activity assay, 200 µL of the reconstituted sarcoplasmic protein (2.2 mg protein) was mixed with 500 µL of 50 mM Tris-HCl (pH 7.5), while 500 µL of 50 mM Tris-HCl (pH 7.5) containing 1 mM β-mercaptoethanol was used for papain activity assay. One hundred µL of enzyme solution (0.2 mg/mL trypsin, 0.2 mg/mL α-chymotrypsin and 0.1 mg/mL papain) was

added to each reaction mixture. The solution was pre-incubated for 10 min at 37 °C and then 1% (w/v) casein solution in 50 mM Tris-HCl, pH 7.5 was added and further incubated at 37 °C for 1 h. The reaction was terminated by adding 400 µL of cold 50% trichloroacetic acid (TCA). The supernatant containing oligopeptides was obtained by centrifuging at 10000×g for 10 min at 4 °C. A blank was prepared in the same manner but with the addition of TCA before casein. TCA-soluble oligopeptide content in the supernatant was analyzed by Lowry method (Lowry, Rosebrough, Farr, and Randall, 1951) using tyrosine as a standard. Degree of inhibition (%) was calculated as follows:

$$\text{Inhibition (\%)} = \frac{(C_0 - C_b) - (I_0 - I_b)}{(C_0 - C_b)} \text{x100}$$

where C_0 is TCA-soluble oligopeptide content of control (without sarcoplasmic protein), C_b is TCA-soluble oligopeptide content of control blank, I_0 is TCA-soluble oligopeptide content of sample with sarcoplasmic protein, and I_b is TCA-soluble oligopeptide content of sample blank.

6.3.5 Inhibitory activity staining

Inhibitory activity staining was conducted under a nonreducing condition according to the modified method of Garcia-Carreno, Dimes, and, Haard (1993). Sarcoplasmic protein solution was mixed with the treatment buffer (12.5 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol) at a ratio of 1:1 (v/v). Sarcoplasmic proteins (30 µg) without boiling were loaded onto 10% polyacrylamide gel copolymerized with 0.12% casein. Gel was run at a constant voltage setting of 120 V on ice. After electrophoretic separation, the gel was washed with 2.5% Triton X-100 for 15 min to remove SDS and then washed with deionized water. Subsequently, the

gel was flooded with 50 ml trypsin solution (0.4 mg/ml) and incubated at 37 °C for 1 h. The gel was then rinsed with deionized water twice and stained in 0.125% Coomassie Brilliant Blue R-250 and destained in a solution containing 25% ethanol and 10% acetic acid to develop inhibitor zone. Blue bands on a clear background indicated the presence of protein exhibiting trypsin inhibitory activity.

6.3.6 GeLC-MS/MS

A 20 μg aliquot of YC-SP was separated on a 4-12% Bis-Tris Novex mini-gel (Invitrogen, Carlsbad, CA, USA) under nonreducing condition using N-morpholinopropane sulfonic acid (MOPS) buffer system. The gel was stained with Coomassie Brilliant Blue R-250 and the target regions were excised into 20 segments at molecular weight range of 25-50 kDa and 75-150 kDa (Figure 6.3d). Gel pieces were washed with 25 mM ammonium bicarbonate followed by acetonitrile. The gels were reduced with 10 mM dithiothreitol at 60°C followed by alkylation with 50 mM iodoacetamide at room temperature. Sequentially, samples were digested with trypsin (Promega, Madison, WI, USA) at 37°C for 4 h. After trypsin digestion, it was quenched with formic acid and the supernatant was submitted to nano LC-MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. Peptides were loaded on a trapping column and eluted over a 75-μm analytical column at 350 nL/min; both columns were packed with Jupiter Proteo resin. The mass spectrometer was operated in data-dependent mode, with MS and MS/MS performed in the Orbitrap at 70,000 FWHM and 17,500 FWHM resolution, respectively. The fifteen most abundant ions were selected for MS/MS. Data were searched using a local copy of Mascot with the following parameters: enzyme (trypsin); database (uniprot vertebrate); fixed modification (carbamidomethyl); variable modifications

(oxidation , acetyl, deamidation); mass values (monoisotopic); peptide mass tolerance (10 ppm); fragment mass tolerance (0.015 Da); max missed cleavages (2). Mascot DAT files were parsed into the Scaffold software for validation, filtering and to create a nonredundant list per sample. Data were filtered using a minimum protein value of 99%, a minimum peptide value of 50% (Prophet scores) and requiring at least two unique peptides per protein.

6.3.7 Trypsin activity assay

Trypsin activity was determined according to the method of Barrett and Kirschke (1981) and Ishida, Sugiyama, Sato, and Nagayama (1995) with slight modifications. The reaction mixture containing 0.7 mL of 50 mM Tris–HCl (pH 8), 0.1 mL of either phosphorylase, glyceraldehydes-3-phosphate dehydrogenase, creatine kinase, or ovomucoidat at 50 µg/mL. One hundred microliters of trypsin solution (0.01 mg/mL) was added and pre-incubated at 37 °C for 5 min. After pre-incubation, 0.1 mL of 10 µM of Boc-Asp(oBzl)-Pro-Arg-AMC was added and the reaction was further incubated at 37 °C for 10 min. The reaction was terminated by addition of 1.5 mL of stopping solution (methanol:n-butanol: deionized water = 35:30:35 (v/v/v)). Fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) was measured using a Luminescence Spectrometer LS 50B (Perkin Elmer, Waltham, MA, USA) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. One unit of trypsin activity was defined as the amount of enzyme that released 1 nmol of AMC per min.

6.3.8 Thermal and NaCl stability

To determine thermal stability, lyophilized sarcoplasmic proteins were reconstituted with 50 mM Tris-HCl (pH 7.5) to contain 11 mg/mL and were incubated

at different temperatures (30, 40, 50, 60, 65, 70 and 80 °C) for 30 min. The samples were immediately cooled in iced water for 10 min. Remaining inhibitory activity was calculated by taking the inhibitory activity of fractioned sarcoplasmic proteins without pre-incubation as 100%.

For NaCl stability, sarcoplasmic protein solution (11 mg/mL) was incubated in 50 mM Tris-HCl (pH 7.5) containing various NaCl concentrations (0-1.2 M) at 37 °C for 30 min.The residual inhibitory activity against trypsin was then determined as described above. Remaining inhibitory activity was calculated by taking the inhibitory activity of fractioned sarcoplasmic proteins without NaCl as 100%.

6.3.9 Effect of fish sarcoplasmic protein on autolytic degradation

TCA-soluble oligopeptide contents of washed mince samples were determined according to Yongsawatdigul and Piyadhammaviboon (2004). Three grams of threadfin bream washed mince were incubated with lyophilized sarcoplasmic proteins of 3 species at varied concentrations of 0, 0.5, 1 and 2% of total weight in either the presence or absence of 2% NaCl. The mixtures were incubated at either 37 or 65 °C for 1 h. Subsequently, 27 ml of 5% TCA was added to the samples to stop the reaction. The mixtures were homogenized for 1 min and centrifuged at 10000×g for 10 min at 4 °C. Supernatant was analyzed for oligopeptide content using Lowry method (Lowry et al., 1951). Autolytic activity was expressed as µmol of tyrosine/g sample. The sample blanks were kept in ice and were treated in the same manner as the samples.

Protein patterns of the samples were analyzed by SDS-PAGE according to the method of Laemmli (1970). The samples were mixed with 27 mL of 5% hot

sodium dodecylsulfate (SDS) solution, homogenized (IKA Works Asia, Bhd, Malaysia), and then heated at 90 °C for 30 min. The homogenate was centrifuged at 10000×g (Sorvall LegendMicro 17R, Thermo Electron LED GmbH, Lengensellbold, Germany) for 10 min at room temperature. Supernatant was mixed with the treatment buffer containing β-mercaptoethanol in a 1:1 ratio and boiled for 3 min. Protein sample (25 µg) was loaded on polyacrylamide gel (4% stacking gel and 10% running gel). Gels were run at a constant voltage setting of 120 V. Gels were stained with 0.125% Coomassie Brilliant Blue R-250 for 1 h and destained in a solution containing 25% ethanol and 10% acetic acid.

