EFFECT OF SARCOPLASMIC PROTEINS FROM THREADFIN BREAM AS GEL ENHANCER OF LIZARDFISH SURIMI GEL

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ผลของโปรตีนซาร์โคพลาสมิกจากปลาทรายแดงต่อการเพิ่มคุณภาพเจลซูริมิปลาปากคม

เพ็ญประภา ปิยธรรมวิบูลย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECT OF SARCOPLASMIC PROTEINS FROM THREADFIN BREAM AS GEL ENHANCER OF LIZARDFISH SURIMI GEL

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

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เพ็ญประภา ปิยธรรมวิบูลย์ : ผลของโปรตีนซาร์โพลาสมิกจากปลาทรายแดงต่อการเพิ่มคุณภาพเจลซูริมิปลาปากคม (EFFECT OF SARCOPLASMIC PROTEINS FROM THREADFIN BREAM AS GEL ENHANCER OF LIZARDFISH SURIMI GEL) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 111 หน้า

ปลาทรายแดง (*Nemipterus* spp.) เป็นปลาสายพันธุ์หลักที่ใช้เป็นวัตถุดิบหลักในการผลิตซูริมิในประเทศไทย มีปริมาณโปรตีนซาร์โคพลาสมิกจากปลาทรายแดงเป็นจำนวนมากเกิดขึ้นจากขั้นตอนการล้างของการผลิตซูริมิ ดังนั้นวัตถุประสงค์ของการศึกษานี้ คือ เก็บเกี่ยวโปรตีนซาร์โคพลาสมิกจากน้ำล้างปลาทรายแดง (TBSP) ศึกษาคุณลักษณะของเอนไซม์ทรานสกลูทามิเนสและตรวจวัดกิจกรรมของการยับยั้งเอนไซม์โปรตีเนสที่มีอยู่ในโปรตีนซาร์โคพลาสมิก จากปลาทรายแดง โปรตีนซาร์โคพลาสมิกจากปลาทรายแดงทำให้เข้มข้นด้วยอัลตร้าฟิลเตรชั่นที่มีเยื่อกรองขนาด 30 กิโลดาลตัน แสดงกิจกรรมของเอนไซม์ทรานสกลูทามิเนสที่ดีที่อุณหภูมิ 37 องศาเซลเซียส และที่พีเอช 7.5 การเติมแคลเซียมคลอไรด์ ($CaCl_2$) ส่งเสริมกิจกรรมของเอนไซม์ทรานสกลูทามิเนสเมื่อเพิ่มปริมาณแคลเซียมจนถึงความเข้มข้น 5 มิลลิโมลาร์ เอนไซม์ทรานสกลูทามิเนสแสดงกิจกรรมสูงสุดที่ความเข้มข้นของดีทีที (DTT) 1 มิลลิโมลาร์ โปรตีนซาร์โคพลาสมิกจากน้ำล้างปลาทรายแดงสามารถเหนี่ยวนำให้เกิดการเชื่อมโยงโมเลกุลโปรตีนในโปรตีนอัลบูมินจากวัว (BSA) เมื่อทำการบ่มที่ 25 องศาเซลเซียส เป็นเวลา 6 ชั่วโมง การศึกษากิจกรรมของเอนไซม์ทรานสกลูทามิเนสบนแผ่นเจลอะคริลาไมด์สภาพธรรมชาติ (Native-PAGE) โดยทำปฏิกิริยากับโมโนแดนซิลคาดาเวอรีน (Monodansylcadavarine) และไดเมททิลเคซีน (di-Methylated casein) พบว่ามีโปรตีน 2 แถบ เรืองแสงภายใต้แสงฟลูออเรสเซนต์ โดยมีขนาด 78 และ 189 กิโลดาลตัน โปรตีน 2 แถบที่เรืองแสงภายใต้แสงฟลูออเรสเซนต์มีขนาดโมเลกุล 66 กิโลดาลตัน เมื่อวิเคราะห์โดยเทคนิคอิเล็กโตโฟเรซิสแบบสูญเสียสภาพธรรมชาติ (SDS-PAGE)

โปรตีนซาร์โคพลาสมิกจากน้ำล้างปลาทรายแดงแสดงความสามารถในการเพิ่มค่าความแข็งแรงในซูริมิปลาปากคม การเติมแคลเซียมคลอไรด์เข้มข้น 0.1 เปอร์เซ็นต์ ร่วมกับการเติมโปรตีนซาร์โคพลาสมิกจากน้ำล้างปลาทรายแดงเข้มข้น 1.6 เปอร์เซ็นต์ในซูริมิปลาปากคมแสดงค่าแรงและค่าระยะทาง ณ จุดแตกหักสูงที่สุดเมื่อทำการบ่มที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 20 นาที

การศึกษากิจกรรมการยับยั้งเอนไซม์โปรตีเนสต่อเอนไซม์ทริปซิน ปาเปน และไคโมทริปซินพบว่าโปรตีนซาร์โคพลาสมิกสามารถยับยั้งการทำงานของทริปซินได้ และการยับยั้งลดลงเมื่อบ่มที่ 55 องศาเซลเซียส เป็นเวลา 15 นาที ผลของ SDS-PAGE ภายใต้สภาวะไม่ถูกรีดิวซ์ (Non-

reducing) ย้อมด้วยสารละลายทริปซิน แสดงแถบโปรตีน 3 แถบ ขนาดโมเลกุล 95, 41 และ 37 กิโลดาลตัน ที่สามารถทนการย่อยของสารละลายทริปซิน ค่าแรงและระยะทาง ณ จุดแตกหักของซูริมิปลาปากคมที่บ่มที่ 37 องศาเซลเซียส เป็นเวลา 20 นาที เพิ่มขึ้นเมื่อเติมโปรตีนซาร์โคพลาสมิก และพบว่าปริมาณโอลิโกเปบไทด์ (TCA-oligopeptide content) ของเจลซูริมิลดลงเมื่อเติมโปรตีนซาร์โคพลาสมิก 0.4 เปอร์เซ็นต์ การคงอยู่ของมัยโอซินสายหลัก (MHC) เพิ่มขึ้นเมื่อมีการเติมโปรตีนซาร์โคพลาสมิกเพิ่มขึ้น เมื่อทำการบ่มซูริมิปลาปากคมที่ 37 องศาเซลเซียส การเติมโปรตีนซาร์โคพลาสมิกมีผลลดการเสื่อมสลายมัยโอซินสายหลักแต่ไม่พบปรากฏการณ์ดังกล่าวเมื่อทำการบ่มปลาปากคมที่ 65 องศาเซลเซียส อย่างไรก็ตามการเพิ่มค่าความแข็งแรงเจลของซูริมิปลาปากคมอาจเกิดจากบทบาทของกิจกรรมเอนไซม์ทรานสกลูทามิเนสและสารยับยั้งเอนไซม์โปรตีเนสที่มีอยู่ในโปรตีนซาร์โคพลาสมิก

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2551 ลายมือชื่ออาจารย์ที่ปรึกษา_____

PENPRABHA PIYADHAMMAVIBOON : EFFECT OF SARCOPLASMIC PROTEINS FROM THREADFIN BREAM AS GEL ENHANCER OF LIZARDFISH SURIMI GEL. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 111 PP.

THREADFIN BREAM / SARCOPLASMIC PROTEIN/ PROTEINASE INHIBITOR/TRANSGLUTAMINASE/ SURIMI

Threadfin bream (*Nemipterus* spp. ; TB) is the main species used as raw material for surimi production in Thailand. Large amount of sarcoplasmic proteins is typically eliminated during washing step of surimi production. The objectives of this study were to recover sarcplasmic protein from TB wash water, characterize TGase contained in the sarcplasmic protein and investigate the proteinase inhibitory activity. Threadfin bream sarcoplasmic proteins (TBSP) were concentrated by ultrafiltration using a membrane with molecular weight cut-off 30 kDa. Optimum TGase activity from TBSP was at 37^{o}C, pH 7.5. An addition of $CaCl_2$ promoted TGase activity and reached the maximum at 5 mM $CaCl_2$. The highest TGase activity was found at 1 mM dithiothreitol (DTT). TBSP induced cross-linkings of bovine serum albumin when incubated at 25^{o}C for 6 h. TGase activity staining by monodansylcadavarine (MDC) on native-polyacrylamide gel electrophoresis showed 2 distinct fluorescent bands with molecular weight (MW) of 78 and 189 kDa. Two proteins showing fluorescence bands exhibited MW of 66 kDa on SDS-PAGE. An addition of 0.1% $CaCl_2$ in combination with 1.6% TBSP exhibited the highest breaking force and deformation of

lizardfish surimi when pre-incubated at 37°C for 20 min. TBSP showed potential to enhance gel strength of lizardfish surimi.

The inhibitory activity of TBSP was also investigated against three proteinases, namely papain, trypsin and α-chymotrypsin. TBSP exhibited an inhibitory activity toward trypsin and its activity diminished when incubated at 55^{o}C for 15 min. SDS-PAGE under non-reducing condition stained by trypsin revealed three protein bands with MW of 95, 41 and 37 kDa. Breaking force and deformation of lizardfish surimi gel added TBSP and pre-incubated at 37^{o}C for 20 min increased with the addition of TBSP (p<0.05). TCA-oligopeptide content of lizardfish surimi gel added TBSP decreased at 0.4% TBSP (p<0.05). Retention of myosin heavy chain (MHC) increased when TBSP concentration increased. TBSP effectively protected MHC from proteolysis when incubated at 37^{o}C, but an efficacy of TBSP on the degradation of MHC was not observed at 65^{o}C. However, both TGase activity and proteinase inhibitory activity of TBSP played an important role in contributing gel enhancing effects to lizardfish surimi.

School of Food Technology Student' Signature_____

Academic Year 2008 Advisor' Signature_____

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LIST OF ABBREVIATIONS

AC	=	Actin
Ala	=	Alanine
α_2-M	=	α_2-macroglobulin
ANOVA	=	Analysis of variance
Arg	=	Arginine
Asp	=	Aspartic acid
BASI	=	Barley α-amylase/subtilisin inhibitor
BBI	=	Bowman-Birk proteinase inhibitor
BBBI	=	Barley Bowman-Birk proteinase inhibitor
BPP	=	Beef plasma protein
BSA	=	Bovine serum albumin
BSE	=	Bovine spongiform encephalopathy
Carb	=	Carbonic anhydrase
CD	=	Circular dichroism
CEA	=	Chicken egg albumin
CI	=	Chymotrypsin/subtilisin inhibitor
CP	=	Cross-linked protein
CS	=	Casein
C-terminal	=	Carboxyl terminal
°C	=	Degree Celsius
Da	=	Dalton

LIST OF ABBREVIATIONS (Continued)

DMC	=	N,N'-Dimethylated casein
DMRT	=	Duncan's multiple range test
DTT	=	Dithiothreitol
EDTA	=	Ethylenediaminetetraacetic acid
EW	=	Egg white
Gly	=	Glycine
×g	=	Relative centrifugal force
h	=	Hour
kDa	=	Kilodalton
Leu	=	Leucine
LLDPE	=	Linear low density polyethylene
Lys	=	Lysine
M	=	Molar
MDC	=	Monodansylcadaverine
MHC	=	Myosin heavy chain
MW	=	Molecular weight
MWCO	=	Molecular weight cut-off
mA	=	Milliampere
min	=	Minute
mg	=	Milligram
mL	=	Milliliter
mM	=	Millimolar

LIST OF ABBREVIATIONS (Continued)

MTGase	=	Microbial transglutaminase
µL	=	Microliter
µm	=	Micrometer
N-termianl	=	Amino terminal
native-PAGE	=	Native polyacrylamide gel electrophoresis
nm	=	Nanometer
nmole	=	Nanomole
PI	=	Proteinase inhibitor
Phe	=	Phenylalanine
PPI	=	Plant proteinase inhibitor
s	=	Second
SDS	=	Sodium dodecyl sulfate
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	=	Serine
SpC	=	Sarcoplasmic protein concentrate
SSI	=	*Streptomyces* subtilisin inhibitor
STI	=	Soy bean trypsin inhibitor
TB	=	Threadfin bream
TBSP	=	Threadfin bream sarcoplasmic protein
TCA	=	Trichloroacetic acid
TGase	=	Transglutaminase
tTGase	=	Tissue transglutaminase

LIST OF ABBREVIATIONS (Continued)

Thr	=	Threonine
TM	=	Tropomyosin
TPCK	=	N-tosyl-L-phenylalanine chloromethyl ketone
Tris	=	Tris (hydroxymethyl) aminomethane
Tyr	=	Tyrosine
UV	=	Ultraviolet
Val	=	Valine
WPC	=	Whey protein concentrate

CHAPTER I

INTRODUCTION

1.1 Introduction

Thailand is the second largest surimi producer of the world. In 2005, production of surimi in Thailand were 150,000 metric tons (MT) and 36 % of surimi was produced from threadfin bream (TB) (Pangsorn, Laong-manee and Siriraksophon, 2007). In surimi production, mince fish is repeatedly washed with water to remove sacroplasmic proteins and other components, such as lipid and myoglobin. It has been recognized that sarcoplasmic proteins hinder gel-forming ability of myofibrillar proteins and sarcoplasmic proteins were not recovered because they have lower functionality than myofibrillar protein fractions (Kawai, Ohno, Wakameda, Inoue and Shinano, 1995). However, it has recently been reported that sarcoplasmic proteins can enhance gel strength of myfibrillar proteins. The gel strength of lizardfish gel increased with addition of tilapia sarcoplasmic proteins (Yongsawatdigul and Piyadhammaviboon, 2007). Morioka and Shimizu (1993) suggested that gels prepared from sarcoplasmic proteins with MW of 94, 40, and 26 kDa showed high gel strength. Contribution of freeze-dried rockfish sarcoplasmic proteins in increasing breaking force of pollock surimi was reported (Kim, Yongsawatdigul, Park and Thawornchinsombut, 2005). Karthikeyan, Mathew, Shamasundar and Prakash (2004) also reported that sarcoplasmic proteins from oil sardine (*Sardinella longiceps*) fractionated by ammonium sulfate were able to

enhance storage modulus (G') of washed sardine meat gel. These evidences suggested that sarcoplasmic proteins did not interfere with gel-forming of myofibrillar proteins.

Transglutaminase (TGase) (glutaminyl-peptide γ-glutamyl transferase, EC 2.3.2.13) is an enzyme catalyzing the acyl-transfer reaction introducing covalent cross-links between proteins as well as peptides and various primary amines. (Folk, 1980) TGase widely distributed in fish species (Kishi, Nozawa and Seki, 1991; Kumazawa, Nakanishi, Yasueda and Motoki, 1996; Woratao and Yongsawatdigul, 2005; Yasueda, Kumazawa, and Motoki, 1994). These TGases require Ca^{2+} for activation. The setting phenomenon in surimi is induced by endogenous transglutaminase activity, resulting in higher gel strength. (Kamath, Lanier, Foegeding and Hamann, 1992; Kimura, Sugimoto, Toyoda, Seki, Arai, and Fujita, 1991). Furthermore, Yongsawatdigul and Piyadhammaviboon (2007) found that tilapia sarcoplasmic proteins concentrated using an ultrafiltration membrane of 30 kDa molecular weight cut off (MWCO) contained TGase activity approximately 498.6 U/mL. TGase activity of the second wash water seemed to be comparable to that of TB mince. (Yongswatdigul, Worratao and Park, 2002). This implied that TGase was contained in sarcoplasmic proteins which were removed during washing step.

Lizardfish (*Saurida* spp.) is one of the main species used to produce surimi in Thailand and Southeast Asia. Gel-forming ability of lizardfish surimi is poor because of its high endogenous proteolytic activity. (Suwansakornkul, Itoh, Hara and Obatake, 1993). In the presence of endogenous proteinase, myofilbrillar proteins underwent degradation upon heating, which is known as "modori" phenomenon (Matsumoto and Noguchi, 1992). Protein food additives have also been used in other species for

improving gel-strength in fish muscle or surimi during heating process, such as egg white powder (EW), whey protein concentrate (WPC), beef plasma protein (BPP), and potato extract (Hamann, Amato, Wu, and Foegeding, 1990; Morrissey, Wu, and An, 1993). Recently, the use of BPP in food application was limited by the spread of bovine spongiform encephalopathy (BSE) or ‘‘mad cow disease’’. Addition of BPP more than 1 % result in off-flavor (Akazawa, Miyauchi, Sakurada, Wasson and Reppond, 1993). Similarly, EW has undesirable odor as sulfur at high concentration and also has high cost (Porter, Koury and Kudo, 1993). It has been reported that carp (*Cyprinus carpio*) muscle containing with calpastatin and a trypsin inhibitor (Toyohara, Makinodan, Tanaka and Ikeda, 1983). Trypsin inhibitor in white croker (*Micropogon opercularis*) skeletal muscle extracts could inhibit proteinase I isolated from white croker skeletal muscle (Busconi, Folco, Martone, Trucco and Sánchez, 1984; Folco, Busconi, Martone, Trucco and Sánchez, 1984). Therefore, inhibitor from sarcoplasmic protein might be a new choice for surimi industry.

In surimi processing, high volume of water was utilized to eliminate sarcoplasmic proteins. There were estimated that every 1 kg of surimi production required water approximately 10 liter. Thus, Thai surimi industry which produces approximately 150,000 MT produced about 1,500 million liter of wash water per year. Lin, Park and Morrissey (1995) reported that surimi wash water consisted of 1.4% soluble proteins. Based on their study, 150,000 MT of surimi produced in Thailand annually discards about 21,000 MT soluble proteins in wash water. Therefore, recovering sarcoplasmic proteins from surimi wash water would not only reduce the environmental problem, but also increase the overall production yield of the industry.

1.2 Research objectives

(1) To characterize transglutaminase from threadfin bream sarcoplasmic proteins (TBSP) and their effects on lizardfish surimi.

(2) To investigate proteinase inhibitory activity of TBSP and effect of TBSP on lizardfish surimi textural properties.

1.3 Research hypothesis

Sarcoplasmic protein from threadfin bream (TBSP) contributes to gel enhancing effect on lizardfish surimi gel. The enhancing ability is likely due to the activity of endogenous transglutaminase (TGase), which exhibits similar characteristics to other tissue TGases. The gel-enhancing effect might also be caused by the proteinase inhibitory activity contained in TBSP.

1.4 Expected results

Results from this research will lead to more understandings on the effect of sarcoplasmic proteins on surimi gelation. Biochemical characteristics of TGase from TBSP will be obtained. Also, more information about proteinase inhibitory activity contained in TBSP will be revealed. This knowledge could be applied to develop a functional ingredient from sarcoplasmic proteins recovered from surimi industry.

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CHAPTER II

LITERATURE REVIEWS

2.1 Threadfin bream

Threadfin breams (TB, *Nemipterus* spp.) belong to the family of Nemipteridae. TB are benthic, inhabit marine water on sandy or on muddy bottoms usually in depth of 10-80 mater, Most species are carnivorous, eating small fishes or small benthic invertebrates (Merrissey and Tan, 2000; Sritakon, Vibunpant and Chotithamm, 2007). TB are found in shallow coastal water in Thailand, Indonesia, India, Philippines and Malaysia. TB are caught in the Andaman sea and the Gulf of Thailand, by bottom trawls (Merrissey and Tan, 2000; Holmes, Noguchi and MacDonald, 1992). There are 39 species of *Nemipterus* and 13 of them found in Thailand (Sritakon, Vibunpant and Chotithamm, 2007).

Figure 2.1 Threadfin beam

Source : Pangsorn, Laong-manee and Siriraksophon (2007).

Thailand is the largest tropical fish surimi producer. In 2005, it is estimated that about 150,000 metric tons (MT) of surimi were made from tropical fish. Main raw material species used in the processing plants are threadfin bream (*Nemipterus* spp.), bigeye snapper (*Priacanthus* spp.), lizardfish (*Saurida* spp.), croaker (*Pennahia* spp.), goatfish (*Upeneus* spp.). There are also some small pelagic species such as sardine (*Sardinella* spp.) and Rainbow runner (*Elagatis bipinnulata*) (Pangsorn, Laong-manee and Siriraksophon, 2007). The quantity of fish supplied to the surimi industry was 530,000 MT/ in 2005. About 36% of surimi production was from TB, 18% of lizardfish and 17% of croaker (Pangsorn, Laong-manee and Siriraksophon, 2007). TB surimi is widely used as a raw material for Japanese kamaboko and surimi based crabstick. Because of TB meat is white with good flavor, strong gel-forming ability and its myofibrillar proteins are highly stable in frozen storage (Guenneugues and Morrissey, 2005).

Table 2.1 Protein distribution of fish muscle

Source	Sarcoplasmic protein (%)	Myofibril protein (%)	Stroma protein (%)
Fish, general	10-25	70-90	3-10
Atlantic cod	21	76	3
Carp	24	71	5
Flounder	21	76	3
Beef	16-28	39-68	16-28

Source : Haard (1992).

2.2 Muscle proteins of fish

Fish muscle proteins can be divided into 3 groups based on their solubility (Haard, 1992; Mackie, 1994). These are water-soluble or sarcoplasmic proteins, salt-soluble proteins or myofibrillar proteins and the insoluble proteins or stroma proteins. Fish muscle contains a relatively high concentration of myofibrillar proteins and a low concentration of insoluble proteins compared to muscles from land animal (Table 1).

2.2.1 Myofibrillar proteins

Myofibrillar proteins are the structural proteins containing myosin, actin, tropomyosin, troponin, and actinin. The proportion of myofibrillar proteins in fish meat is about 66-77% of the total protein content. Myofibrillar protein is extracted with neutral salt solutions of ionic strength above 0.15 (Scopes, 1994; Zayas, 1997). Myofibrillar proteins perform a significant role in the coagulation and gel formation of fish meat.

It is well known that myofibrillar proteins are largely responsible for the textural properties of processed meat products (Xiong, 2000; Yasui et al., 1980). The adequate extraction of myofibrillar proteins is particularly important for promoting gel formation in meat products (Li-Chan et al., 1987). The two main components of myofibrillar proteins are myosin and actin. They are responsible for muscle contraction in the living animal, as well as many functional characteristics in processed meat products (Xiong, 2000).

2.2.2 Stroma proteins

Stroma proteins are the connective tissues and are composed primarily of collagen, elastin, and lipoproteins of the cell membrane. Stroma proteins are insoluble

and cannot be extracted by water, acid or alkaline solution, and 0.01-0.1 M neutral salt solutions (Lawrie and Ledward, 2006; Xiong, 2000).

Collagen is generally associated with the toughness of the meat. Collagen is made of three helically twisted polypeptide chains stabilized by intramolecular and intermolecular bonds. As animals age, more covalent bonds are formed inside and between collagen molecules which contribute to the toughness of the meat (Xiong, 2000). Ziegler and Acton (1984) indicated that the stroma proteins exhibited no gelation ability as the fraction only coagulated upon heating to 80°C.

2.2.3 Sarcoplasmic proteins

Sarcoplasmic proteins, the proteins of the sarcoplasm, comprise about 30% of the total amount of protein and can be extracted by homogenizing the fish muscle with water or neutral salt solutions with ionic strengths below 0.15 (Lawrie and Ledward, 2006). The exact number of sarcoplasmic proteins cannot be established, but it is estimated around 100 to 200. The sarcoplasmic proteins consist of the enzymes involved in glycolytic pathway and may be present in more than one form (isozymes) (Lawrie and Ledward, 2006; Xiong, 2000).

Czok and Bücher (1960) estimated that at least 70% of rabbit muscle sarcoplasmic proteins were accounted for the known qualities of 15 enzymes, including phosphorylase, lactose dehydrogenase and creatine kinase. They also obtained an unidentified nucleotide-containing protein, which co-crystallized with two of glycolytic enzymes. Myoglobin is one of the most important sarcoplasmic proteins in term of meat quality. Myoglobin is responsible for the red color in fresh meat. The molecule consist of two portions: the protein portion called “globin”, and non protein portion called “heme” (Xiong, 2000). Some enzymes from fish

sarcoplasmic proteins can be described as follows :

Calpains

The calpains, intracellular cysteine proteinases, are calcium-dependent. These enzymes are in two forms: μ-calpain and m-calpains, which differ in calcium ions concentration necessary for activation. They require respectively micromolar (μM) and millimolar (mM) concentrations of calcium, (Koohmaraie, 1992) Moreover, the third isoform has been identified in sea bass muscle (Ladrat, Chaplet, Verrez-Bagnis, Noelle and Fleurence, 2000). Calpains are heterodimers composed of a large subunit and a small subunit that have molecular masses of about 80 and 28 kDa, respectively.

Calpains are the most active at neutral pH (6.9-7.5) (Kolodziejska and Sikorski, 1996), but are still quite active at pH 5. Optimum temperature for calpain from carp and tilapia was 30°C and 20% of the activity was retained at 0°C, pH 7.5 (Wang and Jiang, 1991). Ladrat et al. (2000) studied calpains from sea bass muscle (*Dicentrarchus labrax* L.). Two of their partially purified enzymes had optimum activity between 19 and 25°C and the activity decreased with decreasing temperature. The activity against bovine skin gelatin was very low, suggesting that calpains were not able to break down collagen in muscle. Geesink, Morton, Kent and Bickerstaffe, (2000) studied calpains from chinook salmon (*Oncorhynchus tshawytscha*) muscle. They found a few proteolysis of myofibrillar proteins during storage.

Cathepsins

Cathepsins are acid proteinases located in the lysosomes. Lysosomes are known to harbor about 13 types of cathepsins (Goll, Otusuak, Nagainis, Shannon, Sathe, and Mururuma, 1983). The lysosomal cathepsins B, L, H, L-like, S and X are cysteine proteinases (Jiang, Lee, and Chen, 1994; Jiang, Lee and Chen, 1996; Lee,

Chen and Jiang, 1996). These enzymes are present in the circulatory system (Foegeding et al., 1996). Many types of cathepsins have been identified with different amino acids at the catalytic site, and cathepsins B and L are probably the most important for deterioration of muscle texture (Aoki, Yamashita, and Ueno, 2000; Jamdar and Harikumar, 2002; Kolodziejska and Sikorski, 1996). The activities differ between muscle fractions and fish species (Aoki et al., 2000; Yamashita and Konagaya, 1990a). The optimum activity is reported to be at about 40-50°C, and the activity decreases with reducing temperatures. Generally, they can perform best at pH 3-4, some of them retain fairly high activity at pH 6.0-6.5. Cathepsins B and L are activated by reducing thiol compounds. Cathepsins B, H and L are inhibited in vivo by a proteinase inhibitor called cystatin (Anastasi et al., 1983; Turk and Bode, 1991).

The cathepsins are involved in deterioration of muscle texture, and different cathepsins may act in concert to autolyze fish muscle (Ashie, Simpson, and Smith, 1996; Kolodziejska and Sikorski, 1996). Yamashita and Konagaya (1990b) reported that degradation pattern of myofibrillar proteins of softened chum salmon muscle was revealed when myofibrils were treated with purified cathepsin L. This indicated that cathepsin L is the most probable enzyme responsible for extensive muscle autolysis and softening observed in mature chum salmon. Cathepsin B from chum salmon in spawning migration hydrolyzed carp myofibrillar proteins in solution, but was not able to hydrolyze carp collagen (Yamashita and Konagaya, 1991).

2.3 Transglutaminase(TGase)

Transglutaminase (TGase) (glutaminyl-peptide γ-glutamyl transferase, EC 2.3.2.13) is a transferase enzyme which catalyses the acyl transfer reaction. Recently,

Yongsawatdigul and Piyadhammaviboon (2007) found that tilapia sarcoplasmic proteins concentrated using an ultrafiltration membrane of 30 kDa molecular weight cut-off (MWCO) contained TGase activity approximately 498.6 U/mL. The purified TGase from tilapia (*Oreochromis niloticus*) was estimated to be 85 kDa using SDS-PAGE and pI was 6.53. Optimal temperature and optimal pH of tilapia TGase were 37–50°C and 7.5, respectively. Water soluble sarcoplasmic proteins from threadfin bream (*Nemipterus* spp.) contained TGase activity (Yongsawatdigul, Worratao and Park, 2002). In addition, TGase and other sarcoplasmic proteins were mainly removed during the first washing step of small scale mud carps (*Cirrhiana microlepis*), resulting in a lower TGase specific activity (112 unit/mg protein) when compared with fresh mince (182 unit/mg protein) (Yongsawatdigul, Piyadhammaviboon and Singchan, 2006).

2.3.1 Characteristics of TGase

Transglutaminse-catalysed reactions can be categorized into 3 reactions with different acyl acceptors. The acyl donors are the γ-carboxyamide groups of peptide or protein bound glutamyl residues. When transglutaminase uses the primary amine as an acyl acceptor, the acyl-transfer reaction occurs (Figure 2.2a). When the ε-amino groups of lysine residues in proteins are acyl acceptor, ε-(γ-Glu)-Lys cross-links are formed intra-and intermolecular (Figure 2.2b). Without primary amines, water can act as the acyl acceptor and the γ-carboxyamide groups of glutamine residues are deaminated and the process called deamidation (Figure 2.2c). The diagram of transglutaminse-catalysed reactions are shown in Figure 2

2.3.2 Sources of TGase

TGase was firstly found in guinea pig livers since 1957. The enzyme is also

found in animals, plant tissues, fish, invertebrates and microorganisms. TGase is widely distributed in various tissues and body fluids (Folk, 1980). It has been purified from various animals tissues and organs, such as guinea pig liver (Connellan, Chung, Whetzel, Bradley and Folk, 1971), pig plasma (Jiang and Lee, 1992) and placenta (De, Traore and Meunier, 1992). It was also isolated from carp and tilapia muscle (Kishi, Nozawa and Seki, 1991; Worratao and Yongsawatdigul, 2005). This enzyme is known to contribute to gelation of fish muscle.

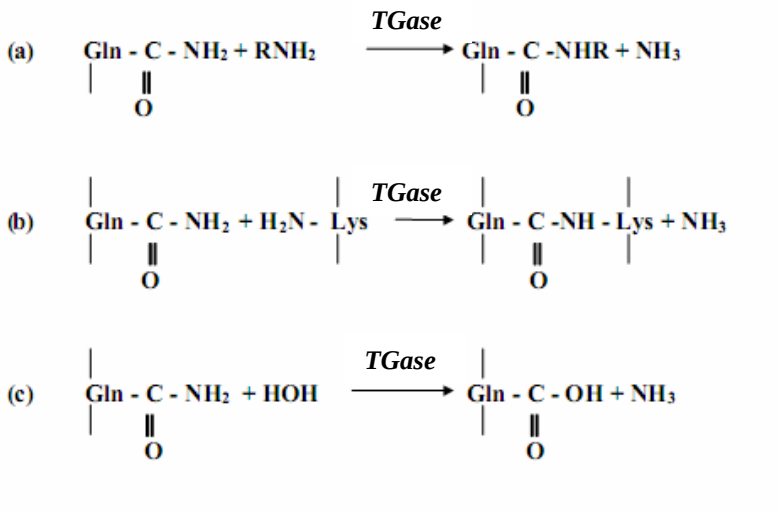

Figure 2.2 The reactions catalyzed by TGase

(a) acyl-transfer reaction

(b) crosslinking reaction between Gln and Lys residues

(c) deamidation

Source : Zhu, Rinzema and Tramper (1995).

TGase was also found in a culture of *Streptoverticillium* sp. and *Streptomyces* sp. (Zhu et al., 1995). The advantages of using microbial transglutaminase (MTGase) can be obtained in mass volume through microbial cultivation which results in a lower cost than purified TGase from guinea pig liver. In addition, the MTGase

is Ca^{2+}-independent while TGase derived from liver is Ca^{2+}-dependent.

2.3.3 Types of TGase

2.3.3.1 Circulating zymogen Factor XIII

It is also known as Laki-Lorand factor or fibrin stabilizing factor**.** Factor XIII is an enzyme of the blood coagulation system that cross-links fibrin. Factor XIII is expressed as a zymogen in plasma, placenta and platelets (Bohn, 1972) and is activated by thrombin into factor XIIIa, its activation into Factor XIIIa requires calcium as a cofactor (Lorand, 1986). Factor XIII has a heterotetrameric structure, which consists of two a- and two b-subunits. The a-subunit (Factor XIIIa) contains the active site of the enzyme (Carrell, Erickson and McDonagh, 1989). Factor XIIIa catalyzes the covalent linking of α-2-plasmin inhibitor to the α-chain of fibrin (Tamaki and Aoki, 1982) and also catalyzes the polymerization of fibrin monomers into γ-γ-(fibrin) dimers and α-polymers during hemostasis (Chen and Doolittle, 1971).

2.3.3.2 Keratinocyte TGase (type 1 TGase)

Keratinocyte TGase exists in membrane-associated and soluble forms, is activated several folds by proteolysis. The enzyme is involved in the terminal differentiation of keratinocytes. The keratinocyte enzyme is the most similar to factor XIII. The keratinocyte transglutaminase is its 105-residue extension beyond the N terminus and 28 residues beyond the C terminus of the tissue transglutaminase. This extension and the unrelated activation peptide of factor XIII (a 37-residue extension) appear to be added for specialized functions after divergence of the tissue transglutaminase from their common lineage. (Philips et al., 1990).

2.3.3.3 Ubiquitous tissue TGase (tTGase or type 2 TGase)

tTGase is expressed ubiquitously. It requires calcium as a cofactor for

transamidation activity. Transcription is increased by retinoic acid. Among many supposed functions, it appears to play a role in wound healing, apoptosis and extracellular matrix development (Griffin, Casadio and Bergamini, 2002). tTGase has been found in numerous fish species, such as red sea bream, mackerel, sardine, carp, pollock, threadfin bream and tilapia (An, Peters and Seymour,1996; Araki and Seki, 1993; Worratao and Yongsawatdugul, 2005; Yasueda, Kumazawa and Motoki, 1994; Yongsawatdigul, Worratao and Park, 2002).

2.3.3.4 Epidermal TGase (hair follicle TGase or type 3 TGase)

Epidermal TGase is a monomer and zymogen which requires proteolysis to become active and it is involved in the formation and assembly of the cornified cell envelope of epidermis and hair follicle (Folk, 1983; Lorand and Conrad, 1984). Both of human and mouse cDNA cloning contain 692 amino acids of molecular weight about 77 kDa (Kim, Gorman, Park, Chung and Steiner, 1993).

2.3.3.5 Microbial transglutaminase (MTGase)

MTGase is Ca^{2+}-independent enzyme. Ando et al. (1989) screened about 5000 strains isolated from soil collected from various locations. Among these strains, *Streptoverticillium* S-8112 was found to have the capability of producing transglutaminase. In addition, other *Streptoverticillium* strains, such as S.*griseocarneum*, S.*cinnamoneum* ssp. *cinnanoneum* and S.*mobaraense*, also have the ability to produce transglutaminase. Recently, transglutaminase activity has also been found in a culture of *Streptomyces* sp. (Ando, Matsura and Susumu,1992). MTGase was also found in *Bacillus subtilis* and in *Physarum polycephalum*. as intracellular forms (Klein, Gozman and Koehn, 1992).

2.3.4 Application of TGase

The transglutaminase enzyme has been applied to various protein-based products, such as meat, fish, dairy, and vegetables (Nielsen, 1995). The enzyme has been used for modifying the functionalities of various proteins including soy proteins, myosin, gluten, globulin, casein and whey (Abourmahmoud and Savello, 1990; Siu, Ma, Mock and Mine, 2002; Takinami, Nio and Motoki, 1984; Truong, Clare, Catignani and Swaisgood, 2004). In meat products, Factor XIIIa has been found to crosslink the proteins within the meat itself, such as fibrin and actin, myosin and actin. Due to its excellent cross-linking ability, TGase can be also used as a binding agent in products when salt reduction is desired (Tsao, Kao, Hsieh and Jiang, 2002).

Moreover, it has been reported that TGase was used as a biocatalysts for processing of wool textiles. This application resulted in a reduction of shrink of wool fabric, while maintaining, and possibly even enhancing, fabric strength (Cortez, Bonner and Griffin, 2004). Guinea pig liver TGase or MTGase from S. *mobaraense* was either directly used with wool or was used after protease treatment. The TGase-treated wool showed an increase in fabric strength of up to 25% compared to the control (Cortez et al., 2004).

Recently, numerous studies demonstrated application of TGase in surimi. Addition 0.47 unit/g of MTGase in mince mackeral increased 3 times of gel strength when compared with control (Jiang, Leu and Tsai, 1998). Addition of MTGase in Chilean jack mackerel (*Trachurus murphyi*) surimi improved the textural quality. Gel strength increased of 364% when incubated at 25°C for 2 h and increased particularly at TGase levels between 0.1 and 0.5%w/w (Dondero, Curotto and Figueroa, 2002). Jiang, Hsieh, Ho and Chung (2000) reported that MTGase from *Streptoverticillium*

ladakanum were used to improve surimi gels from threadfin bream and pollack. The optimal amounts of MTGase and setting conditions were a 0.3 unit/g surimi for threadfin bream and 0.2 unit/g surimi for pollack. The textural properties of lizardfish surimi significantly improved when 0.1 unit of MTGase/g surimi was added and pre-incubated either at 25 and 40°C (Yongsawadigul and Piyadhammaviboon, 2005). In addition, Ca^{2+} was used for activating of endogenous TGase in surimi gelation. The level of Ca^{2+} required for maximum gel strength various with species of fish at its incubating temperature. Increasing of textural properties of threadfin bream surimi gel revealed after adding of 0.2% Ca^{2+} and pre-incubated at 40°C for 2 h. It also showed the increasing of MHC cross-linking on SDS-PAGE pattern (Yongswatdigul et al., 2002). Endogenous TGase from tilapia was added into lizardfish surimi for improving gel properties. At 0.15% $CaCl_2$ addition, lizardfish surimi with 1% concentrated tilapia (*Oreochromis niloticus*) sacroplasmic proteins added and pre-incubated at 37°C for 1 h exhibited 91.6% and 26.7% increase in breaking force and deformation, respectively. Based on SDS-PAGE, cross-linking product from MHC and troponin were noticed (Yongsawatdigul and Piyadhammaviboon, 2007).