6.3.10 Effect of sarcoplasmic protein on textural properties

Thawed threadfin bream surimi was mixed with salt and water to obtain surimi paste with 2% NaCl and 80% moisture content. Lyophilized TB-SP which was found to possess the highest inhibitory activity against trypsin and autolytic degradation of washed mince was added at concentrations ranging from 0-1% of total weight. Paste preparation was carried out using a mortar and pestle pasted in ice bath to control temperature during mixing. Surimi paste was filled into a microplate with a diameter of 5 mm and depth of 10 mm. The filled micro-plates were vacuum-packed in a linear low-density polyethylene (LLDPE)/nylon bag. The samples were pre-incubated at either 25 °C for 2 h, 37 °C for 30 min, or 65 °C for 30 min prior to heating at 90 °C for 30 min. Samples were also heated at 90 °C for 30 min.. After heating, gels were cooled in iced water for 20 min and stored in a refrigerator (5-8 °C) overnight. Breaking force (g) and deformation distance (mm) were recorded using a Texture Analyzer TA-XT2 (Stable Micro System, Surrey, U.K.) with a 2-mm cylindrical probe. Penetration test was performed at a probe speed of 1 mm/s until

breaking. For each treatment, mean values were obtained from at least 8 measurements.

6.3.11 Statistical analysis

Degree of variation and significance of difference were analyzed using an analysis of variance (ANOVA) and differences among treatment means were evaluated by Duncan's multiple range test (DMRT). The statistical analysis was performed using SPSS Statistic Program Version 17.0 (SPSS Inc., Chicago, IL., U.S.A.). Significance of difference was defined at $P < 0.05$.

6.4 Results and discussion

6.4.1 Composition of sarcoplasmic proteins

Crude sarcoplasmic proteins of threadfin bream, bigeye snapper and yellow croaker showed different protein patterns with molecular mass ranging from 7 to 95 kDa under non reducing condition (Figure 6.1, lane 2, 5, 8). The most abundant protein bands of three species exhibited mass between 30-50 kDa. The band intensity of a protein with molecular mass of 95 kDa distinctly appeared in threadfin bream and yellow croaker but rather faint intensity was observed in bigeye snapper. In addition, proteins with molecular mass lower than 20 kDa were rarely found in bigeye snapper, but observed in threadfin bream with mass of 7, 10 and 15 kDa and in yellow croaker at 16 kDa. SDS-PAGE pattern of sarcoplasmic proteins has been found to vary with species (Huang, Jeng, Chen, and Hwang, 2008).

Ammonium sulfate fractionation increased proteinase inhibitory activity of TB-SP, BS-SP, and YC-SP by 2.9, 2.4, and 2.7 folds, respectively. Ammonium sulfate precipitation at 50-70% removed a 95-kDa protein band of BS-SP (Figure 6.1,

lane 6). In TB-SP, a protein with molecular mass of 7 kDa was removed by ammonium sulfate while intensity of protein bands with molecular mass around 15 kDa increased. The higher intensity of a 16-kDa protein was observed in YC-SP after ammonium sulfate precipitation. Individual sarcoplasmic proteins from varied species showed different salting in behavior. No difference of protein patterns between before and after lyophilization was observed in all species**,** implying that lyophilization did not induce cross-linking and/or degradation of sarcoplasmic proteins.

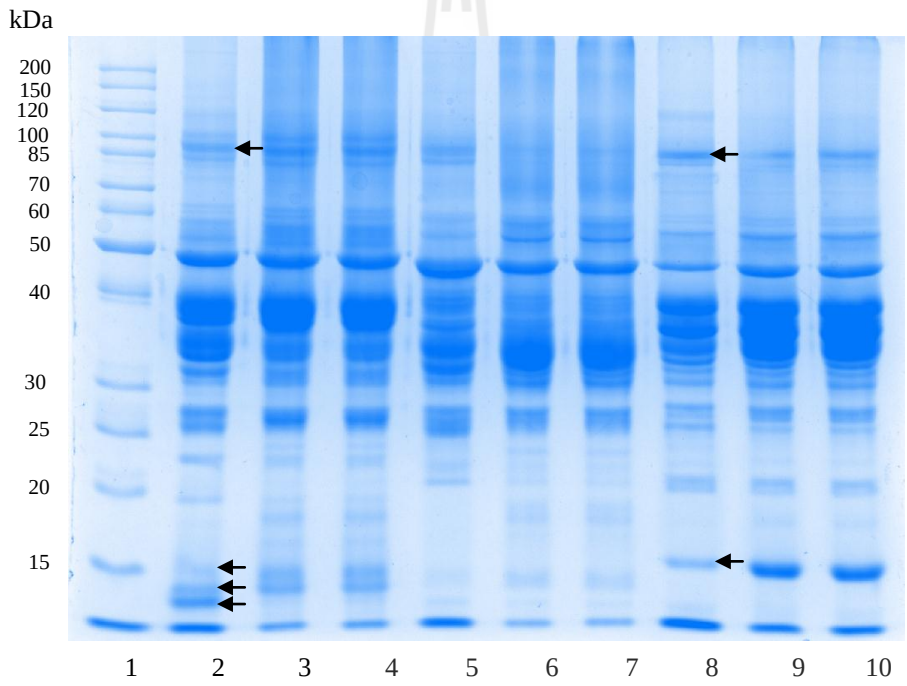

Figure 6.1 SDS-PAGE (10% polyacrylamide) patterns under nonreducing condition of sarcoplasmic proteins from threadfin bream (lane 2-4), bigeye snapper (lane 5-7) and yellow croaker (lane 8-10). Standard marker (lane 1), crude sarcoplasmic proteins (lane 2, 5, 8), 50-70% $(NH_4)_2SO_4$ precipitation before freeze drying (lane 3, 7, 9), 50-70% $(NH_4)_2SO_4$ precipitation after freeze drying (lane 4, 8, 10).

6.4.2 Inhibitory activity of fish sarcoplasmic proteins

Inhibitory activity toward various proteinases of sarcoplasmic proteins from three species was comparable (Figure 6.2). Trypsin was strongly inhibited, whereas a slight inhibition towards papain was observed and α-chymotrypsin utilized sarcoplasmic proteins as a substrate. TB-SP showed the highest trypsin inhibitory activity while the lowest inhibitory activity was observed in BS-SP ($P < 0.05$). This result indicated that major endogenous inhibitors in these species are trypsin-like inhibitor(s). Sarcoplasmic protein from common carp also exhibited the main inhibitory activity against trypsin (Siriangkanakun and Yongsawatdigul, 2012). Muscle of tropical fish predominantly contains trypsin-like proteinases (Kinoshita, Toyohara, and Shimizu, 1990; Cao et al., 2000; Benjakul, Leelapongwattana, and Visessanguan, 2003).

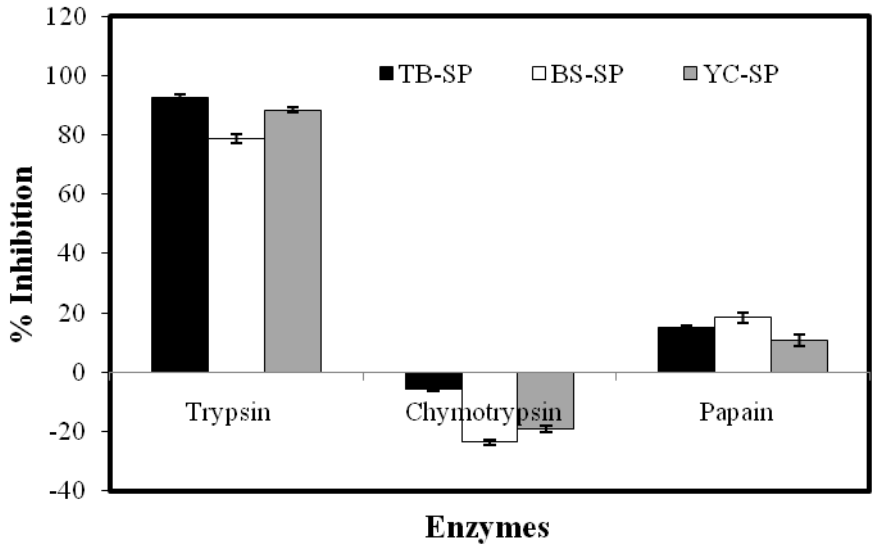

Figure 6.2 Inhibitory effect of sarcoplasmic proteins toward various proteinases at 37 °C. TB-SP, BS-SP, and, YC-SP are lyophilized fractionated sarcoplasmic protein of threadfin bream, bigeye snapper, and yellow croaker, respectively.