2.4 Recovery of sarcoplasmic protein (SP)

In surimi processing, fish mince was repeatedly washed with chilled water to remove sarcoplasmic proteins and other components. The sarcoplasmic proteins removed during washing process are not recovered because they have lower functionality than myofibrillar protein fractions (Kawai, Ohno, Wakameda, Inoue and Shinano, 1995). Recently, some studies reported the use of sarcoplasmic proteins from surimi wash water. Karthikeyan, Mathew, Shamasundar and Prakash (2004)

recovered oil sardine (*Sardinella longiceps*) sarcoplasmic proteins by ammonium fractionation. Five different fractions were obtained by addition of ammonium sulfate of 0 to 20%, 20-40%, 40-60%, 60-80% and 80-100%. The protein yields were 2.5, 3.5, 19, 32 and 31%, respectively. From SDS-PAGE pattern revealed that all fractions composed with protein in the range of 97 to 29 kDa. Rockfish sarcoplasmic proteins were recovered using pH shifting method. The largest SP loss in the supernatant was found in pH10 treatment, while the significantly higher recovery was found at pH 2 and 3. The protein pattern of SP fractions was determined using SDS-PAGE. The major bands were 43, 40, 17, 11 and 8 kDa in both of acidic or alkaline pH shift treatment (Kim, Youngsawatdigul, Park and Thawornchinsomnut, 2005). The recovery of sarcoplasmic proteins from threadfin bream (*Nemipterus hexodon*), TBSP showed maximum precipitation at pH 3.5 with protein concentration of 66%. In addition, recovery of TBSP by ethanol was highest protein precipitation at 60% (w/w) of ethanol with collected protein concentration of 65%. (Bourtoom, Chinnan, Jantawat and Sanguandeekul, 2009). Moreover, the recovery protein from TBSP had MW in range of 71.6 to 23.2 kDa. In addition, sarcoplasmic protein from porcine were fractionated using ammonium sulfate. Precipitation at 0-50%, 50-75% and supernatant at 75% were carried out and referred as SP-f1, SP-f2 and SP-f3, respectively. Based on SDS-PAGE result, whole SP had several protein distributed in range of 94-17 kDa. SP-f1 contained protein band at 94 kDa while SP-f2 contained protein with MW of 30-60 and SP-f3 exhibited main protein band with MW of 35 kDa (Miyaguchi, Hayashi and Nagayama, 2004).

The classical method for protein concentrates and isolates is ultrafiltration (UF). Mireles DeWitt and Morrissey (2002b) recovered catheptic proteases from

Pacific whiting (*Merluccius productus*) surimi wash water. Pretreatment condition, with 60°C heat and acidification to pH 6 were improved protease purity by reducing a majority of the 35–205 kDa proteins. Band patterns for both UF membranes, 30 and 50 kDa were qualitatively similar. The lab-scale ultrafiltration was not usable to purify proteinase from surimi wash water. However, protease recovery was maximized using either a 30- or 50-kDa membrane but concentration of protease using 50-kDa UF membranes was successful in recovering about 80% of original protease activity (Mireles DeWitt and Morrissey, 2002a). Concentrated tilapia (*Oreochromis niloticus*) sacroplasmic proteins (SpC) was recovered using 30-kDa membrane (Youngsawadigul and Piyadhammaviboon, 2007). TGase activity increased about 3.6 folds after ultrafiltration whereas specific activity of TGase in the SpC was not significantly increased when compared to crude extract. It was found that, UF only SpC without increasing TGase purity (Youngsawadigul and Piyadhammaviboon, 2007). The results were similar to that of Mireles DeWitt and Morrissey (2002a) study.

2.5 Effect of sarcoplasmic protein on textural properties

In conventional surimi processing, washing step typically removes sarcoplasmic proteins, blood, fat and other nitrogenous compounds in fish muscle. This is because it has been recognized that sarcoplasmic proteins hinder the gel formation of fish meat by adhering to the myofibrillar protein. Yanagihara, Nakaoka, Hara and Ishihara (1991) purified trypsin-type serine proteinases from the sarcoplasmic fraction of white croaker. These serine proteinases degraded myosin heavy chain in the presence of NaCl. Nevertheless, sarcoplasmic proteins extracted

from fish were recently reported to contain transglutaminase which could increased of gel strength (Lanier, 2000). Ko and Hwang (1995) reported that adding of SP (1%) recovered by UF from milk to thermal gelation of meat paste and myofibrillar proteins improved thermal gelation. Yongsawatdigul and Piyadhammaviboon, (2007) reported that SpC from tilapia contained TGase activity and these enzyme can catalyze protein cross-linking of lizardfish surimi. Some of sarcoplasmic proteins ammonium sulfate fraction from oil sardine (*Sardinella longiceps*) enhanced gel strength of washed sardine meat gel (Karthikeyan, Mathew, Shamasundar and Prakash, 2004).

Kim at el., (2005) reported that sarcoplasmic proteins from rockfish positively contributed to gelation of pollock surimi. Breaking force significantly increased when adding of 2% sarcoplasmic proteins. At 2.5% porcine sarcoplasmic proteins addition, gel strength of water-washed pork meat emulsion increased with approximately 10 folds when adding of porcine sarcoplasmic proteins which is the supernatant at 75% ammonium sulfate saturation fraction (SP-f3) compared to W-SP (whole SP, control) (Miyaguchi et al., 2004). These are evidences suggesting that sarcoplasmic proteins do not interfere gel forming of myofibrillar proteins.

2.6 Proteinase inhibitors (PIs)

Proteinases are the enzymes that catalyze the irreversible breakdown of peptide bonds in proteolysis. Proteinases can be assigned to four groups based on the basis of their catalytic sites. These groups of proteinases are serine proteinases, aspartic proteinases, cysteine proteinases, and metallo-proteinases (Barrett, 2001).

PIs are presented in both intra and inter-cellular. The best known PIs presented in intercellular are inhibitors in blood. Vertebrate blood contains various serine proteinases that are involved in coagulation, fibrinolysis, complement activation and inflammation process. The activated enzymes are regulated by the action of various inhibitors that are circulating in the blood (Whitaker, 1994). In humans, deficiency of inhibitors circulating in the blood leads to various clinical disorders. For example, deficiency of α-1 antitrypsin, a serine proteinase inhibitor of neutrophil elastase, leads to emphysema (Takahara and Sinohara, 1982). Intracellular proteinase inhibitors are located in the cytoplasm or in the nucleus (Grigoryev, Bednar and Woodcock, 1999) and regulate various intracellular proteinases. The lysosomal proteinases are regulated by PIs if they are released into the cytoplasm (Turk, Turk, Guncar, Turk and Kos, 2002). Protease inhibitors are widely distributed in various tissues of animals, plants and microorganisms (Laskowski and Kato, 1980). In plants protease inhibitors are found to be induced in response to insect attack (Ryan, 1990).

Söderhall and Cerenius (1998) reported that melanization is one of the defensive mechanisms adopted by insects against various pathogens. Melanization process involves in activation of proteinases leading to conversion of prophenoloxidase (PPO) to phenoloxidase. The activated enzyme catalyzes formation of quinones which polymerize to form melanin. The quinones and reactive oxygen species produced during melanization are toxic to insect cells and microorganisms. Normal conditions to bypass unwanted-production, insects have released various serine proteinase inhibitors to control the activity of proteolytic enzymes (Boigregrain, Mattras, Brehelin, Paroutaud and Coletti-Previero, 1992; Jiang and Kanost, 1997; Sugumaran, Saul and Ramesh, 1985).

PIs are of common occurrence in the plant. It was first investigated at 1947, by Mickel and Standish who observed that the larvae of certain insects were unable to develop normally on soybean products (Lawrence and Koundal, 2002). Trypsin inhibitor presenting in soybean were shown to be toxic to the larvae of beetle four (*Tribolium confusum*) (Lipke, Fraenkel and Liener, 1954). PIs can be divided into 2 general categories based upon their specification of activity. These are non-specific proteinase inhibitors and the class-specific proteinase inhibitors (Salvesen and Nagase, 2001)

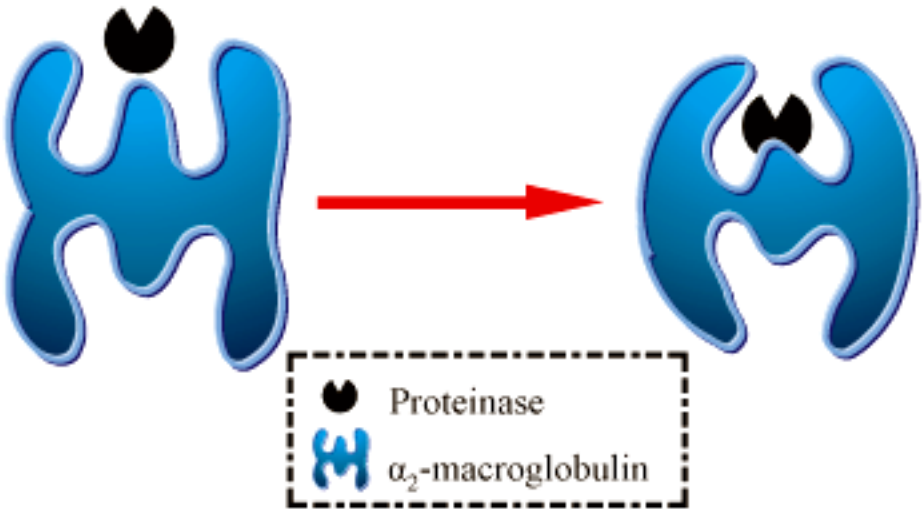

Figure 2.3 Alpha$_2$-macroglobulin mechanism of action

Source : Adapted from Hibbetts, Hines and Williams (1999)

2.6.1 Non-specific inhibitors (α-Macroglobulins)

Non-specific proteinase inhibitors are capable of inhibiting members of all 4 groups of proteinases. This groups of inhibitors consists of the α-macroglobulins, including human α_2-macroglobulin (α_2-M). The members of this group are very large proteins with low specificity for target proteinases. They bind and inhibit mostly endopeptidase (Salvesen and Nagase, 2001). α_2-M is a glycoprotein of MW 725000

Da and composed of two non-covalently subunits. Figure 2.3 shows "trap hypothesis", which a proteinase binds to a bait region on the α_2-M, resulting in a conformational change in the α_2-M. The active site of the proteinase is not bound directly and retains very limited proteolytic activity. (Hibbetts, Hines and Williams, 1999)

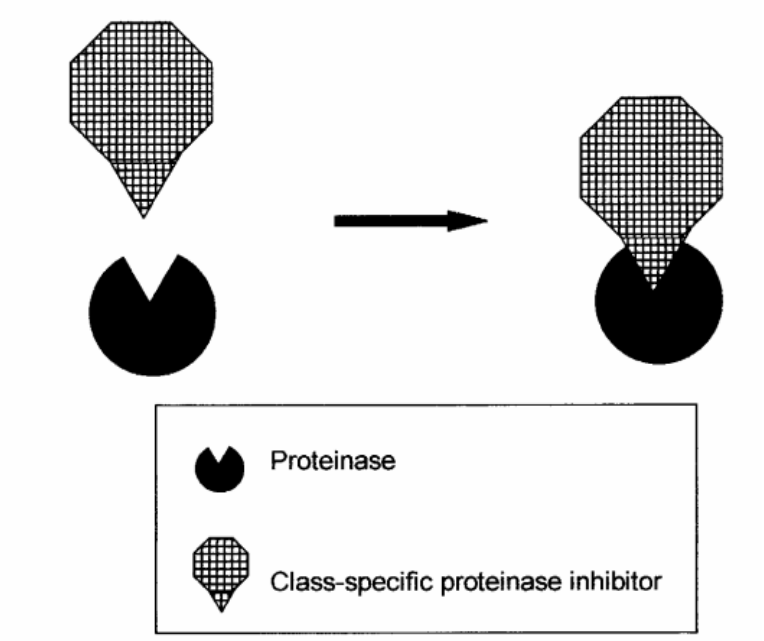

Figure 2.4 Class-specific proteinase inhibitor mechanism of action.

Source : Hibbetts, Hines and Williams (1999)

2.6.2 Class-specific proteinase inhibitors

The class-specific proteinase inhibitors are capable of inhibiting proteinases from each groups of proteinases. These are the serine proteinase inhibitors, aspartic proteinase inhibitors, cysteine proteinase inhibitors and metalloproteinase inhibitors. The proteinase inhibitors of these mechanistic classes are further classified into different families. The class-specific proteinase inhibitors have lower molecular weights and higher specificity (Figure 2.4) when compared with α-macroglobulins (Hibbetts et al., 1999).

2.6.3 Cysteine proteinase inhibitors

The cysteine proteinases contain a sulfhydryl group in their active sites. These proteinases participate in intracellular digestion and include cathepsins H, L, B, and S. Cysteine proteinases inhibitors included cystatins, stefins, and kininogens. These inhibitors act as a protective mechanism against cysteine proteinases released into circulation after cell death.

2.6.4 Aspartic proteinase inhibitors

Aspartic proteinases contain aspartic residues in their active sites. They can be divided into endogenous and exogenous proteinases. Endogenous aspartic proteinases include gastric proteinases, such as pepsins, gastricsins, and chymosins, and nongastric proteinases such as renin, lysosomal cathepsin D, and nonlysosomal cathepsin E. Exogenous aspartic proteinases are found in fungi, bacteria, yeasts, and viruses. The major inhibitor of aspartic proteinases is α_2-macroglobulin. Pepstatin A, which is the natural inhibitor of aspartic proteinases, is poorly characterized (Hibbetts, Hines and Williams, 1999; Salvesen and Nagase, 2001).

2.6.5 Metalloproteinase inhibitors

Metalloproteinases are metal-containing (especially zinc) endopepti-dases at the catalytic site. In mammals, metalloproteinases are synthesize and secreted by connective tissues. The metalloproteinase inhibitors collectively are called tissue inhibitors of metalloproteinases. Metalloproteinases are inhibited by metal chelators which remove metals from the active site. However, some metalloproteinase inhibitors are involved in tissue remodeling. These were called tissue inhibitors of metalloproteinases (TIMPs). They can be found in all connective tissues in the body. TIMPs regulate destruction of extracellular matrix (Hibbetts, Hines and Williams,

1999; García-Carreño and Hernández-Cortés, 2000).

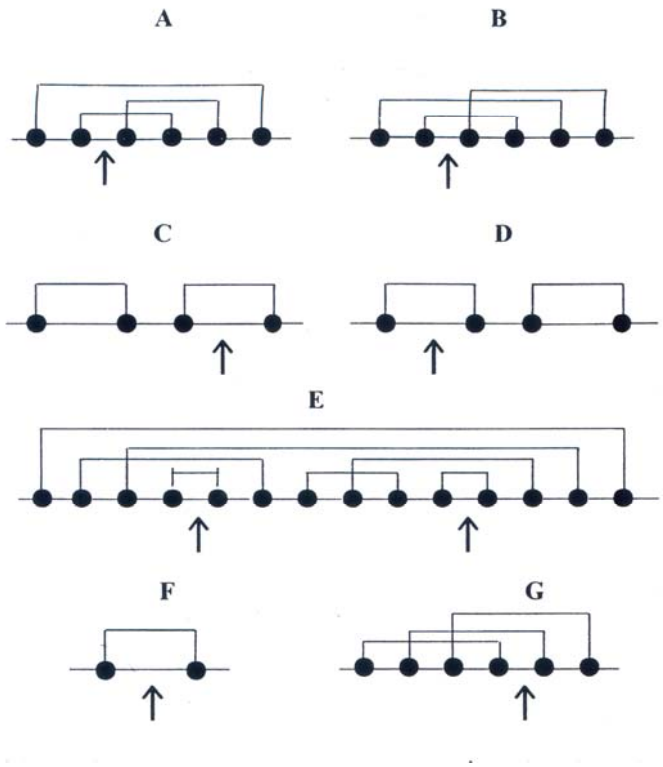

Figure 2.5 Topological structures of proteinase inhibitors. Solid circle and arrow indicate, respectively, a half cystine residue and a reactive site. A, bovine pancreatic trypsin inhibitor (Kunitz) family; B, Kazal serine protease inhibitor family; C, *Streptomyces* subtilisin inhibitor family; D, Soybean trypsin inhibitor (Kunitz) family; E, Soybean trypsin inhibitor (Bowman-Birk) family; F, Potato inhibitor I family; G, Potato inhibitor II family.

Source : García-Carreño and Hernández-Cortés, (2000)

2.6.6 Serine proteinase inhibitors

Serine proteinases contain serine residues in their active sites. These proteinases play important roles in many physiologic processes, including digestion, blood coagulation, immune reactions, and fertilization of the ovum. Serine proteinase inhibitors are very abundant in mammalian plasma.

Table 2.2 Families of protease inhibitors

No.	Name	Approximate domain size and number of domains
1.	Bovine pancreatic trypsin inhibitor (Kunitz)	60 residues
2.	Kazal serine protease inhibitor family	55 residues; up to 7 domains
3.	Soybean trypsin inhibitor (Kunitz) family	180 residues
4.	Bowman-Birk inhibitor family	35 residues; 2 domains
5.	Potato inhibitor I family	70 residues
6.	Potato inhibitor II family	50 residues; 1 or 2 domains
7.	Squash inhibitor family	30 residues
8.	Barley trypsin inhibitor family	120 residues
9.	Thaumatin family	200 residues
10.	*Ascaris* trypsin inhibitor family	60 residues
11.	Locust inhibitor family	35 residues
12.	Ecotin family	140 residues; 2 subunits
13.	Serpin family	400 residues
14.	*Streptomyces* subtilisin inhibitor	110 residues
15.	Hirudin family	65 residues
16.	Cystatin family	110 residues; usually single domain but 3 inhibitory domains in kininogens and 8 in potato mutlistatin
17.	Calpastatin family	140 residues; 4 domains
18.	Potato carboxypeptidase inhibitor family	40 residues
19.	*Ascaris* carboxypeptidase inhibitor family	40 residues
20.	Collagenase inhibitor family	200 residues
21.	*Ascaris* pepsin inhibitor family	150 residues
22.	α_2-macroglobulin family	1500 residues; 2 or 4 subunits

Source : Reeck et al., (1997)

Serine proteinases inhibitors have also been classified in families according to their sequencing and X-ray crystallography. The family characteristics used to identify inhibitor families were the topological relationships between the disulfide bridges and the location of the reactive site (Figure 2.5). With the exception of potato II and *Ascaris* trypsin inhibitor, In each family, the positions of all intra-chain disulfide bridges are completely conserved (Laskowski and Kato, 1980).

According to Laskowski and Kato (1980) families criteria, the protease inhibitor families were expanded. Reeck, Kramer, Baker, Kanost, Fabrick and Behnke, (1997) reported a complete list and grouped inhibitors into 22 families (Table 2). According to the classification, 15 of 22 families are serine proteinase inhibitors (Reeck et al., 1997).

The 15 families of serine protease inhibitors are:

2.6.6.1 Bovine pancreatic trypsin inhibitor (Kunitz) family

This was the first inhibitor whose sequence and three-dimensional structure of free inhibitor and enzyme-inhibitor complex was determined. The pancreatic trypsin inhibitor identified from bovine is a typical example of this family. The inhibitors of this family contain 4-8 disulfide bonds and 2-inhibitory domains. Inhibitors with 2-inhibitory domains are called double headed inhibitors (Laskowski and Kato, 1980). The inhibitors of this family have been found from humans, bovine, pig, birds, garden snails, and snake venom.

2.6.6.2 Pancreatic secretory trypsin inhibitor (Kazal) family.

Secretory pancreatic trypsin inhibitor (Kazal) was named after bovine pancreatic secretory trypsin inhibitor (Kazal) which was found in all vertebrates. The inhibitors are stored in zymogen granules and secreted with the zymogens in the pancreatic juice. Consistent with their function is the clear specificity for trypsin inhibition, failure to interact with trypsinogen, failure to inhibit enterokinase and other pancreatic serine proteinases of its own species. These properties are contrast with those of the pancreatic trypsin inhibitor (Kunitz) (Laskowski and Kato, 1980).

The inhibitors were found in both vertebrates and invertebrates. Turtle egg whites contain two proteinase inhibitors. It has been found as a hybrid of a Kunitz

domain and another domain from a new family called Chelonianin. The second is a two domain ovomucoid, testudin, which has two separate domains linked by 1-disulfide bridge. Crude extracts from leeches (*Hirudo medicinalis*), contain numerous trypsin inhibitors, called bdellins (Fritz, Gebhardt, Meister and Fink, 1971; Fritz, Oppitz, Gebhardt, Oppitz, Werle and Marx, 1969).

2.6.6.3 Soybean trypsin inhibitor (Kunitz) family

The soybean trypsin inhibitor (Kunitz) family contains a single inhibitory domain which inhibits single proteinase molecule. This type of proteinase inhibitors is called single-headed. The inhibitor in this family contains 2 disulfide bonds (Laskowski and Kato, 1980). Park, Choi, Kwak, Kang, Lim, Cheong and Hahm (2005) reported that Kunitz-type inhibior was found in potato tubers (*Solanum tuberosum* L. cv. Jopung). The inhibitors showes molecular mass of 8-22 kDa and had two disulfide bridges and one reactive site.

2.6.6.4 Bowman-Birk inhibitor family

Bowman-Birk class (BBI) is a major class in legume seeds which contain 4 cysteines linked into 7 disulfide bridges and two active sites. These called double-headed inhibitors. Generally, they inhibit trypsin on one site and chymotrypsin on the second one (Laskowski and Kato, 1980). In recent years, BBIs have received enormous attention because of their vital role in the defense mechanisms of plants against insect (Habib and Fazili, 2007). The inhibitors have been found in legumes and cereals, such as barley seeds (Song, Kim, Yang, Moon, Lee and Suh, 1999), pea seed (Ferrasson, Quillien and Gueguen, 1997) and *Vigna unguiculata* seeds (Barbosa et al., 2007).

2.6.6.5 Potato inhibitor I family

This family is referred to as chymotrypsin inhibitor I because they

inhibit chymotrypsin very strongly. The inhibitors of this group are noncovalent tetramers with 4 different subunits (Laskowski and Kato, 1980). The molecular mass of each subunit is 10 kDa. Each subunit consists of one intra chain disulfide bond (Melville and Ryan, 1972). The tetramer binds with 4 chymotrypsin molecules thus each subunit possess one chymotrypsin binding site (Habib and Fazili, 2007).

2.6.6.6 Potato inhibitor II family

The amino acid sequences of potato inhibitor II family have been reported for a "low-molecular-weight chymotrypsin inhibitor" (Laskowski and Kato, 1980). Inhibitors in this family have been reported to inhibit chymotrypsin, trypsin, elastase, oryzin, Pronase E and subtilisin (Antcheva et al., 2001).

2.6.6.7 Squash inhibitor family

The inhibitors have small molecular weight proteins approximately 3000 Da. It consists of 27-33 amino acids residues and cross-linked by 3 intra-disulfide bonds. The reactive site is located between amino acid residue 5 and 6. The inhibitors are highly stable (Wieczorek et al., 1985).

2.6.6.8 Barley trypsin inhibitor family

The barley trypsin inhibitor consists of 13 kDa protein and contains five disulfide bonds (Odani, Koide and Ono, 1983). The anionic and cationic inhibitors isolated from buckwheat seeds are highly pH- and thermo-stable (Kiyohara and Iwasaki, 1985).

2.6.6.9 *Ascaris* trypsin inhibitor family

The most prominent character of this inhibitor is the universal presence of 10 cysteine residues that form five disulphide bonds. Two disulfide bonds are located on either side of the reactive-site loop. The inhibitors contain two β-sheets and

each β-sheet is composed of two antiparallel strands (Grasberger, Clore and Gronenborn, 1994). The reactive-site residues are hypervariable, and this hypervariability is due to the selective pressure brought by their host enzymes (Hawley and Peanasky, 1992).

2.6.6.10 Locust inhibitor family

The inhibitors of this group are found in many tissues of brain, fat body, ovaries of the insects (Simonet et al., 2005). Well-characterized inhibitors of this family are from the migratory locust (*Locusta migratoria*) and the desert locust (*Schistocerca gregaria*). There are 35-residues long and consist of three antiparallel β-strands stabilized by 3 disulfide bonds. (Nakakura, Hietter, Van Dorsselaer and Luu, 1992).

2.6.6.11 Ecotin family

Ecotin has wide substrate specificities. It is a *Escherichia coli* periplasmic protein of 142 amino acids. It consists of 2 β-sheets that are sandwiched. Ecotin monomer consists of 1 disulfide bond near to the reactive site loop that provides stability to the molecule. Each individual Ecotin molecule interacts C-terminal to form a dimer (McGrath, Erpel, Browner and Fletterick, 1991).

2.6.6.12 *Streptomyces* subtilisin inhibitor

Streptomyces subtilisin inhibitors (SSI) are found widely in *Streptomyces* species. *Streptomyces antiplasminolyticus* excretes a potent plasmin inhibitor, plasminostreptin. These 2 inhibitors are 70% homologous. They consist of 113 and 109 residues, respectively, with 2 intra-chain disulfide bridges. SSI forms strong noncovalent dimers and dimers inhibit 2 enzyme molecules without dissociation (Kakinuma, Sugino, Mofiya and Isono, 1978; Sugino, Kakinuma and Iwanaga, 1978; Sugino, Nakagawa and Kakinuma, 1978).

2.6.6.13 Serpin family

Serpins were found in both prokaryotes and eukaryotes (Potempa, Korzus and Travis, 1994); Silverman et al., 2001). Serpins are single chain proteins containing a conserved domain structure of 370-390 residues, usually flanked by N- or C-terminal extensions (Huber and Carrell, 1989). They present in plasma are also variably glycosylated, although the carbohydrate side chains are not required for activity (Guzdek, Potempa, Dubin and Travis, 1990). Serpins interact with their target proteinase at a reactive site located within a loop structure 30-40 amino acids from the C-terminus. The reactive site is exposed on the surface of the protein and is susceptible to proteolysis by non-target proteinases (Johnson and Travis, 1977).

2.6.6.14 Hirudin family

Hirudin inhibits thrombin by blocking substrate binding groups. Hirudin is a 65 amino acids protein with MW of approximately 7000 Da. Hirudin consists of an N-terminal globular domain and an extended C-terminal domain. The N-terminal residues 1-3 form parallel β-strand with 214-217 residues of thrombin. The last five residues at C-terminal are in a helical loop. They form many hydrophobic contacts with thrombin (Rydel et al., 1990).

2.6.6.15 α_2-Macroglobulin family

The major plasma protein belongs to this family is human α_2-macroglobulin (α_2-M). It contains 4 identical subunits, each subunit is approximately 185 kDa. Each subunit consists of a bait region of 25-30 amino acid residues and a thioester bond, both located in the middle of the subunit. These two sites play important role in inhibiting the proteinase (Chaudhuri, 1993). Ohisson (1971) identified 2 α-macroglo-bulins from dog plasma. They were named α_1-macroglobulin

and α_2-macroglobulin. Both α-macroglobulins are structurally and functionally similar to the human α_2-M.

2.6.7 Inhibition mechanism of serine proteinases

The serine proteinases, such as trypsin, chymotrypsin and elastase belong to a common protein superfamily. These three types of digestive serine proteinases are distinguished based on their specificity. In trypsin and α-chymotrypsin the binding pocket quite open because of the Gly-216 and Gly-226 residues (Whitaker, 1994b). Therefore, large side chains of amino acid residues can fit into the pocket. However, trypsin has Asp-189 at the bottom of the pocket then trypsin specifically cleaving the C-terminal to residues carrying a basic side chain as Lys and Arg, because of the positive charge of Lys and Arg form an electrostatic bind with the carboxyl group of Asp-189 (Whitaker, 1994b). Whereas α-chymotrypsin has Ser-189, showing a preference for cleaving C-terminal to residues carrying a large hydrophobic side chain as Phe, Tyr and Leu. In addition, the binding pocket of elastase filled with the bulky side chain of Val-216 and Thr-226 then elastase showing a preference for cleaving C-terminal to residues carrying a small neutral side chain as Ala and Gly (Figure 2.6) (Laskowski and Kato, 1980; Whitaker, 1994b). Inhibitors of these serine proteinases have been found in many plant species, and are universal throughout the plant kingdom, with trypsin inhibitors being the most common type (Habib and Fazili, 2007). All serine inhibitor families from plants are competitive inhibitors and all of them inhibit proteinases with a similar standard mechanism (Laskowski and Kato, 1980).

When a protein interacts with the active site of a proteinase in a manner similar to a substrate and blocks the activity, then this protein is called a proteinase inhibitor. According to Laskowski and Kato (1980), standard mechanism referred to

the inhibition mechanism which inhibitor suppressed proteinase activity by acting as substrates of their target enzymes. Inhibitors that follow standard mechanism have a similar reactive site loop configuration. The loop consists of the bond between 2 amino acid residues P1 and P1'on the substrate (Figure 2.7).

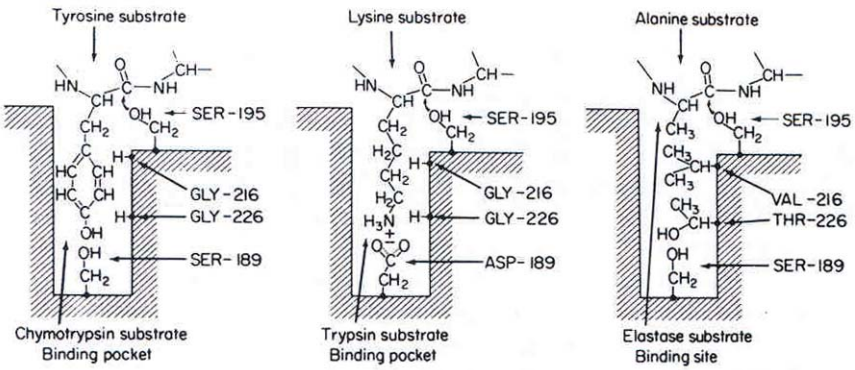

Figure 2.6 Proposed substrate binding loci in α-chymotrypsin, trypsin and elastase .

Source : Whitaker (1994b)

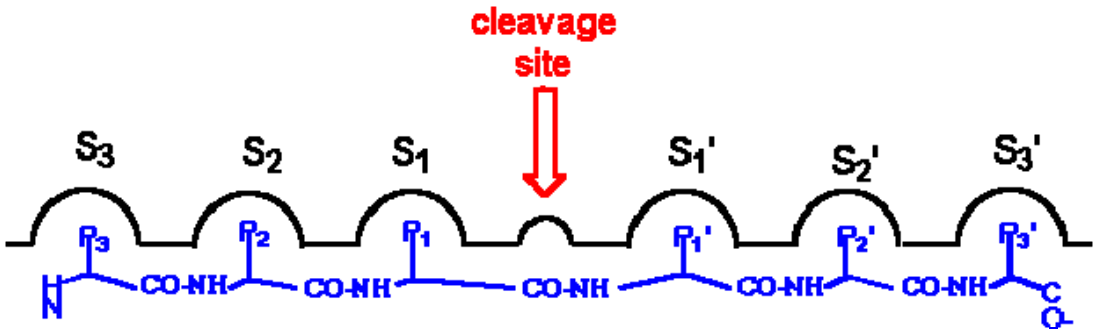

Figure 2.7 Schematic representation of specificity subsites of proteinases and the corresponding complementary sites of substrates. Arrow indicates the cleavage site.

Source : http://www.rosalindfranklin.edu

In standard mechanism model, the enzyme and inhibitor interact to form a stable complex (C) with relatively small conformational change. This stable complex dissociates very slowly to give free enzyme and native (I) or modified inhibitor (I*). In I* the peptide bond at P1-P1' is cleaved (Figure 2.8). In trypsin like proteinase, the segment N- terminal of the peptide bond of the inhibitor fits into the enzyme as an antiparallel β-strand. This is facilitated through main chain hydrogen bond formation between P3 and P1. The C-terminal residue at P2' position interacts to form one more hydrogen bond (Laskowski and Kato, 1980).

$$\mathbf{E + I \rightleftharpoons L \rightleftharpoons C \rightleftharpoons X \rightleftharpoons L^* \rightleftharpoons E + I^*}$$

Figure 2.8 Enzyme-inhibitor interaction. E,enzyme; I, native inhibitors; C, stable enzyme inhibitor complex; X, long lived intermediate in the E+I* reaction; I*, modified inhibitors; L and L*, loose, noncovalent complexes of E with I and I* respectively.

Source : Laskowski and Kato (1980).

On the other hand, α_2-macroglobulin, ecotin and serpins are out of standard mechanism rule. α_2-macroglobulin inhibits the serine proteinases by engulfing the proteinase after cleavage of α_2-macroglobulin molecules by their target enzymes (Figure 2.3) (Sottrup-Jensen and Birkedal-Hansen, 1989). Ecotin from E. *coli* inhibited proteinases which have wide specificities. Each ecotin dimer inhibited 2 enzyme molecules. It has a less typical substrate like primary site and an antibody like secondary site, which allows inhibitor to bind tightly to proteinases and to blok the substrate binding site. The primary site interactions that are less optimal are compensated by the additional secondary site interactions (McGrath et al., 1995).

2.6.8 The serine proteinase inhibitors from plants

PIs are generally occurred in plants. Plant PIs (PPIs) are generally small proteins that have mainly been described as occurring in storage tissues, such as tubers, seeds, in leave and fruits (Xavier-Filho, 1992). They are also induced in plants in response to injury or attack by insects or pathogens (Ryan, 1990). These PPIs act as anti-metabolic proteins, which interfere with the digestive process of insects. The defensive capabilities of PPIs rely on inhibition of proteinases which present in insect guts or secreted by microorganisms. This action affected a reduction in the availability of amino acids which necessary for their growth and development (Lawrence and Koundal, 2002). In addition, a novel trypsin inhibitor (PPTI) was purified from the seeds of the native Brazilian tree *Poecilanthe parviflora*. This inhibitor was stable over a wide range of temperature and pH and in the presence of DTT. The N-terminal sequence of the PPTI showed a high degree of homology with other Kunitz-type inhibitors. And the inhibitor (PPTI) substantially inhibited trypsin-like activity in midguts of larval *Diatraea saccharalis*, *Anagasta kuehniella*, *Spodoptera frugiperda*, and *Corcyra cephalonica* (Garcia, Freire, Novello, Marangoni, and Macedo, 2004). Pereira et al., (2007) reported that a serine proteinase inhibitor belonging to the Bowman-Birk class was purified from a wild accession of *Phaseolus coccineus* L. seeds (PcBBI1) inhibited both trypsin and chymotrypsin from *Hypothenemus hampei* larval midgut. It has been reported that PdKI was purified from *Pithecellobium dumosum* seeds belong to Kunitz-type inhibitors family from the Mimosoideae and Caesalpinoideae subfamilies. PdKI inhibited digestive proteinase from *Zabrotes subfasciatus*, *Ceratitis capitata*, *Plodia interpunctella*, *Alabama argillaceae*, and *Callosobruchus maculates* with 69, 66, 44, 38, and 29% inhibition, respectively (Oliveira et al., 2007).

The most well known of the plant serine proteinase inhibitors is soybean Kunitz trypsin inhibitor (SBTI) (Laskowski and Kato, 1980). It has MW of 21 kDa that complexes with the enzyme with a very high association constant. Moreover, the soybean seed contains another serine proteinase inhibtor. It is the Bowman-Birk proteinase inhibitor (BBI) that inhibit trypsin and chymotrypsin at independent reactive sites. It is often to confused between soybean Kunitz trypsin inhibitor and soybean BBI, but the 2 are strikingly different. The polypeptind chain in single headed Kunitz trypsin inhibitor has 181 residues and only 2 disulfide bridge while soybean BBI with typical double headed has about 70 residues and 7 disulfides bridge. (Laskowski and Kato, 1980).

The main serine proteinase inhibitors that are present in barley seeds are the chymotrypsin/subtilisin inhibitors 1 and 2 (CI-1 and 2), a bifunctional barley α-amylase/subtilisin inhibitor (BASI), a barley Bowman-Birk type trypsin inhibitor (BBBI), some trypsin/α-amylase inhibitors (CM proteins) and serpins (Carbonero and GarcÌa-Olmedo, 1999; Shewry, 1999). The CI, BASI and BBBI proteins strongly inhibit microbial proteinases, but the CM proteins which have been shown to inhibit proteinases only affected trypsin molecules from bovines and from some insects, not those from microbes (Carbonero and GarcÌa-Olmedo, 1999; Shewry, 1999). The BBBI contains amino acid sequences that are homologous with those of the double-headed trypsin-chymotrypsin inhibitors. (Nagasue, Fukamachi, Ikenaga and Funatsu, 1988; Prakash et al., 1996). BBBI strongly inhibited both bovine and bacterial trypsins (Boisen and Djurtoft 1982). BBBI is a single chain protein that contains 2 homologous domains (Nagasue et al. 1988, Prakash et al. 1996). The 2 domains can simultaneously bind 2 trypsin molecules (Nagasue et al., 1988; Song et al., 1999).

Boisen and Djurtoft (1982) purified two BBBI isoforms with molecular masses of about 16 kDa from barley embryos. The molecular mass that was calculated from the amino acid sequence of the rootlet inhibitor was approximately 13.8 kDa. These masses probably differed because they were determined using different analysis methods and the embryonal and rootlet inhibitors were apparently the same protein (Nagasue et al., 1988). BBBI contains 10 disulfide bridges that are highly conserved among the various Bowman-Birk type inhibitors and they probably confer to the protein its resistance to heat and to pepsin treatments at pH 2 (Boisen and Djurtoft, 1982; Nagasue et al., 1988; Song et al., 1999). In addition, crystal structure of BBBI in the free state has been determined by the multiwavelength anomalous method. This is the first report on the structure of a 16 kDa double-headed Bowman-Birk inhibitor (BBI) from monocotyledonous plants. BBBI consists of 125 amino acid residues with two inhibitory loops. The BBBI structure consists of 11 β-strands and the loops connecting these β-strands but it lacks α-helices (Song et al., 1999).

Terras, Schoofs, Thevissen, Osborn, Vanderleyden, Cammue and Broekaert, (1993) reported that CMe and 2 Bowman-Birk type inhibitors have some antifungal properties, but these properties were probably due to the proteins rendering the hyphal plasmalemma permeable. A 14 kDa trypsin inhibitor from maize, whose N-terminal amino acid sequence was homologous to that of the barley CMe, was associated with resistance to the fungus *Aspergillus flavus* (Chen, Brown, Lax, Guo, Cleveland and Russin, 1998). It also inhibited the germination of spores and mycelial growth of various plant pathogens that were grown in potato dextrose broth (Chen, Brown, Lax, Cleveland and Russin, 1999a). However, the inhibitor did not affect the fungal growth if bacterial α-amylase was added to the potato dextrose broth or when the fungus was

grown on a protein-containing (gelatin) medium, implying that the effect was probably due to its ability to interfere with the fungal α-amylase activity (Chen, Brown, Russin, Lax, and Cleveland, 1999b). Additionally, the proteins that inhibited the subtilisin-like *Fusarium* proteinase were chymotrypsin/subtilisin (CI) inhibitors 1A, 1B, and 2A and the barley α-amylase/subtilisin inhibitor (BASI) (Pekkarinen and Jones, 2003).