In addition, trypsin inhibitors have been found in hake (Martone, Busconi, Folco, and Sanchez, 1991) and white croaker skeletal muscle (Folco, Busconi, Martone, Trucco, and Sanchez, 1984). Muscular inhibitor is believed to play an important role in controlling muscle integrity (Carrell, Pemberton, and Boswell, 1987). Mbebi, Hantaï, Jandrot-Perrus, Doyennette, and Verdière-Sahuquè (1999) reported that proteinase nexin I, a serine proteinase inhibitor, in human skeletal muscle played a role after injury or inflammatory pathologies.

Proteins from TB-SP exhibiting trypsin inhibitory activity on activity staining showed molecular mass of 37, 45, 50 and 95 kDa (Figure 6.3a). Trypsin inhibitors with molecular mass of 32, 37, 45 and 50 kDa was noticeable in BS-SP (Figure 6.3b), while those of YC-SP showed molecular mass of 33, 37, 45, 48 and 95 kDa (Figure 6.3c). The highest intensity of protein band on inhibitory activity staining was observed at 37 kDa in TB-SP. Piyadhammaviboon and Yongsawatdigul (2010) reported that sarcoplasmic protein from threadfin bream (*Nemipterus* spp.) contained three trypsin inhibitors with molecular mass of 37, 41 and 95 kDa, which is in agreement with this study. Trypsin inhibitors from common carp (*Cyprinus carpio*) sarcoplasmic protein also showed molecular mass of 35, 41, 44, 50 and 69 kDa (Siriangkanakun and Yongsawatdigul, 2012).

A trypsin inhibitor has been reported in various organs of fish. Molecular mass of trypsin inhibitor from white croaker (*Micropogon opercularis*) skeletal muscle was 65 kDa, as estimated by SDS-PAGE and gel filtration (Sangorrín, Folco, Martone, and Sánchez, 2001). Molecular mass of trypsin inhibitor from cod (*Gadus morhua*) muscle and serum was 60 kDa (Hjelmeland, 1983). Purified trypsin inhibitor from the egg of skipjack tuna (*Katsuwonus pelamis*) showed molecular mass

of 78 kDa as estimated by gel filtration and 39 kDa by SDS-PAGE, implying the dimer characteristic. Nagashima, Takeda, Ohta, Shimakura, and Shiomi (2004) reported that two trypsin inhibitors were isolated from the skin mucus of *Takifugu pardalis* with molecular mass of 47 and 57 kDa on SDS-PAGE. Molecular mass of trypsin inhibitor varied with sources and fish species.

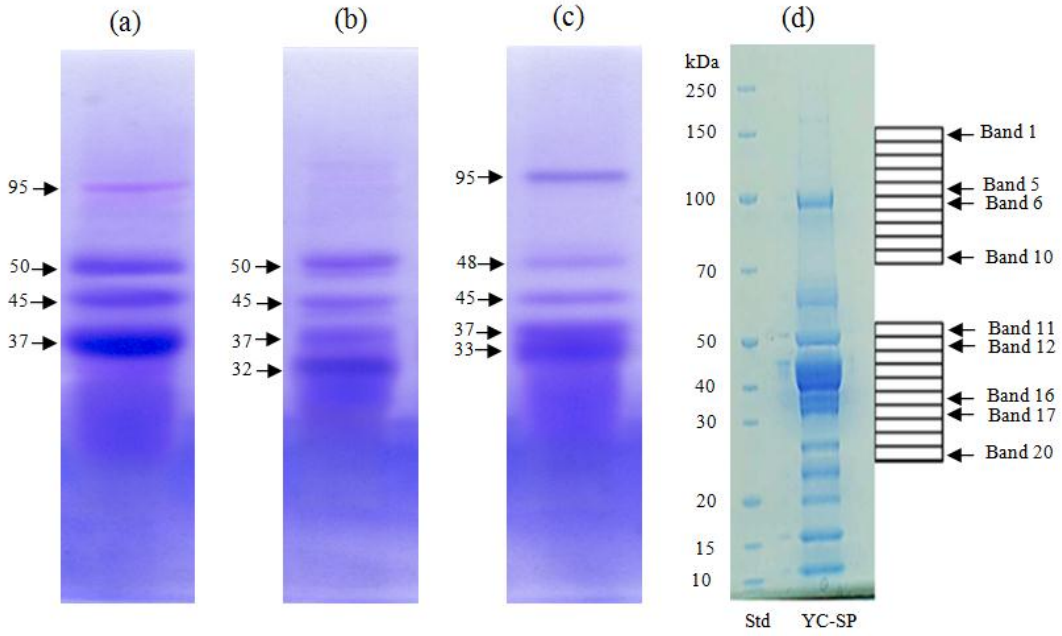

Figure 6.3 Trypsin inhibitory activity staining of lyophilized fractionated sarcoplasmic protein of threadfin bream (a), bigeye snapper (b) and yellow croaker (c) and protein staining under nonreducing condition of yellow croaker sarcoplasmic protein, YC-SP (d). Std, standard markers; Boxed areas in (d) indicate gel bands excised for mass spectrometric analysis.

6.4.3 GeLC-MS/MS

Proteomic of lyophilized fractioned sarcoplasmic proteins of YC-SP was carried out using GeLC-MS/MS since it showed the most abundant proteinase inhibitors based on trypsin inhibitory activity staining (Figure 6.3c). GeLC-MS/MS was performed in the range of 25-50 kDa and 75-150 kDa with a total number of 20 gel segments, covering mass of proteinase inhibitors shown at 33-48 and 95 kDa (Figure 6.3d). LC–MS/MS analysis of the peptides generated from in-gel digestion of all 20 gel segments resulted in the identification of a total of 465 proteins. Because amino acid sequences of YC-SP proteins have not been reported in literatures, protein identification was based on similar amino acid sequences in the database using the Mascot search engine.

The excised bands 11 and 12 coinciding with molecular mass of 45-50 kDa were identified to be β-enolase with the highest spectral count (SpC), while alpha-1-antitrypsin, also known as alpha-1-proteinase inhibitor (α1-PI), was identified with lower SpC (Table 6.1). Low SpC value of α1-PI suggested that it was present in smaller amount than β-enolase in both band 11 and 12. β-Enolase is an enzyme that catalyzes a reaction of glycolysis, and has not been reported to exhibit trypsin inhibitory activity. Thus, trypsin inhibitors of YC-SP with molecular mass of 45 and 48 kDa were likely to be α1-PI. α1-PI belongs to serpin family with molecular mass ranging from 40-60 kDa and is highly specific for serine proteinase inhibition (Patterson, 1991). Based on GeLC-MS/MS of YC-SP, protein bands exhibiting trypsin inhibitory activity with molecular mass of 45 and 50 kDa found in TB-SP and BS-SP (Figure 6.3a and b) could also be assumed to be α1-PI.

A major protein at molecular mass 84-150 kDa (band 1-8) was identified to be phosphorylase with the most abundance observed in band 5 and 6 (~95-105 kDa) (Table 6.1). A 95-kDa protein was, thus, likely to be phosphorylase, which was found to be resistant to trypsin digestion in both TB-SP and YB-SP (Figure 6.3a and c), but not BS-SP (Figure 6.3b). Major proteins of band 16 (Figure 6.3d) with molecular mass about 35-40 kDa were tentatively identified to be glyceraldehyde-3-phosphate dehydrogenase, creatine kinase and aldolase, while creatine kinase and L-lactate dehydrogenase were dominant proteins in band 17 (~32-35 kDa).

Since phosphorylase, glyceraldehyde-3-phosphate dehydrogenase, and creatine kinase showed high abundance, they were tested for trypsin inhibitory capacity. Retention of all 3 proteins was observed on gel stained for trypsin inhibitor activity (Figure 6.4b). However, no inhibitory activity was detected when trypsin assay was performed (Table 6.2). Ovomucoid which was used as a positive control showed inhibitory activity in both trypsin inhibitor staining gel and trypsin activity assay. Positive results of phosphorylase, glyceraldehyde-3-phosphate dehydrogenase, and creatine kinase observed on trypsin activity staining gel appeared to be artifactual. These 3 proteins are unlikely to be a muscular trypsin inhibitor.

Phosphorylase, glyceraldehyde-3-phosphate dehydrogenase, and creatine kinase contain 31, 17, and 8 β-sheet structure, respectively (Fritz-Wolf, Schnyder, Wallimann, and Kabsch, 1996; Venkaiah and Kumar, 1991). This could limit trypsinolytic degradation during trypsin inhibitor staining.

Table 6.1 Protein identification from YB-SP using GeLC-MS/MS method.

Band	Proteins	Species	Accession Number	SpC
1	Phosphorylase	*Tetraodon nigroviridis*	Q4SFP9_TETNG	36
2	Phosphorylase	*Tetraodon nigroviridis*	Q4SFP9_TETNG	45
3	Phosphorylase	*Tetraodon nigroviridis*	Q4SFP9_TETNG	40
4	Phosphorylase	*Tetraodon nigroviridis*	Q4SFP9_TETNG	60
5	Phosphorylase	*Tetraodon nigroviridis*	Q4SFP9_TETNG	205
6	Phosphorylase	*Tetraodon nigroviridis*	Q4SFP9_TETNG	269
7	Phosphorylase	*Gasterosteus aculeatus*	Q4SFP9_TETNG	54
8	Phosphorylase	*Gasterosteus aculeatus*	Q4SFP9_TETNG	21
9	Transferrin	*Larimichthys crocea*	C3KEE6_LARCR	14
10	Transferrin	*Larimichthys crocea*	C3KEE6_LARCR	39
11	Beta-enolase	*Epinephelus coioides*	D6PVP1_EPICO	46
	Alpha-1-antitrypsin homolog	*Cyprinus carpio*	A1AT_CYPCA	9
12	Beta-enolase	*Epinephelus coioides*	D6PVP1_EPICO	305
	Alpha-1-antitrypsin homolog	*Cyprinus carpio*	A1AT_CYPCA	2
13	Beta-enolase	*Epinephelus coioides*	D6PVP1_EPICO	292
14	Muscle-type creatine kinase	*Siniperca chuatsi*	C7ASM1_SINCH	246
15	Fructose-bisphosphate aldolase	*Tetraodon nigroviridis*	H3DCM1_TETNG	317
16	Glyceraldehyde-3-phosphate dehydrogenase	*Oplegnathus fasciatus*	B5AAJ5_9PER	173
	Creatine kinase	*Poecilia reticulata*	A3RH11_POERE	108
17	Creatine kinase	*Poecilia reticulata*	A3RH11_POERE	89
	L-lactate dehydrogenase	*Lates calcarifer*	C7SJ29_LATCA	80
18	Fructose-bisphosphate aldolase	*Takifugu rubripes*	H2TGY6_TAKRU	98
19	Phosphoglycerate mutase	*Gasterosteus aculeatus*	G3Q0I0_GASAC	130
20	Muscle-type creatine kinase	*Siniperca chuatsi*	C7ASM1_SINCH	137

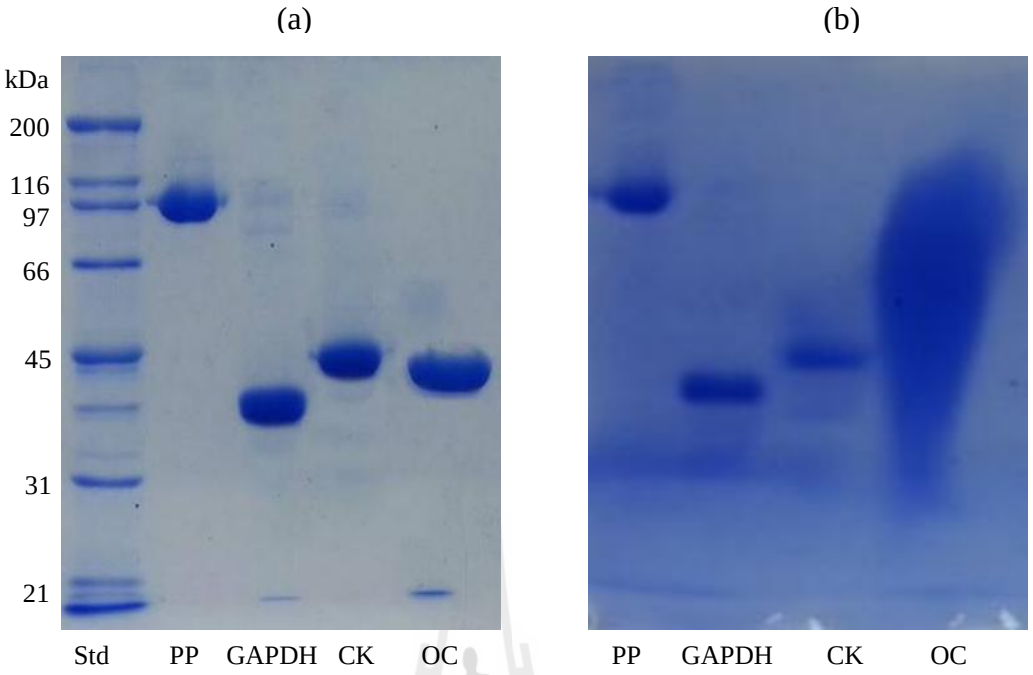

Figure 6.4 SDS-PAGE patterns on 10% polyacrylamide gel stained with Coomassie blue (a) and trypsin inhibitory activity under non-reducing condition (b). Std, standard markers; PP,phosphorylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CK, creatine kinase; OC, ovomucoid.

This study indicated that positive results observed on trypsin activity staining might not absolutely indicate “true inhibitory activity”, but rather suggest “resistance to proteolysis” of such a protein. Inhibition should be confirmed by enzyme assay. Discrepancy between trypsin inhibitor staining and enzyme assay was also found in bovine serum albumin containing 17 disulfide bonds (Piyadhammaviboon, and Yongsawatdigul, 2010). Therefore, the active trypsin inhibitor present in lyophilized fractionated sarcoplasmic protein of these 3 species is likely to be only α1-PI.

Table 6.2 Residual trypsin activity in the presence of various proteins.

Protein	Trypsin activity (Unit/mL)
Control (no protein)	22.80 ± 0.42^{bc}
Phosphorylase	22.35 ± 0.01^{b}
Glyceraldehyde-3-phosphate dehydrogenase	23.09 ± 0.03^{c}
Creatine kinase	22.89 ± 0.04^{c}
Ovomucoid	1.52 ± 0.02^{a}

Values are given as means ±SD from triplicate determinations.

* Different letters indicate differences at $P < 0.05$.

6.4.4 Thermal and NaCl stability

Sarcoplasmic proteins from three species displayed similar thermal stability. Trypsin inhibitory activity slightly decreased at temperature higher than 50 °C and completely diminished at 70-80 °C (Figure 6.5a). Common carp (*Cyprinus carpio*) sarcoplasmic proteins underwent thermal denaturation at temperature higher than 60 °C (Siriangkanakun and Yongsawatdigul, 2012). Purified trypsin inhibitor from egg of skipjack tuna *Katsuwonus pelamis* was stable in the pH ranging from 4.0 to 10.0 and at temperature below 40 °C (Choi, Park, and Kim, 2002). Inhibitory activity against trypsin considerably decreased when purified trypsin inhibitor from white croaker skeletal muscle (*Micropogon opercularis*) was heated to temperature above 60 °C (Sangorrínet al., 2001).

Surimi paste from tropical species is typically set at 40 °C, while those from cold and temperate species are performed at 5-25 °C, for promoting catalytic reaction of endogenous transglutaminase. Proteinase-laden surimi could undergo

textural degradation at this stage due to proteolytic activity of endogenous proteinases (Yongsawatdigul, Worratao, and Park, 2002). Thermal stability of trypsin inhibitor at 40 °C suggested that fractionated sarcoplasmic proteins could have potential to minimize proteolytic degradation during setting of proteinase-laden surimi.