2.6.9 The serine proteinase inhibitors from animals

A major role for serine PIs in animals is limiting the activity of endogenous proteinases in tissues where this activity would be harmful, as in case of pancreatic trypsin inhibitors found in mammals. It has long been known that ovomucoids are the major glycoprotein proteinase inhibitors from avian egg whites (Saxenaa and Tayyab, 1997). Three different proteins of the avian egg white are known to possess trypsin inhibitory activity as ovomucoid, ovoinhibitor and ovostatin (Kato, Kohr and Laskowski, 1978; Kitamoto, Nakashima and Ikai, 1979; Lineweaver and Murray, 1947). Kato et al., (1978) studied the primary structure of chicken ovomucoid. They found that a 24-amino acid residue signal sequence is removed during the synthesis of the protein. The single polypeptide chain is organized into 3 homologous Kazal-type domains. Study on circular dichroism (CD) showed that Ovomucoid is rich in β-structure (46%) compared with α-structure (26%) (Watanabe, Matsuda and Sato, 1981). Chicken ovoinhibitor is a 49 kDa, 447 amino acid residues single polypeptide comprising 7 homologous Kazal-type domains with covalently attached carbohydrate moieties (Scott et al., 1987; Yet and Wold, 1990). In nature, about 51% of the amino acid residues are hydrophobic. The amino acid sequence of the nature protein derived from cDNA is identical to that determined from protein sequencing, except that an

Asn at the 12 position of the 5 domain was identified as Asp by protein sequencing. Since this amino acid is closed to Gly, it is possible that spontaneous de-amidation of Asn to Asp occurs after its synthesis (Scott et al., 1987).

In addition, Nagase, Harris, Woessner and Brew (1983) identified a trypsin inhibitory protein in chicken egg white and named it ovostatin (and was later called 'ovomacroglobulin'). The protein has an amino acid composition similar to that of α2-macroglobulin from plasma and has inhibitory activity towards trypsin, papain, subtilisin, rabbit synovial collagenase and thermolysin (Nagase et al., 1983; Nagase and Harris, 1983; Saxenaa and Tayyab, 1997). The native protein, MW 660,000 contains 4 subunits of equal MW 175,000, each pair being disulfide linked. The dimers are held together by noncovalent interactions (Nagase et al., 1983; Nagase and Harris, 1983). The final proteinase inhibitor which was identified from avian egg white was 'cystatin'. Cystatin from chicken egg white was found to inhibit ficin, papain and dipeptidyl peptidase, as well as cathepsins B, H and L (Anastasi et al., 1983; Fossum and Whitaker, 1968). The proteins of the cystatin superfamily are classified into three families (types 1, 2 and 3) on the basis of their molecular structure. Chicken cystatin is a type 2 cystatin. Like the other proteins of this family, chicken cystatin contains two disulfide bridges with MW of 13,000 Da (Anastasi et al., 1983; Schwabe, Anastasi, Crow, McDonald and Barrett, 1984).

Takahara and Sinohara (1982) isolated trypsin inhibitors from mouse serum. This high inhibitory capacity of mouse serum was accounted for mainly by 2 glycoproteins. The first one is α_1-antitrypsin whose molecular properties are very similar to those of α_1-antitrypsin purified from human and rat (Ikehara, Miyasato, Ogata and Oda, 1981; Morii, Odani, Koide and Ikenaka, 1978; Musiani and Tomasi,

1976). The second, contrapsin, have MW of 55,000 Da. Murinoglobulin was glycoprotein inhibitor which did not correspond to any of the known plasma proteinase inhibitors that have been well characterized in human or other mammals. Murinoglobulin contained about 7.6% carbohydrate. The MW of 180,000 Da as judged by the equilibrium sedimentation analysis and SDS-PAGE under reducing conditions (Saito and Sinohara, 1985). Recently, it has been reported that an endogenous trypsin inhibitor isolated from the mouse muscle cytoplasmic fraction is a glycoprotein with MW of 110 kDa. This inhibitor inhibited protease M activity on myofibrillar proteins from mouse (Sangorrín, Martone and Sánchez, 2000; Sangorrín, Martone and Sánchez, 2002).

Calpastatins inhibit Ca^{2+}-activated neutral proteinase (CANP) were found in bovine cardiac muscle (Waxman and Krebs, 1978), carp ordinary muscle (Toyohara, Makinodan, Tanaka and Ikeda, 1983) and grass prawn muscle (*Penaeus monodon*) (Jiang, Wu, Su and Tzeng, 2000). Hara and Ishihara (1987) isolated serine proteinase inhibitor from carp (*Cyprinus carpio*) ordinary muscle. The inhibitor was stable over the range of pH 7.0-9.5 at 5°C for 15 hr, but unstable below pH 4.5. The isoelectric point was about 5.3. This inhibitor strongly inhibited trypsin, α-chymotrypsin and elastase. The MW of the inhibitor was about 100,000 by gel filtration and was about 56,000 Da by SDS-PAGE under reducing condition. In contrast, Toyohara, Makinodan, Tanaka and Ikeda, (1983) reported that trypsin inhibitor from carp (*Cyprinus carpio*) muscle had MW of 70,000 Da and inhibited the caseinolytic activities of trypsin and α-chymotrypsin. Similarly, white croaker (*Micropogon opercularis*) trypsin inhibitor has MW of 65 kDa and its thermal stability decreased at temperature greater than 60 °C (Folco, Busconi, Martone, Trucco and Sánchez, 1984;

Sangorrín, Folco, Martone, and Sánchez, 2001).

A novel serine proteinase inhibitor has been purified from the white croaker (*Argyrosomus argentatus*) skeletal muscle and specifically inhibited a myofibril-bound serine proteinase (MBSP) isolated from the skeletal muscle of lizardfish (*Saurida wanieso*). No inhibition, was detected toward other serine proteinases, such as bovine trypsin, bovine chymotrypsin and a myofibril bound serine proteinase from carp (*Cyprinus carpio*) muscle (Cao, Osatomi, Matsuda, Ohkubo, Hara and Ishihara, 2000). While, a novel myofibril-bound serine proteinase inhibitor (MBSPI) from the skeletal muscle of lizardfish (*Saurida wanieso*) reveals high inhibition specificity toward a myofibril-bound serine proteinase (MBSP) purified from lizardfish muscle. No inhibition was detected toward bovine trypsin, bovine chymotrypsin, 2 trypsins from carp hepatopancreas and a serine proteinase isolated from the sarcoplasmic fraction of white croaker muscle and also a myofibril-bound serine proteinase from carp muscle (Cao, Osatomi, Hara and Ishihara, 2001). Furthermore, the sequences of tryptic digested peptide fragments of MBSPI revealed high identity (76%) to porcine phosphoglucose isomerase (PGI) The purified MBSPI also exhibited PGI activity. The rabbit muscle PGI also specifically inhibited the activity of MBSP. It was strongly suggested that MBSPI was actually PGI and PGI is a specific inhibitor toward MBSP purified from lizardfish muscle (Cao et al., 2000). Purified trypsin inhibitor from white croaker (*Micropogon opercularis*) exhibited inhibitory activity against trypsin, chymotrypsin and elastase. It was not inhibit proteinase belonging to other groups, indicating that it is highly specific for serine proteinases (Sangorrín et al., 2001).

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CHAPTER III

PROTEIN CROSS-LINKING ABILITY AND GEL-ENHANCING PROPERTIES OF SARCOPLASMIC PROTEINS FROM THREADFIN BREAM (*Nemipterus* spp.)

3.1 Abstract

Threadfin bream sarcoplasmic proteins (TBSP) were concentrated by ultrafiltration using a membrane with molecular weight cut-off 30 kDa. Optimum TGase activity from TBSP was at 37^{o}C, pH 7.5. TGase activity increased with $CaCl_2$ concentration and reached the maximum at 5 mM $CaCl_2$. The highest TGase activity was found at 1 mM dithiothreitol (DTT). TBSP induced cross-linkings of bovine serum albumin when incubated at 25^{o}C for 6 h. TGase activity staining by monodansylcadavarine (MDC) on native-polyacrylamide gel electrophoresis (PAGE) showed 2 distinct fluorescent bands with molecular weight (MW) of 78 and 189 kDa. Two proteins from fluorescent bands exhibited MW of 66 kDa on SDS-PAGE. Addition of 0.1% $CaCl_2$ in conjunction with 1.6% TBSP exhibited the highest breaking force and deformation of lizardfish surimi when pre-incubated at 37°C for 20 min. TBSP showed potential to enhance gel strength of lizardfish surimi.

Key words: Sarcoplasmic protein, transglutaminase , cross-linking, threadfin bream, activity staining

3.2 Introduction

Sarcoplasmic protein is defined as water or low ionic strength soluble proteins. The conventional surimi process, therefore, removes sarcoplasmic proteins in fish muscle during washing. It has been recognized that sarcoplasmic proteins hinder the gel-forming ability of myofibrillar proteins (Haard, Simpson and Pan, 1994). However, many studies have recently reported that sarcoplasmic proteins can enhance gel strength of myfibrillar proteins. Karthikeyan, Mathew, Shamasundar and Prakash (2004) reported that sarcoplasmic proteins from oil sardine (*Sardinella longiceps*) fractionated by ammonium sulfate were able to enhance storage modulus of washed sardine meat gel. The gel strength of lizardfish gel increased with addition of tilapia sarcoplasmic proteins (Yongsawatdigul and Piyadhammaviboon, 2007). Morioka and Shimizu (1993) suggested that gels prepared from sarcoplasmic proteins with MW of 94, 40, and 26 kDa showed high gel strength. Contribution of freeze-dried rockfish sarcoplasmic proteins in increasing breaking force of pollock surimi has also been reported (Kim, Yongsawatdigul, Park and Thawornchinsombut, 2005). These studies suggested that sarcoplasmic proteins did not interfere gel-forming of myofibrillar proteins.

Transglutaminase (TGase) (glutaminyl-peptide γ-glutamyl transferase, EC 2.3.2.13) is an enzyme catalyzing the acyl-transfer reaction introducing covalent cross-links between proteins as well as peptides and various primary amines. (Folk, 1980) TGase widely distributed in fish species (Kishi, Nozawa and Seki, 1991; Kumazawa, Nakanishi, Yasueda and Motoki, 1996; Woratao and Yongsawatdigul, 2005; Yasueda, Kumazawa, and Motoki, 1994). These TGases require Ca^{2+} for activation. The setting phenomenon in surimi is induced by endogenous

transglutaminase activity, resulting in higher gel strength. (Kamath, Lanier, Foegeding and Hamann, 1992; Kimura et al.,1991)

Thailand is the second largest surimi producer of the world. In 2006, production of surimi in Thailand were 150,000 metric tons and 50 % of surimi was produced from threadfin bream (TB). Yongsawatdigul and Piyadhammaviboon (2007) found that tilapia sarcoplasmic proteins concentrated using an ultrafiltration membrane of 30 kDa molecular weight cut-off (MWCO) contained TGase activity approximately 498.6 U/mL. TGase activity of the second wash water seemed to be comparable to that of TB mince. (Yongswatdigul, Worratao and Park, 2002). This implied that TGase was removed during washing step. Therefore, objectives of this study were to elucidate biochemical characteristics of TGase contained in TBSP and to investigate the gel enhancing efficacy of TBSP on lizardfish surimi.

3.3 Materials and Methods

3.3.1 Chemicals

Monodansylcadavarine (MDC), di-methylated casein (DMC), casein, standard protein for native-PAGE and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethylenediaminetetraacetic disodium salt (EDTA) and bovine serum albumin (BSA) were purchased from Fluka (Buchs, Switzerland). Reagents used for gel electrophoresis were purchased from Promega (Madison, WI, USA). All other chemicals were of reagent grade.

3.3.2 Materials

Fresh threadfin bream (*Nemipterus* spp.) were purchased from Rayong province. Fish were packed in a polystyrene foam box filled with ice and transported

to Suranaree University laboratory approximately within 8 h after catch. Samples were vacuum-packed and kept at –40°C until use. Frozen lizardfish (*Saurida* spp.) surimi was obtained from a surimi plant at Samutsakorn province. Surimi samples were packed in a polystyrene foam box filled with ice, and immediately transported to the Suranaree University laboratory. Frozen surimi was cut into 1-kg blocks. Surimi were vacuum-packed and kept at –18°C until used.

3.3.3 Preparation of TBSP

TB mince was mixed with 3 volumes of cold drinking water (reverse osmosis) and homogenized for 1 min then centrifuged at 5,000xg, 4°C for 15 min. Supernatant was collected and subsequently centrifuged at 35,000xg, 4°C for 30 min. Supernatant was referred as TBSP.

TBSP were concentrated using an ultrafiltration membrane of 30-kDa MWCO (Vivaspin concentrator; Vivascience AG, Hannover, Germany). The ultrafiltration was carried out by centrifuging at 4°C until samples were concentrated to 8-10 folds. Retentates were analyzed for protein content, TGase activity, SDS-PAGE, TGase activity staining and protein cross-linking.

3.3.4 Determination of TGase activity

TGase activity was assayed using the method of Yongsawatdigul, Worratao and Park (2002) with slight modifications. The mixture (2 mL) contained 2 mg DMC, 15 μM MDC, 5 mM $CaCl_2$, 3 mM DTT , 70 mM Tris-HCl buffer pH 7.5 and 100 μL of TBSP. After incubated at 37°C for 10 min, the reaction was terminated by adding 400 μL of 0.1 M EDTA. The fluorescence intensity of MDC incorporated into DMC was measured using a spectrofluorophotometer (RF-1501, Shimadzu, Kyoto, Japan) at excitation and emission wavelength of 350 and 480 nm, respectively. The enhancing

factor as described by Takagi et al. (1986) used for this study was 1.17. One unit of TGase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of MDC into DMC per min (U). The activity was calculated as follows:

$$\text{TGase activity (U)} = \frac{(FI_s\text{-}FI_b) \times 15{,}000 \text{ nmol} \times 2 \text{ mL}}{10 \text{ min} \times 1.17 \times FI_o \times 1{,}000 \text{ mL}}$$

Where, FI_s = the fluorescence intensity of sample, FI_b = blanks with inactivated enzyme, FI_o = blanks without enzyme (substituting with deionized water).

3.3.5 Biochemical characteristics of TBSP TGase

3.3.5.1 Optimal temperature

The temperature profile on catalytic activity was conducted using the reaction mixture as described previously. TBSP were pre-incubated for 5 min and assayed at various temperatures; 20, 25, 30, 35, 37 and 40°C for 10 min. The reactions were carried out at 70 mM Tris-Cl (pH 7.5), 5 mM $CaCl_2$ and 3 mM DTT.

3.3.5.2 Optimal pH

The optimal pH was carried out with various buffers; 50 mM Tris-maleate (pH 7.0), 70 mM Tris-HCl (7.5-9.0), and 70 mM glycine-NaOH buffer (9.5-10). All reactions were assayed at 1 mg/ml DMC, 15 μM MDC, 5 mM $CaCl_2$ and 3 mM DTT at 37°C for 10 min.

3.3.5.3 Effect of $CaCl_2$

Effect of $CaCl_2$ was performed at 0, 0.1, 0.5, 1 and 5 mM. The assay were investigated at 37°C for 10 min and contained 1 mg/mL DMC, 15 μM MDC, 3 mM DTT, and 70 mM Tris-HCl, pH 7.5.

3.3.5.4 Effect of DTT

The effect of DTT was assayed at 37°C for 10 min at various final

concentrations of 0, 1, 2, 3, 5, 7 and 10 mM. The reaction mixture contained 1 mg/mL DMC, 15 μM MDC, 5 mM $CaCl_2$, and 70 mM Tris-HCl, pH 7.5.

3.3.5.5 Effect of NaCl

Effect of NaCl was tested at various final concentrations of 0, 0.2, 0.3, 0.4, 0.6, 0.8 1.0 and 1.2 M in the presence of 1 mg/mL DMC, 15 μM MDC, 5 mM $CaCl_2$, 3 mM DTT, and 70 mM Tris-HCl, pH 7.5. The reaction was incubated at 37^{o}C for 10 min.

3.3.6 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were mixed with the solubilizing buffer (125 mM Tris–HCl pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol) at a ratio 1:1 and boiled for 3 min. Thirty micrograms protein were loaded on 10 %(w/v) polyacrylamide gel according to the method of Laemmli (1970). Gels were run at a constant voltage set at 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.

3.3.7 TGase activity staining

Activity staining of TGase was performed according to the procedure of Lorand et al. (1979) with slight modifications. Standard native markers (BSA, chicken egg albumin, α-lactalbumin and carbonic anhydrase) and concentrated TBSP were loaded on native-PAGE using 6.5, 7.0, 7.5 and 8.0 % polyacrylamide gel. Gels were run at a constant voltage of 100 V. After electrophoresis, the gels were stained by two methods. Firstly, gels with standard markers were stained with Coomassie Brilliant blue as mentioned earlier. Another gel was stained in the activity staining solution (2 mM MDC, 5 mg/mL DMC, 5 mM $CaCl_2$, 5 mM DTT and 50 mM Tris-

HCl, pH 7.5) in a shaking incubator set at 40°C for 2 h. Consequently, gels were rinsed with distilled water twice and immersed in 10 % TCA for 2 h. Free MDC was removed by soaking in 7.5% acetic acid for 10 min twice. MDC-bound proteins were detected by ultraviolet (UV) illuminator (Gel Doc, Bio-Rad Laboratories, Hercules, CA).

3.3.8 Determination of TGase molecular weight

The fluorescent protein bands were cut and chopped into small pieces using a clean razor blade. The cut samples were filled with 500 µL of elution buffer containing 0.1% SDS, 150 mM NaCl and 250 mM Tris-HCl (pH7.5) and were shaken overnight. The mixtures were centrifuged at 3,000xg for 5 min using Nanosep® MF centrifugal devices (Pall Life Science, Ann Arbor, MI, USA) equipped with 0.2-µm membrane. The permeate was mixed with 500 µL of exchange buffer containing 0.1% SDS, 250 mM Tris-HCl (pH7.5) and were centrifuged at 3,000xg for 7 min (3 times) using Nanosep® centrifugal devices with 10-kDa MWCO membrane (Pall Life Science, Ann Arbor, MI, USA). Molecular weight of the retentate were determined using SDS-PAGE.

3.3.9 Protein cross-linking study

The reaction mixture (2 mL) contained 10 mg of protein substrate (casein or BSA), 5 mM $CaCl_2$, 5 mM DTT, 100 mM Tris-HCl, pH 7.5 and 18.5 unit of TBSP TGase. Protein substrates were pre-incubated at 50°C for 10 min and added into the reaction mixture. Subsequently, it was incubated at either 37°C for 30 min or 25°C for 6 h. The sample without incubation was used as a control. The reaction was terminated by adding 2 mL of 10% hot SDS. The mixtures were centrifuged at 10,000xg for 10 min. Cross-linked products were monitored using 7.5% polyacrylamide gel.

3.3.10 Effect of TBSP on textural properties of lizardfish surimi

Lizardfish surimi pastes containing 2% NaCl, 1.6% TBSP (selected concentration studied in Chapter IV) and 80% moisture content were prepared with various additions of $CaCl_2$ at 0, 0.1, 0.2, 0.3 and 0.4 %. The surimi without adding TBSP were compared. The pastes were stuffed into a microplate with a well diameter of 6.86/6.35 mm (top/bottom) and depth of 10.67 mm using a syringe. The filled microplates were vacuum-packed in a linear low density polyethylene (LLDPE)/nylon bag. Samples were pre-incubated at 37°C for 20 min, and subsequently heated at 90°C for 10 min. Samples heated at 90°C for 10 min were used as a control. Samples were immediately cooled in iced water for 20 min and stored in a refrigerator (5-8°C) overnight. Breaking force (g) and deformation (mm) were determined using a 2-mm cylindrical probe at a test speed of 1 mm/s (Stable Micro System, Surrey, England).