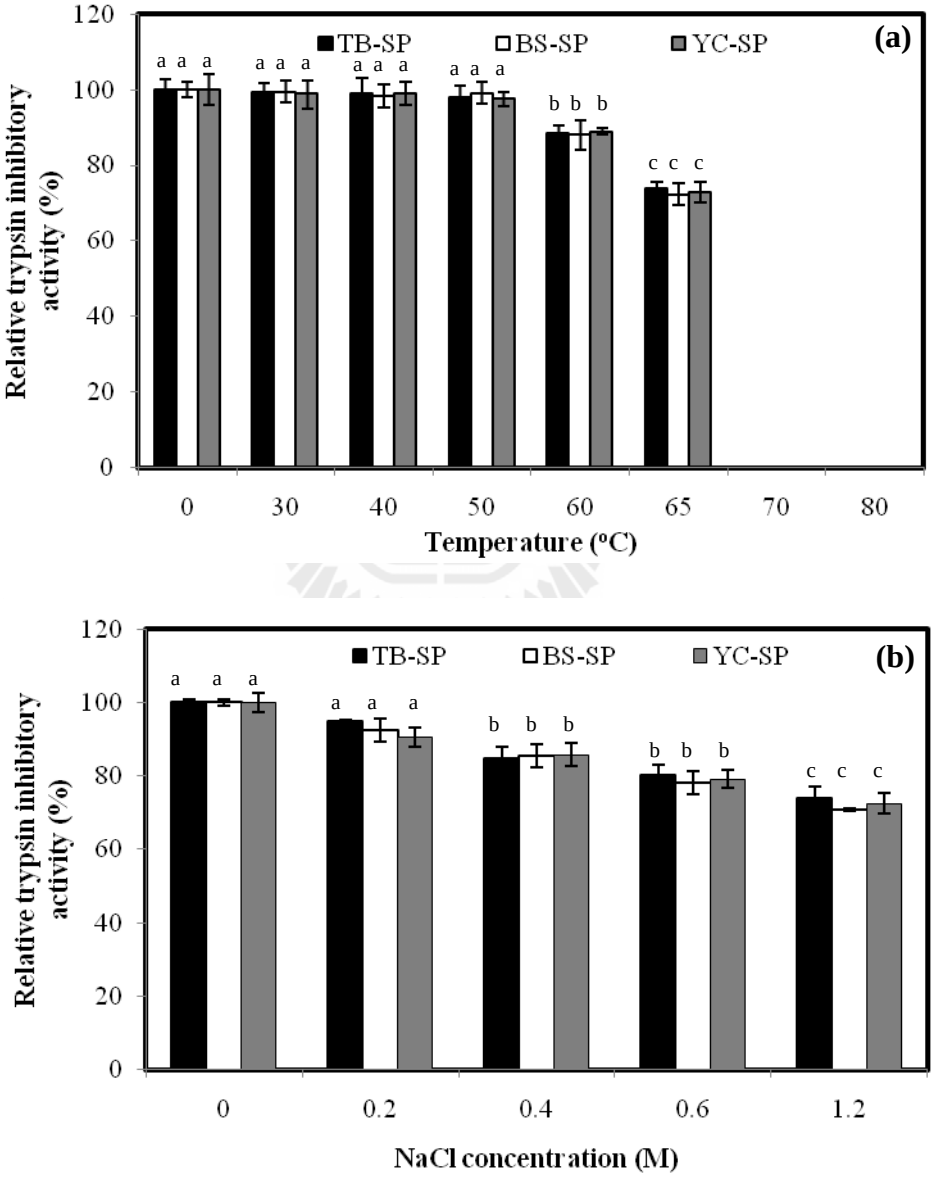

Figure 6.5 Stability of trypsin inhibitor in fractionated sarcoplasmic proteins from 3 fish species towards temperature (a) and NaCl (b). Different letters within the same fish species indicate difference at $P < 0.05$.

Relative inhibitory activity of TB-SP decreased as NaCl concentration increased (Figure 6.5b). The similar trend was also observed in BS-SP and YC-SP. These implied that NaCl might induce conformational changes of the inhibitor, resulting in a decrease of trypsin inhibitory activity. However, the remaining relative inhibitory activity was in the range of 78-86% at 0.4-0.6 M NaCl, the typical salt concentration in surimi paste. Therefore, salt content used in surimi gel preparation slightly reduce inhibitory capacity of fractionated sarcoplasmic proteins of these 3 species.

6.4.5 Effect of sarcoplasmic proteins on autolytic degradation

Washed threadfin bream mince incubated at 37 °C showed lower TCA-soluble oligopeptide content than at 65 °C regardless the amount of salt and fractionated sarcoplasmic protein added (Table 6.3). Heat-activated proteinases exhibiting high proteolytic activity at 60-65 °C were found in threadfin bream surimi (Rawdkuen and Benjakul, 2008). In the absence of fractionated sarcoplasmic proteins, TCA soluble oligopeptide content of samples added 2% NaCl was higher than those in the absence of NaCl at both studied temperatures. This could be due to salt solubilizing effect of myofibrillar proteins, leading to an increase in substrate accessibility to endogenous proteinases. TCA-soluble oligopeptide content decreased as addition level of sarcoplasmic protein content increased at both incubation temperatures ($P < 0.05$). At 2% addition, TB-SP and YC-SP showed higher inhibition of 61-65% as compared to BS-SP, which showed about 50% inhibition at 65 °C. Residual proteolytic activity was only found in BS-SP, which limited its use as functional ingredient for controlling proteolysis of surimi. The combined addition of SP and 2% NaCl showed higher TCA soluble oligopeptide than samples added SP

alone ($P < 0.05$). This confirmed the effect of NaCl on reduction of inhibitory activity (Figure 6.5b).

Table 6.3 TCA-soluble oligopeptide of washed threadfin bream mince added with lyophilized fractionated sarcoplasmic proteins

Sarcoplasmic protein	Addition level (%)	TCA-soluble oligopeptide (µmol/g sample)			
		37 °C		65 °C	
		0% NaCl	2% NaCl	0% NaCl	2% NaCl
No inhibitor	0	0.265±0.004^{a}	0.298±0.006^{a}	1.392±0.009^{a}	1.965±0.014^{a}
TB-SP	0.5	0.243±0.004^{bc}	0.275±0.006^{bc}	0.693±0.006^{d}	1.102±0.004^{d}
	1	0.232±0.003^{def}	0.263±0.004^{df}	0.587±0.004^{e}	0.941±0.009^{f}
	2	0.223±0.006^{f}	0.255±0.007^{f}	0.489±0.011^{g}	0.905±0.008^{g}
BS-SP	0.5	0.248±0.003^{b}	0.285±0.005^{b}	0.804±0.007^{b}	1.186±0.007^{b}
	1	0.238±0.004^{bcd}	0.272±0.004^{bc}	0.756±0.004^{c}	1.058±0.007^{e}
	2	0.229±0.007^{def}	0.269±0.008^{cd}	0.759±0.014^{c}	1.055±0.010^{e}
YC-SP	0.5	0.245±0.004^{bc}	0.282±0.003^{bc}	0.751±0.004^{c}	1.125±0.010^{c}
	1	0.236±0.004^{cde}	0.269±0.007^{cd}	0.687±0.008^{d}	1.085±0.007^{d}
	2	0.225±0.004^{ef}	0.258±0.003^{df}	0.548±0.007^{f}	0.943±0.011^{f}

Values are given as means ±SD from duplicate determinations.

* Different letters in the same column indicate difference at $P < 0.05$.

Severe degradation of myosin heavy chain (MHC) of washed threadfin bream mince in the absence of sarcoplasmic proteins was evident at 65 °C, suggesting proteolysis was induced by heat activated endogenous proteinases (Figure 6.6). Band intensity of actin was comparable in all samples, indicating that actin was not a preferred substrate of threadfine bream endogenous proteinase(s). A decrease of MHC

of samples added 2% NaCl occurred to a greater extent than that of samples without NaCl addition. This result was in agreement with higher TCA-soluble oligopeptide contents of samples added NaCl (Table 6.3).