3.3.11 Statistical analyses

All experiments were conducted in duplicate. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were used to determine differences among treatments. The statistical analysis was performed using Statistical Analysis System (SAS Inst. Inc., Cary, N.C., USA.). Significance of differences was defined at $p \leq 0.05$.

3.4 Results and Discussion

3.4.1 Optimum temperature and pH of TGase

Optimal temperature of TGase in TBSP was found at 37^{o}C (Figure 3.1a), while the optimal pH was 7.5 (Figure 3.1b). Optimum temperature of TBSP was

similar to that reported in TB (*Nemipterus virgatus*) crude TGase (Tsukamasa, Miyake, Ando and Makinodan, 2002) which was at 40°C. Crude tilapia TGase exhibited optimal activity at 50°C (Woratao and Yongsawatdigul, 2003), while the purified TGase from Japanese oyster showed optimum activity at 25 and 40°C (Kumazawa, Sano, Seguro, Yasueda, Nio and Motoki, 1997). Optimum temperature of scallop hemocyte TGase was 15°C (Nozawa, Mori, Kimura and Segi, 2005).

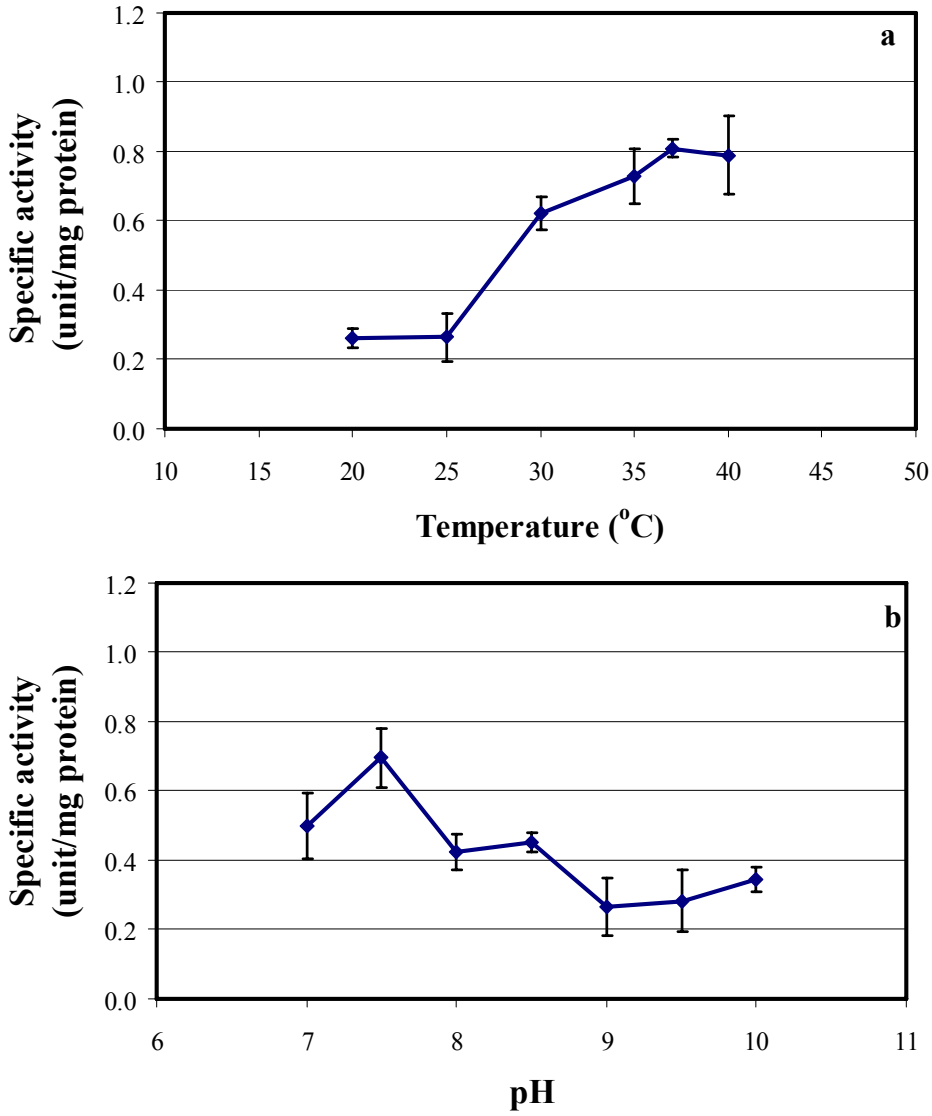

Figure 3.1 Temperature (a) and pH (b) profile of TGase in TBSP.

It was reported that optimum setting temperature of TB gel which is a tropical fish, was at 40°C (Yongsawatdigul et al., 2002) Setting temperature appeared to correspond with the optimum temperature of endogenous transglutaminase activity. The optimum pH found in this study, pH 7-7.5, was similar to that reported in crude tilapia, (Woratao and Yongsawatdigul, 2003). pH optima of carp and scallop TGase was reported to be 8.0 (Nozawa, Mamegoshi and Segi, 1997)

3.4.2 Effect of $CaCl_2$

A minimum TGase activity was found in the absence of $CaCl_2$ (Figure 3.2). TGase activity considerably increased with $CaCl_2$ concentration upto 1 mM and then slowly increased between 1 and 5 mM $CaCl_2$ (Figure 3.2), indicating that TGase in TBSP is a Ca^{2+}-dependent enzyme. Ca^{2+} might cause conformation change in enzyme, resulting in an acyl-enzyme intermediate formation, resulting in full activation of TGase activity. When $CaCl_2$ was added over 5 mM, protein precipitation was observed (data not shown). The excessive addition of $CaCl_2$ may cause "salt bridge" between protein molecules, leading to protein aggregation, interfering with TGase activity measurement. $CaCl_2$ concentration required for full activation of carp muscle TGase was also at 5 mM. (Kishi, Nozawa and Seki, 1991). Kumazawa et al. (1997) reported that activity of the purified Japanese oyster TG-1 increased with $CaCl_2$ concentration upto 100 mM. But Japanese oyster TG-2 required 25 mM $CaCl_2$ for full activation. It is evident that requirement of $CaCl_2$ for TGase activation varied with species.

This result suggested that TBSP could be used as a natural source of TGase particularly for surimi. $CaCl_2$ should be added at a level between 1 and 5 mM for full activation of TGase in TBSP.

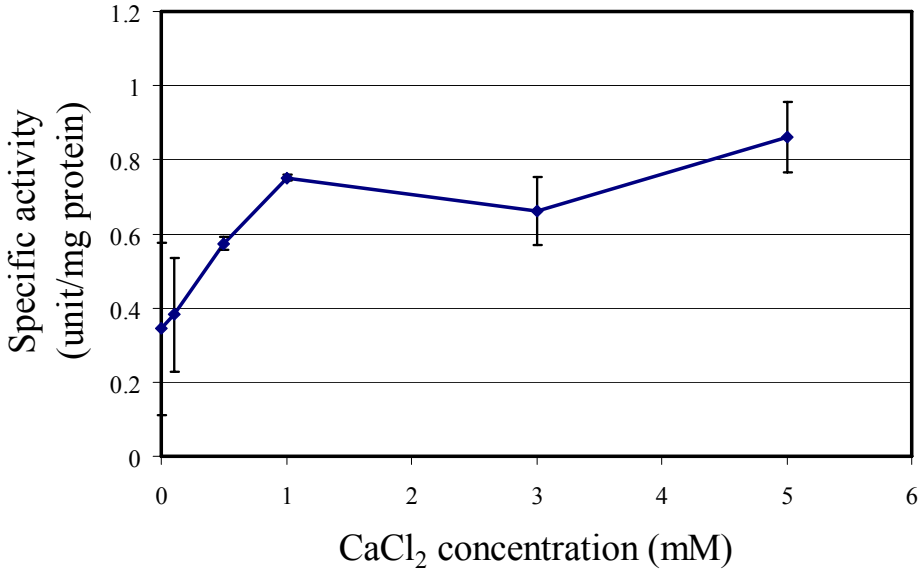

Figure 3.2 Effect of $CaCl_2$ on TGase activity of TBSP.

3.4.3 Effect of DTT and NaCl

TGase activity decreased after addition of 1 mM DTT and remained constant thereafter (Figure 3.3). Generally, DTT was added into TGase reaction mixture for maintaining a reduced form of sulfhydryl group at the active site (Folk, 1980). But this study revealed that TGase in TBSP did not require DTT for protection of its sulfhydryl group. The similar result was found in the purified tilapia TGase whose activity decreased with DTT at concentration greater than 5 mM (Woratao and Yongsawatdigul, 2005). Woratao and Yongsawatdigul (2003) reported that crude tilapia TGase did not require additional DTT for MDC incorporation to DMC. Moreover, DTT concentration at 0-10 mM did not affect TGase activity of partial purified of TB liver (Hemung and Yongsawatdigul, 2008).

The highest activity was found at 0.2 M NaCl (Figure 3.4). The activity continually decreased at 0.2-0.6 M and remained constant thereafter. Worratao and

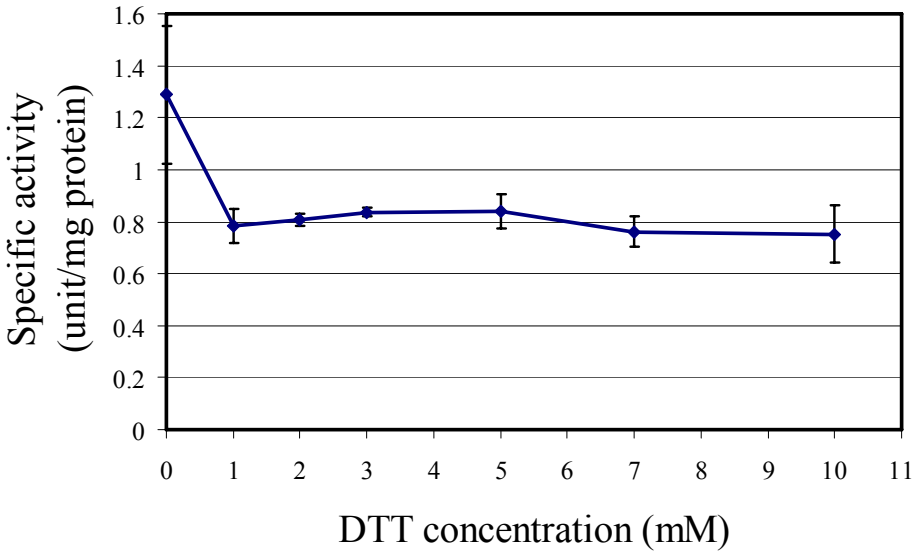

Figure 3.3 Effect of DTT on TGase activity of TBSP.

Yongsawatdigul (2005) reported that the activity of purified tilapia TGase decreased with increasing NaCl concentration. Activity of partial purified TGase from TB liver was not affected by NaCl (Hemung and Yongsawatdigul, 2008). In addition, purified Japanese oyster TGase, TG-1 and TG-2, showed different activity. TGase activity of TG-1 increased with NaCl concentration while TG-2 showed the highest TGase activity at 0.2 M and its activity decreased at higher concentration (Kumazawa et. al., 1997). In contrast, Nozawa et al. (1997) found that TGase activity from marine invertebrates, such as scallop, botan shrimp and squid, was enhanced in the presence of 0.5 M NaCl. While 0.5 M NaCl had no effect on TGase from carp, rainbow trout and Atka mackerel. Marine shellfish TGases were significantly activated by NaCl. TGase activity of abalone, surf clam, whelk, scallop, ark shell, Japanese oyster and Japanese littleneck were activated by NaCl, while TGase activity of Seta-shijimi, yamato-shijimi, ishimakigai and melanian snail which were freshwater shellfish decreased at NaCl greater than 0.1 M (Nozawa, Mori and Segi, 2001). These results

suggested that NaCl activates TGase from marine invertebrates but inactivates TGase from freshwater shellfish. Effect of NaCl on TGase activity seems to well correlate with the environment of habitat.

At 0.4 M NaCl, TGase in TBSP still have activity greater than without added NaCl, indicating that TBSP can be added into surimi to increase textural properties of surimi gel. Therefore, understanding the effect of NaCl concentration would allow the use of TBSP in surimi gels which typically contains 2 % NaCl (approximately 0.4M).

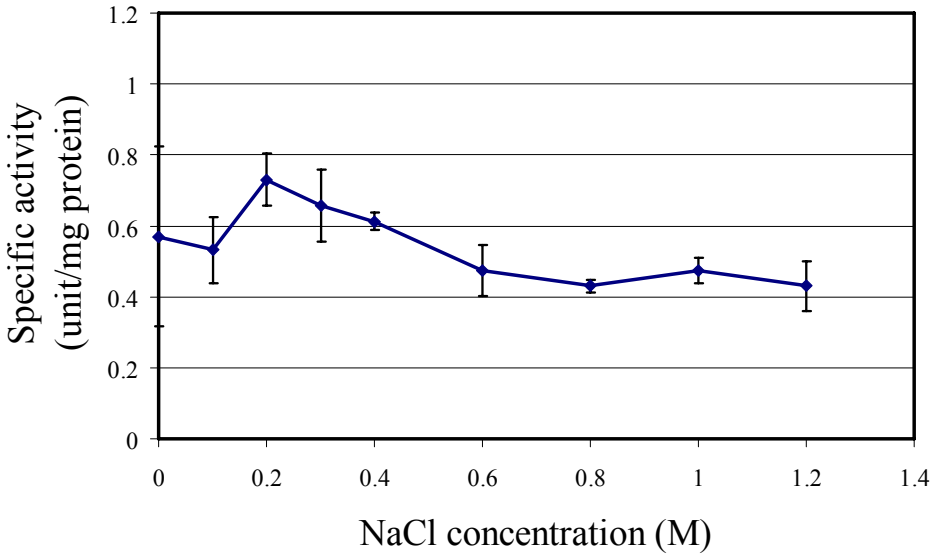

Figure 3.4 Effect of NaCl on TGase activity of TBSP.

3.4.4 Molecular weight and activity staining of TGase

TGase catalyzed incorporation on DMC resulting in fluorescent band on native-PAGE. Molecular weight of TGase in TBSP was estimated from 2 distinct fluorescent bands to be 78 and 189 kDa based on native-PAGE (Figure 3.5). In native-PAGE, proteins were separated based on charge density, rather than molecular mass.

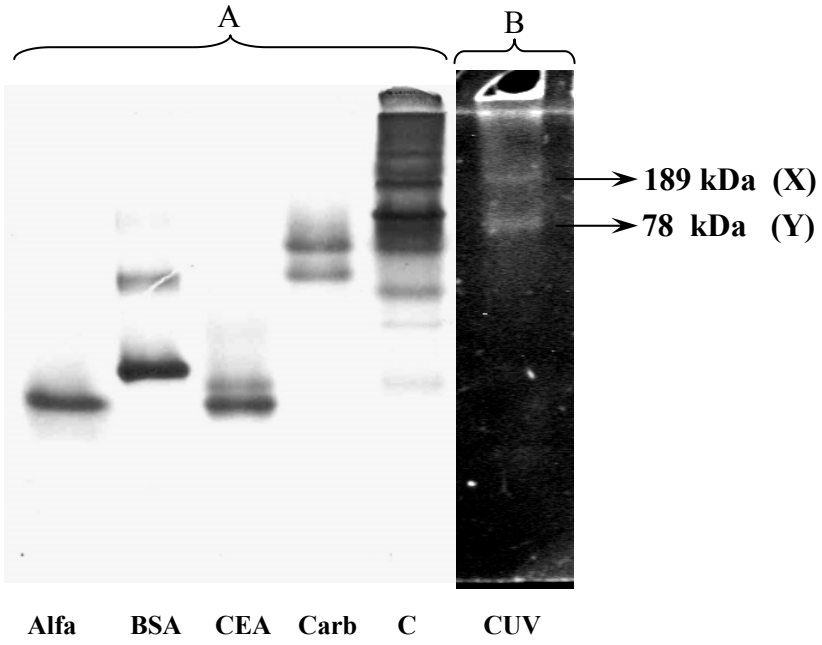

Figure 3.5 Native-PAGE (8.0 % polyacrylamide) pattern (A) and activity staining of TBSP TGase(B): Alfa, α-lactalbumin standard (14.2 kDa); BSA, bovine serum albumin standard (monomer 66 kDa, dimer 132 kDa); CEA, chicken egg albumin standard (45 kDa); Carb, carbonic anhydrase standard (29 kDa); C, crude TBSP TGase; CUV, crude TBSP TGase under UV light.

When the fluorescent bands were cut and separated on SDS-PAGE, a single band with MW of 66 kDa was revealed (Figure 3.6). Based on results, it can be presumed that 2 protein bands on native-PAGE were TGase isoforms, showing the same molecular mass with different charge characteristic. TGases from various sources showed similar mass to that found in TBSP. TGase from carp ordinary muscle was about 80 kDa (Kishi et al., 1991). Kumazawa et al. (1997) reported that MW of a purified TGase from Japanese oyster was 84 kDa and 90 kDa. Purified

tilapia TGase was 85 kDa (Woratao and Yongsawatdigul, 2005) In addition, the purified TGase from limulus hemocyte was 86 kDa.

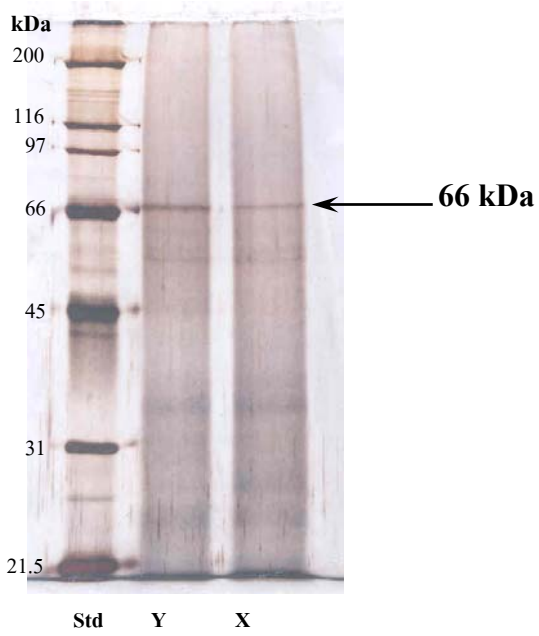

Figure 3.6 SDS-PAGE pattern (10 % polyacrylamide) of eluted protein from native-PAGE. Y and X corresponded with bands shown on Figure 3.5.

3.4.5 Protein cross-linking ability of TBSP

Cross-linked protein (CP) is an important of evidence indicating the catalytic reaction of TGase. CP was observed in BSA incubated at 25^{o}C for 6 h, while CP was not found in casein (Figure 3.7). These results suggested that BSA was a better substrate of TGase in TBSP. de-Jong, Wijngaards, Boumans, Koppelman and Hessing (2001) reported that pig erythocyte TGase was able to cross-link BSA, casein and glycinin. Bovine plasma TGase was able to cross-link BSA, casein, hemoglobin, myosin, α-lactalbumin and β-lactoglobulin whereas bacterial TGase was able to

cross-link BSA, casein, hemoglobin, myosin, α-lactalbumin, β-lactoglobulin and glycinin.