More retention of MHC was observed in the samples added fractionated sarcoplasmic proteins. MHC band intensity increased as concentration of inhibitors increased, corresponding well with low TCA-soluble oligopeptide contents (Table 6.3). At the same concentration, BS-SP showed the lowest inhibitory activity as judged by SDS-PAGE pattern (Figure 6.6b) and higher TCA-soluble oligopeptide content (Table 6.3). MHC band intensity of samples added TB-SP and YC-SP increased with addition levels of fractionated sarcoplasmic protein (Figure 6.6a and c). TB-SP and YC-SP could be applied in washed threadfin bream mince and surimi with effective concentration of 2%. However, addition of 2% BS-SP did not increase retention of MHC. TCA-soluble oligopeptide, inhibitory activity of 1% BS-SP was comparable to that of 2% BS-SP at 65 °C ($P > 0.05$, Table 6.3). Therefore, the effective addition level of BS-SP was 1%.

Although proteinase inhibitory activity appeared to decrease with an increased NaCl when tested in the enzyme assay, protection of MHC by sarcoplasmic proteins of 3 species was observed even in the presence of NaCl. Protective effect of BS-SP appeared to be the least based on TCA-soluble oligopeptide content (Table 6.3) and protein degradation pattern (Figure 6.6b). No differences on muscle protein pattern were found at 37 °C (data not shown). These results indicated that addition of TB-SP and YC-SP effectively inhibited autolytic degradation of washed threadfin bream mince at both temperatures, 37 and 65 °C.

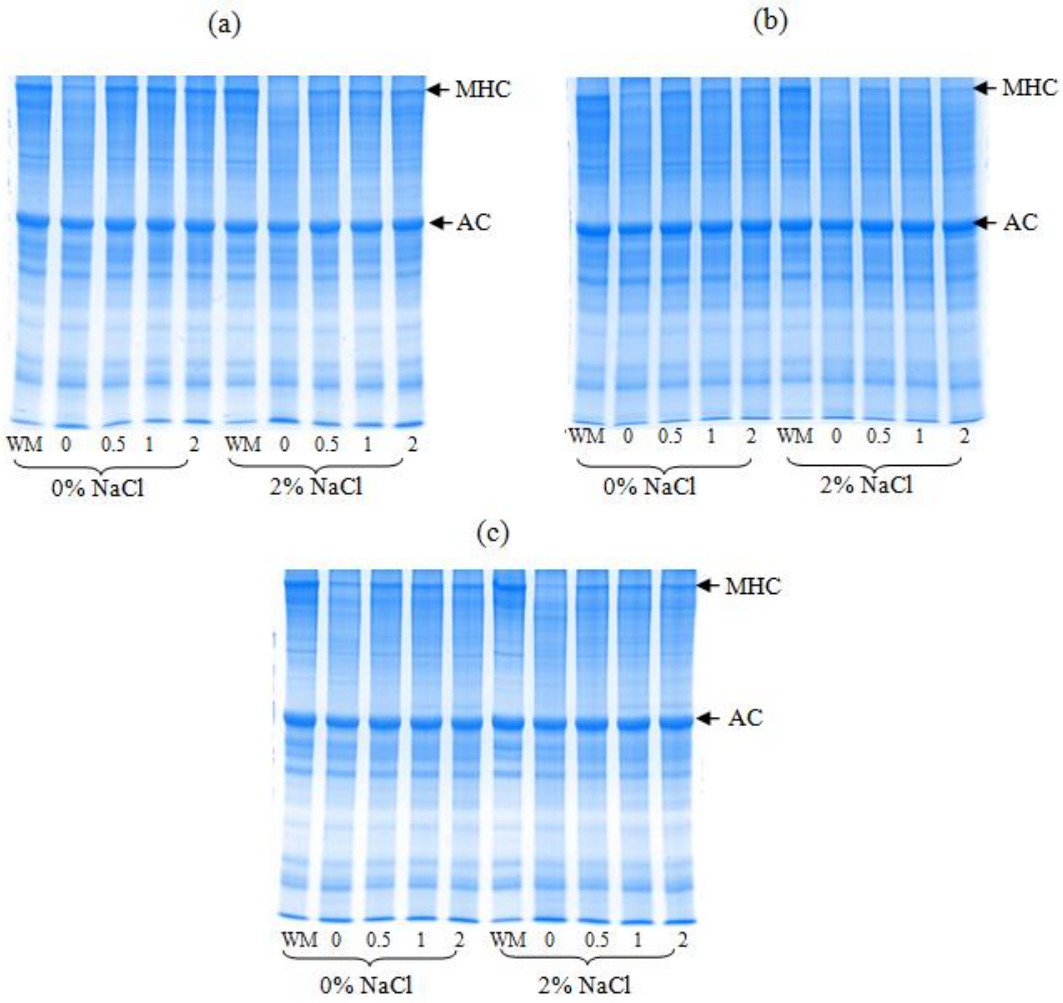

Figure 6.6 Degradation patterns of washed threadfin bream mince containing TB-SP (a), BS-SP (b) and YC-SP (c) at different levels in the absence and presence of 2% NaCl. Samples were incubated at 65 °C for 1 h. Numbers denote the amounts of fractionated sarcoplasmic protein added (%). WM, washed mince without heating; MHC, myosin heavy chain; AC, actin.

6.4.6 Effect of TB-SP on textural properties of surimi

Since TB-SP showed the highest inhibitory activity based on the enzyme assay (Figure 6.2) and protein degradation (Figure 6.6a), its effect on textural properties of surimi was elucidated using commercial threadfin bream surimi. Threadfin bream surimi gels pre-incubated at 65 °C without inhibitors showed the lowest textural properties ($P < 0.05$, Figure 6.7), indicating the evidence of endogenous proteinase activity similar to that of washed mince (Figure 6.6). In the absence of TB-SP, breaking force and deformation values of gels pre-incubated at 37 °C increased by 36.3% and 23.9%, respectively, as compared to those heated at 90 °C without setting. Pre-incubation at 37 °C could induce unfolding and exposure of buried hydrophobic and sulfhydryl groups of actomyosin, leading to formation of hydrophobic interactions and/or disulfide linkages (Hemung and Yongsawatdigul, 2005; Yongsawatdigul and Sinsuwan, 2007). Moreover, it could be attributed from the action of endogenous transglutaminase.

Textural properties of surimi gel increased with addition level of TB-SP. Breaking force of gel added 1% TB-SP and preincubated at 37 °C increased by 46% compared to that of sample without TB-SP. Inhibition of proteolytic activity in conjuction with endogenous TGase activity might contribute to such an improvement.

Textural improvement was also observed at 65 °C, the most deteriorated temperature for gel texture, at the addition level of 0.5 and 1% of TB-SP (Figure 6.7). Addition of EW showed higher textural properties than TB-SP at the same concentrations regardless of the heating regimes applied ($P < 0.05$).