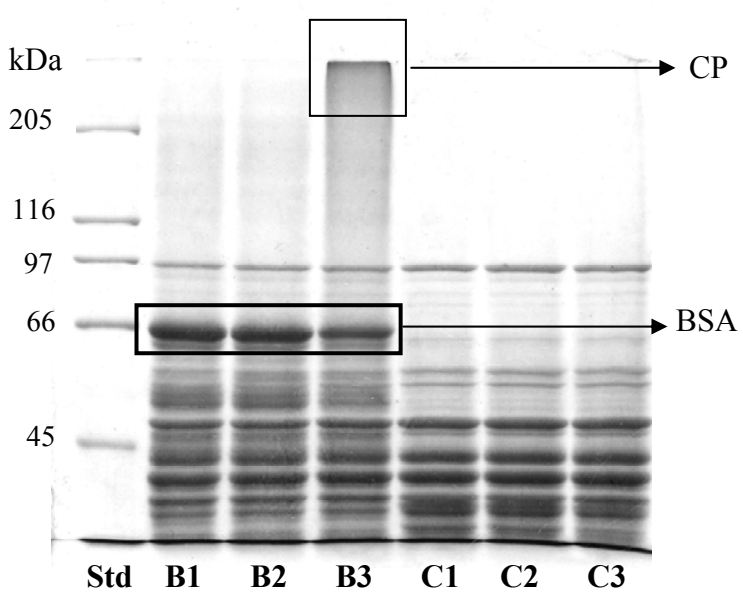

Figure 3.7 SDS-PAGE (7.5 % polyacrylamide) pattern of TBSP TGase-mediated cross-linking of BSA and casein. The reaction mixture contained TBSP TGase 1.85 unit/mg substrate, 5 mg/mL protein substrate, 5 mM $CaCl_2$, 5 mM DTT and 100 mM Tris-HCl, pH 7.5 : Std, broad-range standard marker; B1, BSA without incubation; B2, BSA incubated at 37°C for 30 min; B3, BSA incubated at 25°C for 6 h; C1, casein without incubation; C2, casein incubated at 37°C for 30 min; C3, casein incubated at 25°C for 6 h; CP, cross-linked proteins.

From figure 3.7, the extent of polymerization was greater at 25°C for 6 h, while CP was not shown at 37°C which was the optimum temperature of TGase in TBSP. This result suggested that the enzyme exhibited low thermal stability at optimum temperature (37°C). Therefore, the use of TGase in TBSP should be limited at temperature lower than 37°C.

3.4.6 Gel enhancing effect of TBSP

Addition of 0.1% Ca^{2+} along with 1.6 % TBSP to lizardfish surimi and pre-incubated at 37°C resulted in the highest breaking force ($p<0.05$) when compared with the sample without TBSP (Figure 3.8a). Addition of TBSP and pre-incubation at 37°C showed significant increasing on breaking force ($p<0.05$). Heating at 90°C minimized both proteinase and endogenous transglutaminase activity. Pre-incubation at 37°C promoted TGase activity in TBSP. Breaking force of lizardfish surimi without TBSP decreased when pre-incubated at 37°C. This could be due to proteolytic degradation caused by endogenous proteinase.

Addition of 0.1% Ca^{2+} increased breaking force of gel added TBSP (Figure 3.8a). TGase was likely to be activated in the presence of Ca^{2+}, which subsequently promoted the protein cross-linking and increased breaking force. In the absence of TBSP, addition of Ca^{2+} resulted in a decreased breaking force value. This result may be caused by calcium-dependent proteinase which was activated in the presence of Ca^{2+}. Yongsawatdigul and Piyadhammaviboon (2004) reported that EDTA inhibited autolytic degradation of lizardfish surimi about 45.3% inhibition, suggesting the involvement of Ca^{2+}-dependent proteinase and/or other metallo-proteinase. Deformation value of surimi gels showed the similar trend (Figure 3.8b, d) Recently, Yongsawatdigul and Piyadhammaviboon (2007) reported that addition of tilapia sarcoplasmic protein (1%) to lizardfish surimi and pre-incubated at 37°C resulting in the highest gel strength. An increase of gel strength was caused by the formation of cross-linked product catalyzed by TGase in tilapia sarcoplasmic protein. Protein content of sample added TBSP was increased 1.6% when compared with surimi without adding TBSP. However, increasing of gel strength could not be affected by

higher protein content since gel strength of samples heated at 90°C without pre-incubation.

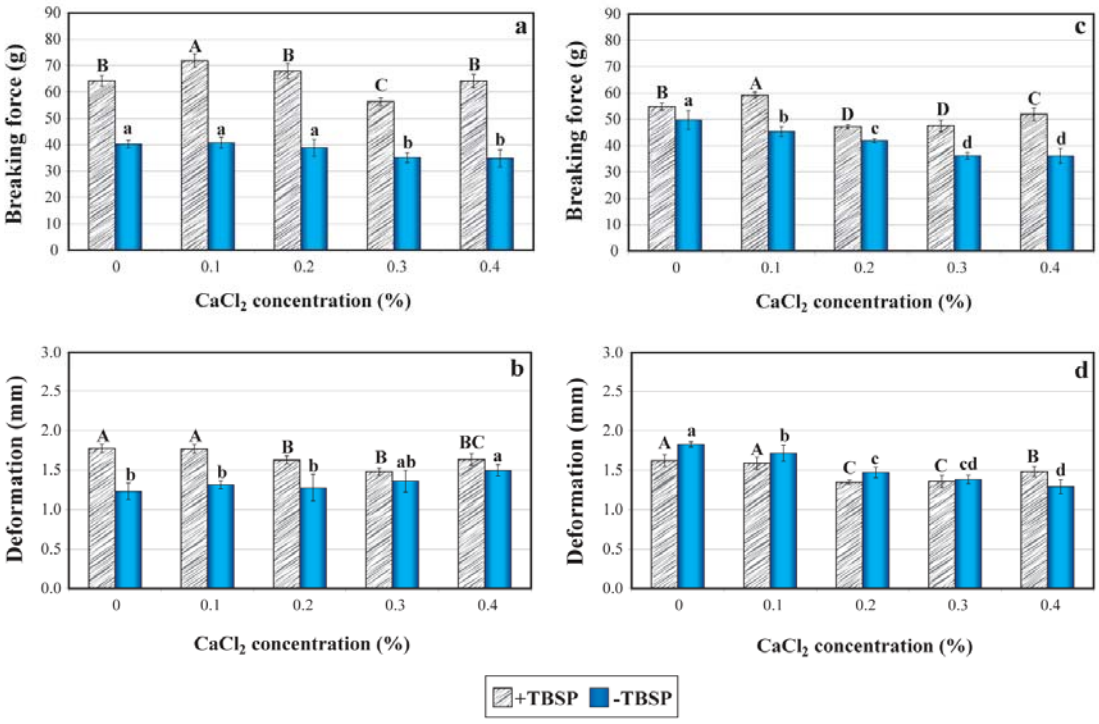

Figure 3.8 Effect of calcium chloride on textural properties of lizardfish surimi pre-incubated at 37°C for 20 min followed by heating at 90°C for 10 min (a,b) or heating at 90°C for 10 min without pre-incubation(c,d). +TBSP indicated lizardfish surimi mixed with TBSP, -TBSP indicated samples without TBSP. Different letters on bars indicate significant difference (p<0.05) in the same samples. Small letters are for gel without adding TBSP, whereas capital letters are for gel with adding TBSP

Generally, endogenous TGase is a Ca^{2+}-dependent enzyme, addition of Ca^{2+} to fish gel has been reported to activate TGase activity, resulting in an improved textural properties of fish protein gel (Lee and Park, 1998; Yongsawatdigul,

Piyadhammaviboon and Singchan, 2006; Yongsawatdigul, Worratao, and Park, 2002). Transglutaminase in TBSP play an important role in improving gel strength of lizardfish surimi. However, effect of Ca^{2+} also have been reported to promote hydrophobic interaction and disulfide bond linkages during setting of myosin and actomyosin (Hemung and Yongsawatdigul, 2005; Yongsawatdigul and Sinsuwan, 2007). Therefore, increasing of gel strength of lizardfish surimi when adding Ca^{2+} may be caused by an increased of hydrophobic interaction and disulfide bond linkages.

3.5 Conclusions

TBSP contained TGase activity with optimum temperature at 37^{o}C and pH 7.5. TBSP TGase is a Ca^{2+}-dependent TGase. Activity staining of TGase revealed 2 distinct fluorescent bands with molecular weight of 78 and 189 kDa. MW of 2 fluorescent bands were 66 kDa based on SDS-PAGE, indicating the presence of isozyme. Concentrated TBSP induced protein cross-linking of BSA when incubated at 25^{o}C for 6 h and also enhanced gel strength of lizardfish surimi in the presence of 0.1% $CaCl_2$ and 1.6% TBSP with pre-incubation at 37^{o}C.

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CHAPTER IV

PROTEINASE INHIBITORY ACTIVITY OF SARCOPLASMIC PROTEINS FROM THREADFIN BREAM (*Nemipterus* spp.)

4.1 Abstract

Sarcoplasmic proteins from threadfin bream (TBSP) were concentrated using ultrafiltration with a 30-kDa molecular weight cut-off membrane. The inhibitory against three proteinases (papain, trypsin and α-chymotrypsin) was investigated. TBSP exhibited inhibitory activity toward trypsin and its activity diminished when incubated at 55^{o}C for 15 min. SDS-PAGE under non-reducing condition stained by trypsin revealed 3 protein bands with molecular weight of 95, 41 and 37 kDa. Breaking force and deformation of lizardfish surimi gel added TBSP and pre-incubated at 37^{o}C for 20 min increased with addition of TBSP (p<0.05). TCA-oligopeptide content of lizardfish surimi gel added TBSP decreased at 0.4% TBSP (p<0.05). Retention of myosin heavy chain (MHC) increased when TBSP concentration increased. TBSP effectively protect MHC from proteolysis when incubated at 37^{o}C, but was not observed at 65^{o}C. Inhibitory effect of TBSP could be a key factor contributing to its gel-enhancing ability.

Key words: threadfin bream, proteinase inhibitor, trypsin, activity staining, surimi

4.2 Introduction

In conventional surimi processing, washing step typically removes sarcoplasmic proteins, blood, fat and other nitrogenous compounds in fish muscle. Therefore, texture of final products are greatly improved. It has been recognized that sarcoplasmic proteins hinder the gel forming ability of myofibrillar proteins. Recently, effect of fish sarcoplasmic proteins on gel properties has been reported. Karthikeyan, Mathew, Shamasundar and Prakash (2004) suggested that gels prepared from sarcoplasmic proteins with MW of 94, 40, and 26 kDa showed high gel strength. It was suggested that oil sardine (*Sardinella longiceps*) sarcoplasmic proteins do not interfere with gel forming ability of myofibrillar proteins. Sarcoplasmic proteins obtained from ammonium sulfate fraction enhanced gel strength of washed sardine meat gel. Yongsawatdigul and Piyadhammaviboon, (2007) found that addition of tilapia sarcoplasmic protein increased gel strength of lizardfish gel. which was enhanced by endogenous TGase from tilapia sarcoplasmic protein. In addition, it was reported that carp (*Cyprinus carpio*) muscle containing calpastatin and a trypsin inhibitor (Toyohara, Makinodan, Tanaka and Ikeda, 1983). Trypsin inhibitor in white croker (*Micropogon opercularis*) skeletal muscle extracts could inhibited proteinase I which was isolated from white croker skeletal muscle (Busconi, Folco, Martone, Trucco and Sánchez, 1984; Folco, Busconi, Martone, Trucco and Sánchez, 1984). This evidence indicated that TBSP served as proteinase inhibitor.

Lizardfish (*Saurida* spp.) is one of the main species used to produce surimi in Thailand and Southeast Asia. Gel-forming ability of lizardfish surimi is poor because of its high endogenous proteolytic activity. (Suwansakornkul, Itoh, Hara and Obatake, 1993). In the presence of endogenous proteinase, myofilbrillar proteins underwent

degradation upon heating, which is know as “modori” phenomenon (Matsumoto and Noguchi, 1992). Protein food additives have also been used in other species for improving gel-strength in fish muscle or surimi during heating process, such as egg white powder (EW), whey protein concentrate (WPC), beef plasma protein (BPP), and potato extract (Hamann, Amato, Wu and Foegeding, 1990; Morrissey, Wu, and An, 1993). However, some food grade proteinase inhibitor such as BPP has been limited by the spread of bovine spongiform encephalopathy (BSE) or ‘‘mad cow disease’’. Addition of BPP more than 1 % result in off-flavor (Akazawa, Miyauchi, Sakurada, Wasson and Reppond, 1993). Similarly, EW has undesirable odor as sulfur at high concentration and also has high cost (Porter, Koury and Kudo, 1993). Therefore, TBSP derived from waste water of surimi process could be an alternative proteinase inhibitor for surimi industry.

Effect of TBSP on textural properties of lizardfish surimi and function of TBSP as related to proteinase inhibitor have not yet been investigated. Objectives of this study were to investigate the proteinase inhibitory activity of TBSP and effect of TBSP on lizardfish surimi textural properties.

4.3 Materials and Methods

4.3.1 Chemicals

Chymotrypsin, trypsin, tyrosine, and bovine serum albumin (BSA) were purchased from Fluka (Buchs, Switzerland). Trichloroacetic acid (TCA) was purchased from BDH Chemical Ltd. (Poole, Dorset, UK). Papain, casein, and soybean trypsin inhibitor (STI) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Reagents used for gel electrophoresis were purchased from Promega (Madison,

WI, USA). All other chemicals were of reagent grade.

4.3.2 Materials

Fresh threadfin bream (TB) (*Nemipterus* spp.) were purchased from Rayong province. Samples were packed in a polystyrene foam box filled with ice and transported to Suranaree University laboratory, approximately within 8 h after catch. Samples were vaccum-packed and kept at –40^{o}C until used. Frozen lizardfish (*Saurida* spp.) surimi was obtained from a surimi plant at Samutsakorn province. Samples were packed in a polystyrene foam box filled with ice, and immediately transported to the Suranaree University laboratory. Frozen surimi was cut into 1 kg blocks. Surimi blocks were vacuum-packed and kept at –18^{o}C until used.

4.3.3 Preparation of threadfin bream sarcoplasmic protein

Threadfin bream mince was mixed with 3 volumes of cold drinking water (reverse osmosis), homogenized for 1 min, and centrifuged at 5,000xg , 4^{o}C for 15 min. Supernatant was collected and centrifuged at 35,000xg , 4^{o}C for 30 min. Supernatant was referred to as threadfin bream sarcoplasmic protein (TBSP) and used throughout the study.

4.3.4 Proteinase inhibitory activity assay

Proteinase inhibitory activity of TBSP was determined against α-chymotrypsin, papain and trypsin using casein as a substrate. Two hundred μL of TBSP was mixed with 500 μL of either 100 mM Tris-HCl (pH 8) for α-chymotrypsin and trypsin, or 100 mM Tris-HCl (pH 7) for papain, and 100 μL of enzyme solution (0.1 mg/mL α-chymotrypsin, 0.1 mg/mL papain and 0.2 mg/mL trypsin). The mixture was pre-incubated at 37°C for 10 min and then added 200 μL of 10 mg/mL casein solution. The mixtures were further incubated at 37°C for 1 h and the reaction

was terminated by adding 400 µL of 50% trichloroacetic acid (TCA). A control was prepared by substituting 200 µL of TBSP solution with 200 µL of deionized water. Blanks were prepared by adding 400 µL of 50% TCA before adding 200 µL of substrate solution. The reaction mixtures were then centrifuged for 15 min at 10,000xg, and TCA-soluble oligopeptide content was determined using tyrosine as a standard (Lowry's assay). The inhibitory activity was calculated as follows:

$$\% \text{ Inhibition} = \frac{(TC-TC_B)-(TS-TS_B)}{(TC-TC_B)} \times 100$$

Where, TC = tyrosine of control, TC_B = tyrosine of control blank, TS = tyrosine of sample (added TBSP), TS_B = tyrosine of sample blank.

4.3.5 Proteinase inhibitory activity staining against trypsin

The study was performed using native-PAGE and non reducing SDS-PAGE electrophoresis. Inhibitory activity staining was conducted according to the method of Garcia-Carreno et al. (1993) with slight modifications. TBPS was mixed with the sample buffer at a ratio of 1:1 (v/v). Thirty µg protein were loaded onto 7.5% polyacrylamide gel. After electrophoresis, the gel was incubated with a gentle shaking in an ice box with casein solution (2%casein in 100 mM Tris-HCl, pH 8) for 30 min and then rinsed with 50 mM Tris-HCl (pH 8). Gel was further incubated with trypsin solution (5mg/ml) at 37°C for 1 h and rinsed with deionized water twice. Finally, gels were stained using Coomassie blue. The apparent blue band in clear background indicated the presence of trypsin inhibitor.

4.3.6 Trypsin inhibitory activity assay

Trypsin inhibitory activity of TBSP was determined. Two hundred µL of TBSP was mixed with 500 µL of 100 mM Tris-HCl (pH 8) and 100 µL of trypsin

solution (2.5 mg/mL). The reaction mixture was assayed and inhibitory activity was calculated as described in 4.3.4.

Trypsin inhibitory activity of TBSP, EW, bovine serum albumin (BSA) and Soy bean trypsin inhibitor (STI) was compared by adding each protein (1.7 mg protein) into 500 µL of 100 mM Tris-HCl (pH 8) and 100 µL of trypsin solution (2.5 mg/mL). The mixtures were pre-incubated at 37 °C for 10 min and then was assayed as described in 4.3.4.

Thermal stability of TBSP on trypsin inhibitory activity also monitored at various temperatures of 30, 35, 40, 45, 50, 55, 60 and 65°C. Sample (1.7mg protein) was pre-incubated at the studied temperatures for 15 min and cool down on ice for 5 min before analysis of trypsin inhibitory activity as described in 4.3.4.

4.3.7 Effect of TBSP on textural properties of lizardfish surimi

Lizardfish surimi pastes containing 2% NaCl, 0.15% $CaCl_2$ and 80% moisture content were prepared with TBSP addition of 0, 0.4, 0.8 and 1.6 g protein/100 g total weight. The pastes were filled into a microplate with a well diameter of 6.86/6.35 mm (top/bottom) and depth of 10.67 mm using a syringe. The filled microplates were vacuum-packed in a linear low density polyethylene (LLDPE)/nylon bag. Samples were pre-incubated at either 25^{o}C for 2 h or 37°C for 20 min, and subsequently heated at 90°C for 10 min. Samples heated at 90°C for 10 min were used as the control. Samples were immediately cooled in iced water for 20 min and stored in a refrigerator (5-8°C) overnight. Breaking force (g) and deformation (mm) were determined using a 2-mm cylindrical probe at a test speed of 1 mm/s (Stable Micro System, Surrey, England).

4.3.8 Polyacrylamide gel electrophoresis (PAGE)

Two grams of lizardfish gel samples were mixed with 18 mL of 10% hot SDS solution, homogenized (IKA Works Asia, Bhd, Malaysia), and heated at 90°C for 30 min. The homogenate was centrifuged at 10,000×g (Centrifuge 5415D, Eppendorf, Hamburg, Germany) for 20 min. Supernatant was collected for SDS-PAGE analyses. The homogenate were mixed with solubilizing buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol) at a ratio of 1:1 and boiled for 3 min. Thirty μg protein was loaded onto 10 % (w/v) polyacrylamide gel according to the method of Laemmli (1970). Gels were run at a constant voltage setting at 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.