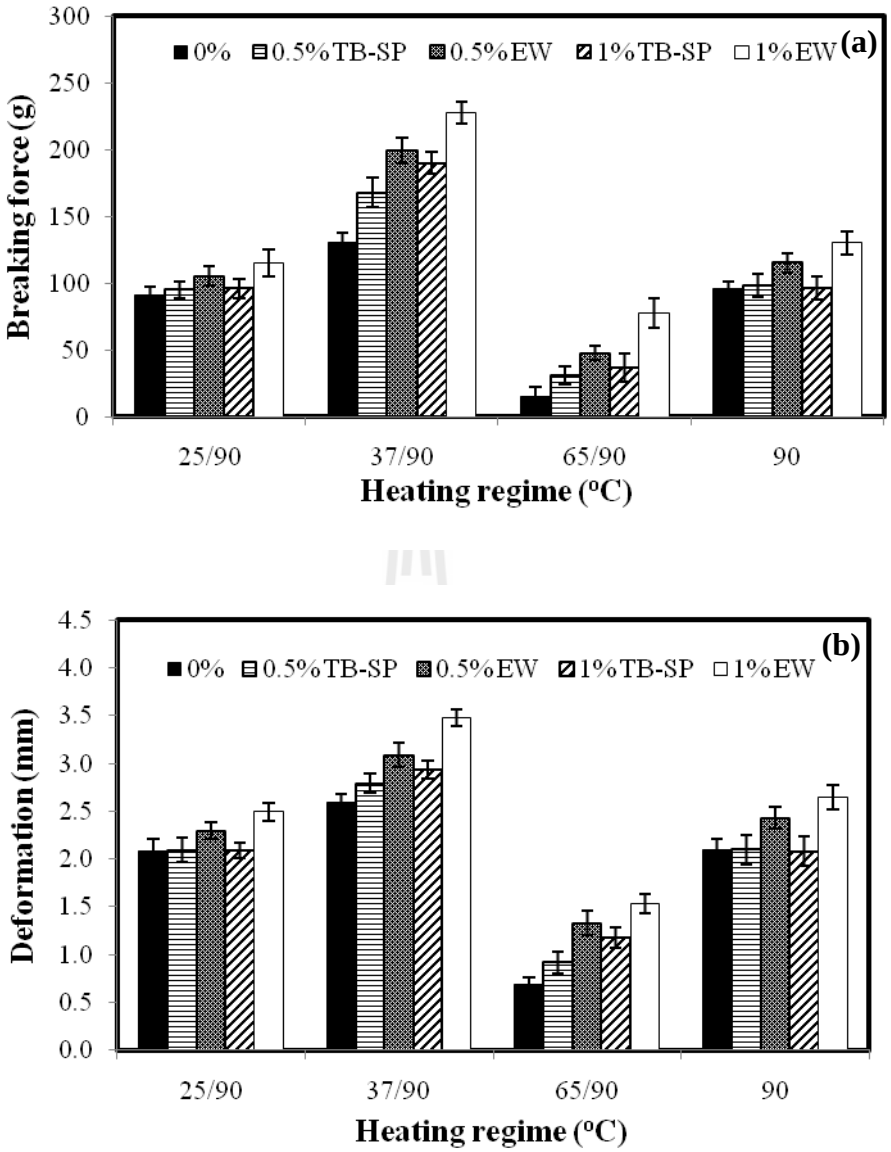

Figure 6.7 Breaking force (a) and deformation (b) of threadfin bream surimi mixed with various concentrations of TB-SP and EW and subjected to various heating conditions. TB-SP, lyophilized fractioned threadfin bream sarcoplasmic proteins; EW, egg white powder; numbers indicate addition level of inhibitors.

An increase of textural properties of samples added TB-SP at 37 and 65 °C is likely resulted from inhibitory activity of α1-PI. EW has been known to be a

highly specific serine and cysteine proteinase inhibitor (Nakamura and Doi, 2000). In addition, EW was also found to have gel-forming ability (Hunt, Park, and Handa, 2009). Addition of 0.5% and 1%TB-SP had no effect on textural properties of threadfin bream surimi gels pre-incubated at 25 °C or those heated directly to at 90 °C. This was likely because proteinase activity in threadfin bream surimi at 25 and 90 °C was less pronounce. In addition, gel-forming ability of TB-SP appeared to be insignificant. Morioka et al (1992) reported that sarcoplasmic proteins from Pacific mackerel were able to form gels at 10% protein or higher. These results indicated that addition of 0.5 and 1% TB-SP along with pre-incubation at 37 and 65 °C was able to improve textural properties of threadfin bream surimi and the main enhancing effect was contributed from proteinase inhibitory of TB-SP.

6.5 Conclusions

TB-SP, BS-SP and YC-SP exhibited inhibitory activity toward trypsin. α1-PI was likely to be responsible for such inhibitory activity. Thermal stability of fractionated sarcoplasmic proteins of 3 species was stable at 30-50 °C. Trypsin inhibitory activity decreased up to 20% at 0.4 M NaCl. TB-SP and YC-SP effectively inhibited the autolytic activity of washed threadfin bream mince. The addition of TB-SP improved textural properties of threadfin bream surimi gel, particularly when pre-incubated at 37 °C. Therefore, fractionated TB-SP could be used as a functional ingredient to control proteolysis and improve gel texture of proteinase-laden surimi.

6.6 References

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CHAPTER VII

SUMMARY

Common carp exhibited much lower autolytic activity than goatfish. Autolytic and transglutaminase activity of common carp and goatfish were inactivated by alkaline pH-shift process. Major proteinases at pH 7, a typical pH of food product, of common carp mince was serine proteinase(s), especially trypsin-like proteinase(s) whereas goatfish mince contained both serine- and metallo- proteinases. Common carp gels showed much higher textural properties than goatfish gels. Conventional washing process was suitable for improving textural properties of common carp muscle proteins. In contrast, breaking force of goatfish gels was enhanced by alkaline pH-shift process. Setting did not improve textural properties of both species gels prepared by alkaline pH-shift process. Therefore, common carp could be used as an alternative raw material for surimi-based products.

Sarcoplasmic protein (SP) from common carp exhibited inhibitory activity toward trypsin. Trypsin activity staining of non-reducing SDS-PAGE showed 5 remaining protein bands with molecular mass of 35, 41, 47, 52, and 69 kDa with incubation at 40 °C while only 2 proteins were found with molecular mass of 35 and 54 kDa incubated at 65 °C. SP alone effectively reduced proteolysis of threadfin bream surimi and increased its textural properties to a greater extent than 0.1% Ca^{2+}. Common carp SP might be a potential alternative food-grade inhibitor applied to enhance textural properties of surimi.

Proteinase inhibitors with molecular mass of 47 and 52 kDa were purified from common carp sarcoplasmic proteins. Both inhibitors were identified to be alpha-1-proteinase inhibitor (α1-PI). Isoelectric point (pI) of both inhibitors were about 5.3 and 5.4. The inhibitors were glycoproteins and had two N-glycosylation sites at both N214 and N226. Trypsin was strongly inhibited by both inhibitors. In addition, α1-PI showed good inhibitory activity towards autolytic degradation of bigeye snapper surimi. Thus, α1-PI could be applied to minimize proteolytic degradation of fish muscle proteins.

Lyophilized sarcoplasmic fraction of threadfin bream (TB-SP), bigeye snapper (BS-SP) and yellow croaker (YC-SP) exhibited inhibitory activity against trypsin. α1-PI was likely to be present in fractionated sarcoplasmic proteins of 3 species. Trypsin inhibitory activity of three species showed good thermal stability in the temperature range of 35-60 °C. NaCl might induce conformational changes of the inhibitor molecule in three species. High textural properties of threadfin bream surimi with addition of TB-SP was probably resulted from the action of α1-PI. Proteinase inhibitors in sarcoplasmic protein could be recovered and used to improve textural properties of surimi products.

APPENDIX

Supporting materials for Chapter V

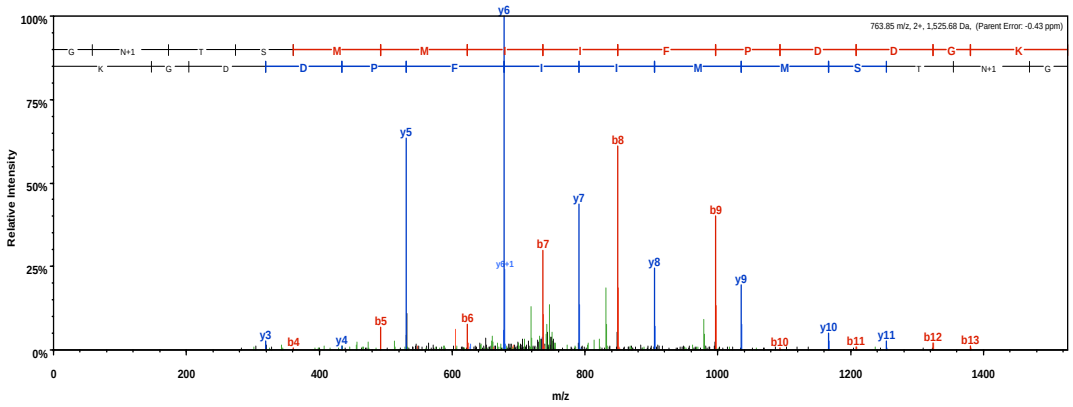

Figure 1A MS/MS ion chromatogram of inhibitor I of the peptide GNTSMMIIFPDDGK with deglycosylated asparagines at N226.

Table 1A Ion series of inhibitor I of the peptide GNTSMMIIFPDDGK with deglycosylated asparagines at N226 upon fragmentation by MS/MS.

B	B Ions	B+2H	AA	Y Ions	Y+2H	Y
1	58.0	-	G	1,526.7	763.8	14
2	173.1	-	N+1	1,469.7	**735.3**	13
3	274.1	-	T	1,354.6	**677.8**	12
4	**361.1**	-	S	**1,253.6**	**627.3**	11
5	**492.2**	-	M	**1,166.6**	583.8	10
6	**623.2**	312.1	M	**1,035.5**	518.3	9
7	**736.3**	368.7	I	**904.5**	452.7	8
8	**849.4**	425.2	I	**791.4**	396.2	7
9	**996.5**	498.7	F	**678.3**	339.7	6
10	**1,093.5**	**547.3**	P	**531.2**	-	5
11	**1,208.5**	**604.8**	D	**434.2**	-	4
12	**1,323.6**	**662.3**	D	**319.2**	-	3
13	**1,380.6**	**690.8**	G	204.1	-	2
14	1,526.7	763.8	K	147.1	-	1

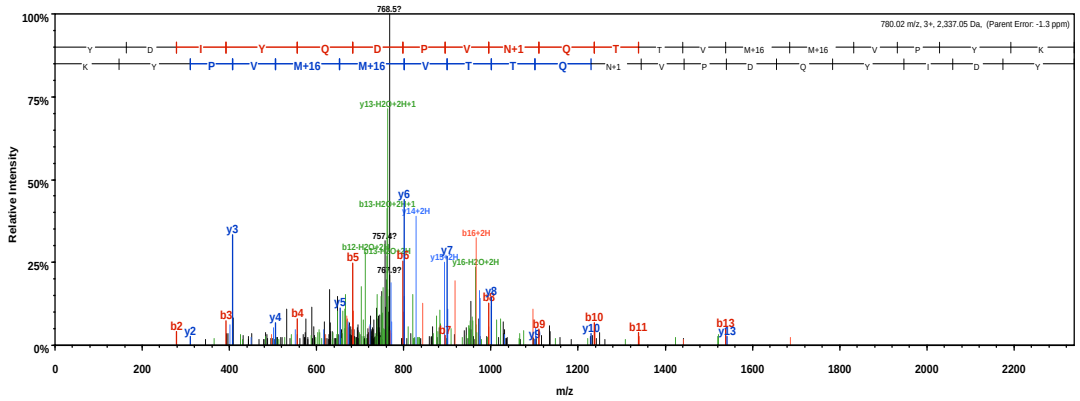

Figure 2A MS/MS ion chromatogram of inhibitor II of the peptide YDIYQDPVNQTTVMMVPYK with deglycosylated asparagines at N214.

Table 2A Ion series of inhibitor II of thevpeptide YDIYQDPVNQTTVMMVPYK with deglycosylated asparagines at N214 upon fragmentation by MS/MS.

B	B Ions	B+2H	AA	Y Ions	Y+2H	Y
1	164.1	82.5	Y	2,338.1	1,169.5	19
2	**279.1**	140.1	D	2,175.0	1,088.0	18
3	**392.2**	196.6	I	2,060.0	**1,030.5**	17
4	**555.2**	278.1	Y	1,946.9	**973.9**	16
5	**683.3**	342.2	Q	1,783.8	**892.4**	15
6	**798.3**	399.7	D	1,655.8	**828.4**	14
7	**895.4**	448.2	P	**1,540.7**	**770.9**	13
8	**994.5**	**497.7**	V	1,443.7	**722.3**	12
9	**1,109.5**	555.2	N+1	1,344.6	**672.8**	11
10	**1,237.5**	**619.3**	Q	**1,229.6**	**615.3**	10
11	**1,338.6**	**669.8**	T	**1,101.5**	**551.3**	9
12	**1,439.6**	**720.3**	T	**1,000.5**	**500.7**	8
13	**1,538.7**	**769.9**	V	**899.4**	**450.2**	7
14	**1,685.7**	**843.4**	M+16	**800.4**	**400.7**	6
15	1,832.8	**916.9**	M+16	**653.3**	327.2	5
16	1,931.8	**966.4**	V	**506.3**	253.7	4
17	2,028.9	1,015.0	P	**407.2**	204.1	3
18	2,192.0	**1,096.5**	Y	**310.2**	155.6	2
19	2,338.1	1,169.5	K	147.1	74.1	1

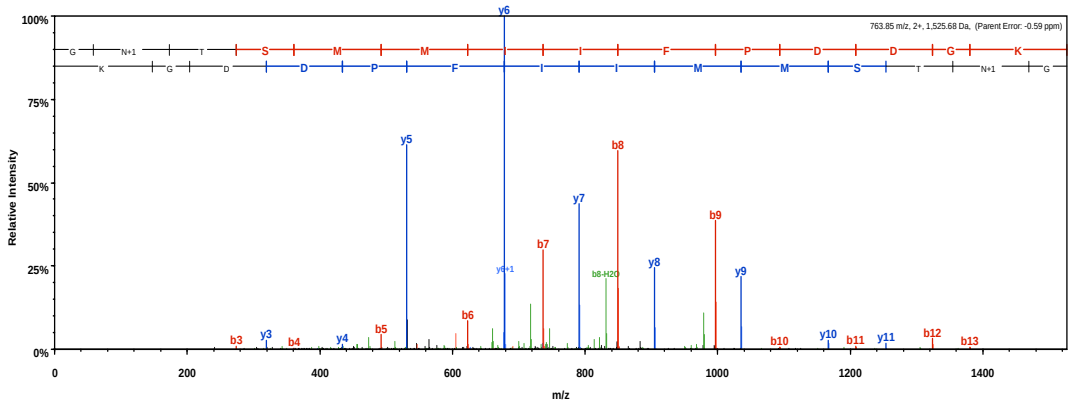

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Table 3A Ion series of inhibitor II of the peptide GNTSMMIIFPDDGK with deglycosylated asparagines at N226 upon fragmentation by MS/MS.

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4	**361.1**	-	S	**1,253.6**	**627.3**	11
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6	**623.2**	312.1	M	**1,035.5**	518.3	9
7	**736.3**	368.7	I	**904.5**	452.7	8
8	**849.4**	425.2	I	**791.4**	396.2	7
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10	**1,093.5**	**547.3**	P	**531.2**	-	5
11	**1,208.5**	**604.8**	D	**434.2**	-	4
12	**1,323.6**	**662.3**	D	**319.2**	-	3
13	**1,380.6**	**690.8**	G	204.1	-	2
14	1,526.7	763.8	K	147.1	-	1

BIOGRAPHY

Siriphon Siriangkanakun was born in August 5, 1983 in Phitsanulok Province, Thailand. She studied at Chalermkwansatree School and graduated in 2002. In 2005, she received the degree of Bachelor of Science (Agro-Industry) with first honor from Naresuan University, Phitsanulok, Thailand. In 2007-2010, she received the Strategic Scholarships for Frontier Research Networks from Commission on Higher Education, Ministry of Education Fund to study for the degree of Doctor of Philosophy (Food Technology) at Suranaree University of Technology. During her graduate study, she obtained opportunities to present her research works including IFT annual meeting and Food expo (Las Vegas, Nevada, June 25-28th, 2012) under the title of "Purification and characterization of trypsin inhibitor from common carp (*Cyprinus carpio*) muscle" and EFFoST Annual Meeting 2013 (Bologna, Italy, November 12-15th, 2013) under the title of "Purification and identification of trypsin inhibitor from common carp (*Cyprinus carpio*) sarcoplasmic proteins for surimi gel improvement". She also published her research work under the title of "Trypsin inhibitory activity and gel-enhancing effect of sarcoplasmic proteins from common carp" in Journal of Food Science (Vol. 77, page C1124-C1130) in 2012.