Band intensity of myosin heavy chain (MHC), actin (AC) and tropomyosin (TM) on polyacrylamide gels were measured using HP ScanJet (model C7716, Hewlett-Packard Co., Singapore). The corresponding peaks were integrated using a LabWorks 4.0 software (UVP Inc., Upland, CA, USA). Protein was quantified as percentage of the area in each protein comparing with its paste.

4.3.9 TCA-soluble oligopeptides

TCA-soluble oligopeptide contents were measured using the method described by Yongsawatdigul and Piyadhammaviboon (2004). Five hundred milligrams of gel sample was added 4.5 mL 5% cold TCA solution, then the mixture was homogenized using an IKA homogenizer (IKA Works Asia, Bhd, Malaysia) and centrifuged at 10,000 xg (Rotor PK 121R, ACCEL Co., Italy) for 15 min at 4^{o}C. Supernatant was analyzed for oligopeptide content using Lowry's assay with tyrosine as a standard. TCA-soluble oligopeptide content was expressed as nmole of tyrosine/g sample.

4.3.10 Statistical analysis

Experiments were conducted in triplicate. Mean values and standard deviations were presented. The effects of TBSP on textural properties and TCA-soluble oligopeptide were analyzed by analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was used to determine differences among treatments. The statistical analysis was performed using Statistical Analysis System (SAS Inst. Inc., Cary, N.C., USA.). Significance of differences was defined at $p \leq 0.05$.

4.4 Results and Discussion

4.4.1 Proteinase inhibitor in TBSP

TBSP showed inhibitory activity toward trypsin with 63% inhibition. In contrast, it served as a substrate for α-chymotrypsin and papain as % inhibition showed negative values (Figure 4.1). Trypsin and α-chymotrypsin are in the class of serine proteinase but TBSP only inhibited trypsin. The result indicated TBSP

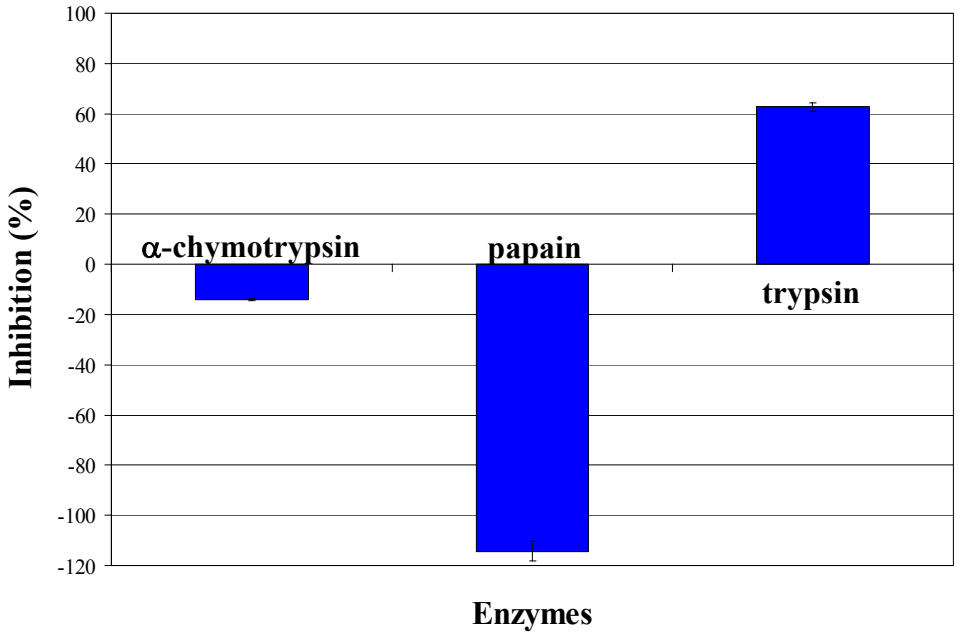

Figure 4.1 Inhibitory effect of TBSP towards various proteinases

specifically inhibited trypsin activity. Hara and Ishihara (1987) reported that purified serine proteinase inhibitor from carp (*Cyprinus carpio*) ordinary muscle strongly inhibited trypsin, chymotrypsin and elastase. Purified trypsin inhibitor from white croaker (*Micropogon opercularis*) exhibited inhibitory activity against trypsin, chymotrypsin and elastase. It does not inhibit proteinases belonging to other groups, indicating that it is highly specific for serine proteinases (Sangorrín, Folco, Martone and Sánchez, 2001).

On native-PAGE, one major band of TBSP was observed on trypsin stained gel (Figure 4.2b,d). Retention of protein was observed in EW, STI and WPC, but no protein band was detected on CS lane (Figure 4.2b,d). Three protein bands of TBSP with molecular weight of 95, 41 and 37 kDa were found on non-reducing SDS-PAGE stained with trypsin (Figure 4.2d). The result suggested that these remaining protein bands could be trypsin inhibitor. A trypsin inhibitor from muscle has been isolated and purified from white croaker (*Micropogon opercularis*) with molecular mass of 65 kDa (Sangorrín et al., 2001). Cao, Osatomi, Matsuda, Ohkubo, Hara and Ishihara, (2000) reported that purified inhibitor (55 kDa) from the skeletal muscle of white croaker (*Argyrosomus argentatus*) specifically inhibited a myofibril-bound serine proteinase (MBSP) isolated from lizardfish (*Saurida wanieso*). And also a purified proteinase inhibitor isolated from carp muscle showed molecular weight of 56 kDa on SDS-PAGE (Hara and Ishihara, 1987). The purified inhibitor from lizardfish (*Saurida wanieso*) skeletal muscle showed molecular mass of 50 kDa on SDS-PAGE and gel filtration (Cao, Osatomi, Hara and Ishihara, 2001). Thus, inhibitor from TBSP appeared to show different MW from other fish inhibitors previously reported.

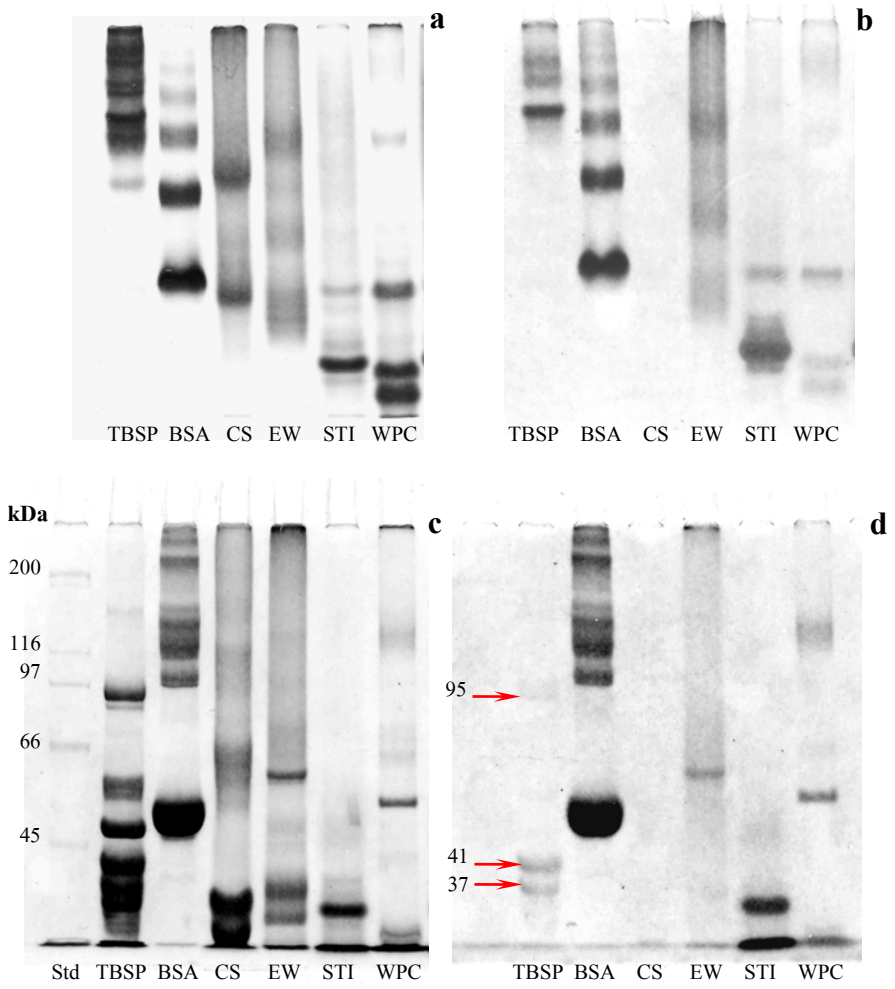

Figure 4.2 Polyacrylamide (7.5%) gel patterns stained with coomassie blue (a,c) and trypsin staining (b,d) using Native-PAGE (a,b) and SDS-PAGE (c,d) system. BSA= bovine serum albumin, CS = casein, EW = egg white, STI = soybean trypsin inhibitor, WPC = whey protein concentrate.

Figure 4.3 shows inhibitory activity of various proteins based on TCA-soluble oligopeptide content. The results indicated that at the same protein concentration, EW and STI showed the highest trypsin inhibitory activity (91 % inhibition), followed by WPC (38% inhibition) and TBSP (24 % inhibition). While BSA and CS showed no inhibitory activity with -22 and -19% inhibition, respectively. BSA and CS seemed to serve as a trypsin substrate. In thus case, CS used to be total substrate for trypsin. In

the same agreement of this study, Weerasinghe, Morrissey and An (1996a) found that BSA showed little hydrolysis by trypsin while BSA was seen on trypsin-stained gels. Therefore, the remaining of BSA lane detected on trypsin-stained gels could not be interpreted as trypsin inhibitor.

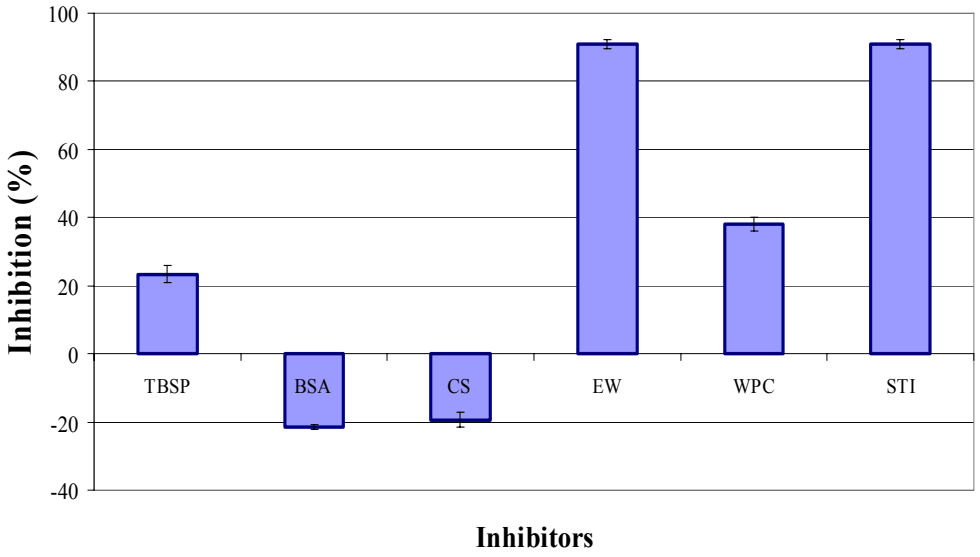

Figure 4.3 Inhibitory activity of TBSP comparing with various proteinase inhibitors. Abbreviations are the same as Figure 4.2.

EW and WPC are the most commonly used as food grade inhibitors. EW was found to exhibit a higher inhibitory against trypsin more than WPC (Weerasinghe et al., 1996a; Nakamura and Doi, 2000). EW contained several inhibitors. Ovomucoid is a trypsin inhibitor appeared as a wide range of MW between 27 and 35 kDa while MW of ovoinhibitor is 49 kDa (Saxenaa and Tayyab, 1997). Other components in EW are ovomacroglobulin or ovostatin, ovalbumin and cystatin (Saxenaa and Tayyab, 1997). WPC also consists of several proteinase inhibitory components including immunoglobulin, β-lactoglobulin and α-lactalbumin with MW of 145, 18.3 and 14 kDa, respectively. (Weerasinghe, Morrissey, Chung, and An, 1996b). However, it has

not been reported about proteinase inhibitor in sarcoplasmic proteins from threadfin bream. Further study on purification of TBSP is needed to characterize its biochemical characteristic.

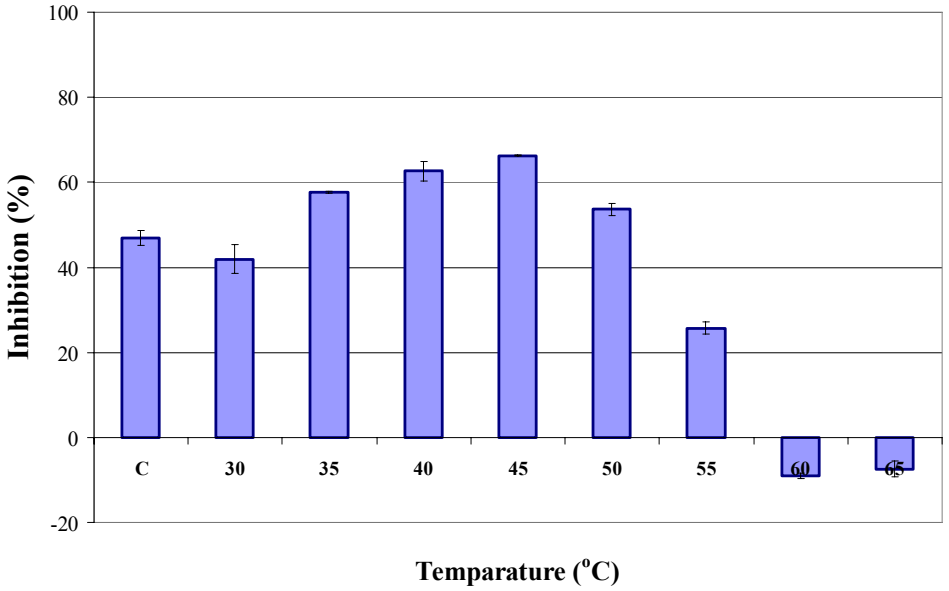

Figure 4.4 Thermal stability of TBSP against trypsin

The trypsin inhibitory activity of TBSP decreased with temperature. The inhibitory activity was not found after pre-incubation at 60 and 65°C (Figure 4.4). At 60 and 65°C, negative values of remaining activity were found. It implied that TBSP acted as a substrate of trypsin. TBSP possibly still contains many kinds of protein, including some proteinase. Therefore, some heat stable endogenous proteinase might be activated at this temperature range, resulting in a degradation of substrate and also itself while inhibitory activity was terminated. It has been reported that several proteinase inhibitors decreased their activities when incubated at high temperature. Sangorrín et al., (2001) reported that the purified inhibitor from white croaker (*Micropogon opercularis*) skeletal muscle was heat-labile and its activity decreased after pre-incubation at temperature above 50°C. Partially purified trypsin inhibitor

from white croaker (*Micropogon opercularis*) skeletal muscle was rapidly inactivated at 60°C, but crude muscle extract retained 50% of its inhibitory activity after being heated at 60°C for 3 h (Folco, Busconi, Martone, Trucco and Sánchez, 1984). In addition, Toyohara, Makinodan, Tanaka and Ikeda, (1983) reported that trypsin inhibitor from carp (*Cyprinus carpio*) muscle completely lost its inhibitory activity after heating at 70°C for 5 min. On the other hand, carp muscle calpastatin showed high thermal stability at 100°C (Toyohara, Makinodan, Tanaka and Ikeda, 1983). Based on these result TBSP could be used as an inhibitor at temperature below 55°C.

4.4.2 Effect of TBSP on autolytic degradation of lizardfish surimi

TBSP inhibited about 52.2 and 33% autolytic activity at 37 and 65°C, respectively (Figure 4.5). At 37°C, the TCA-soluble oligopeptide content of sample added 2% EW was similar to that of sample added 2% TBSP (Figure 4.5). At 65°C, EW showed inhibition activity about 81%. STI showed 80% inhibition at 37°C and 82% inhibition at 65°C. These results suggested that TBSP inhibited proteolysis of lizardfish. Based on densitogram, TBSP did not effectively reduce proteolysis of MHC at 65°C, but it inhibited the degradation of actin and tropomyosin (TM) (Figure 4.6). At 37°C, the retention of MHC was evident in the sample added TBSP. Both EW and STI effectively inhibited degradation of MHC, resulting in a greater retention of MHC at 37 and 65°C (Figure 4.6). Yongswatdigul and Piyadhammaviboon (2004) reported that both MHC and TM were preferred substrates of endogenous proteinase in lizardfish surimi. Therefore, inhibition of MHC and TM degradation could contribute to the improvement of lizardfish surimi gel. Protein additives are widely used as proteinase inhibitor in surimi processing. Food grade inhibitors include beef plasma protein (BPP), EW, WPC and potato powder (PP) (Morrissey, Wu and An,

1993; Weerasinghe et al., 1996b; Yongsawatdigul and Piyadhammaviboon, 2004). TBSP might be used as a proteinase inhibitor in surimi processing at low setting condition.

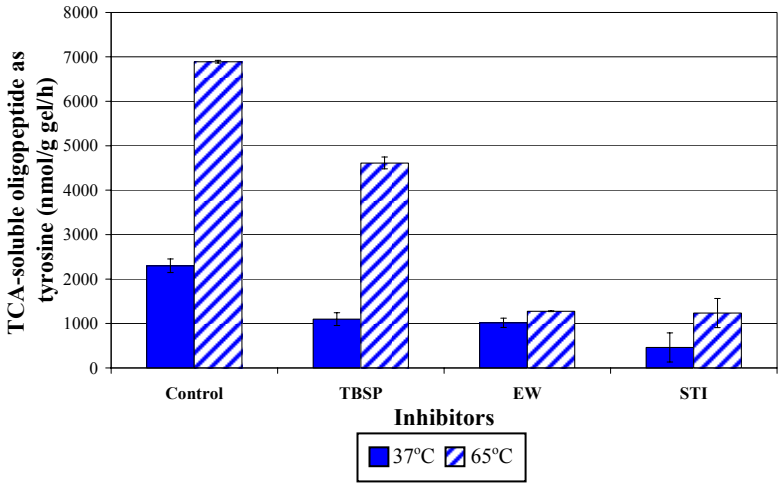

Figure 4.5 TCA-soluble oligopeptide contents of lizardfish surimi mixed with various inhibitors and incubated at 37 and 65°C for 1 h. TBSP = 2%TBSP, EW= 2% egg white and STI = 500 μg soy bean trypsin inhibitor/g surimi.

4.4.3 Effect of TBSP on textural properties of lizardfish surimi

Addition of TBSP at 0.4, 0.8 and 1.6% increased both breaking force and deformation of lizardfish surimi gel (Figure 4.7a,b). Without addition of TBSP, the lowest gel strength was found at 37°C (p<0.05). While, addition of TBSP along with pre-incubation at 37°C resulted in greater gel strength than surimi pre-incubated at 25°C and without pre-incubation (p<0.05) (Figure 4.7a,b). This evidence was in agreement with Yongsawatdigul and Piyadhammaviboon (2004) found that autolysis of lizardfish surimi was found since incubation at 40°C, however, addition of EW and WPC improved textural properties and reduced degradation of lizardfish surimi.

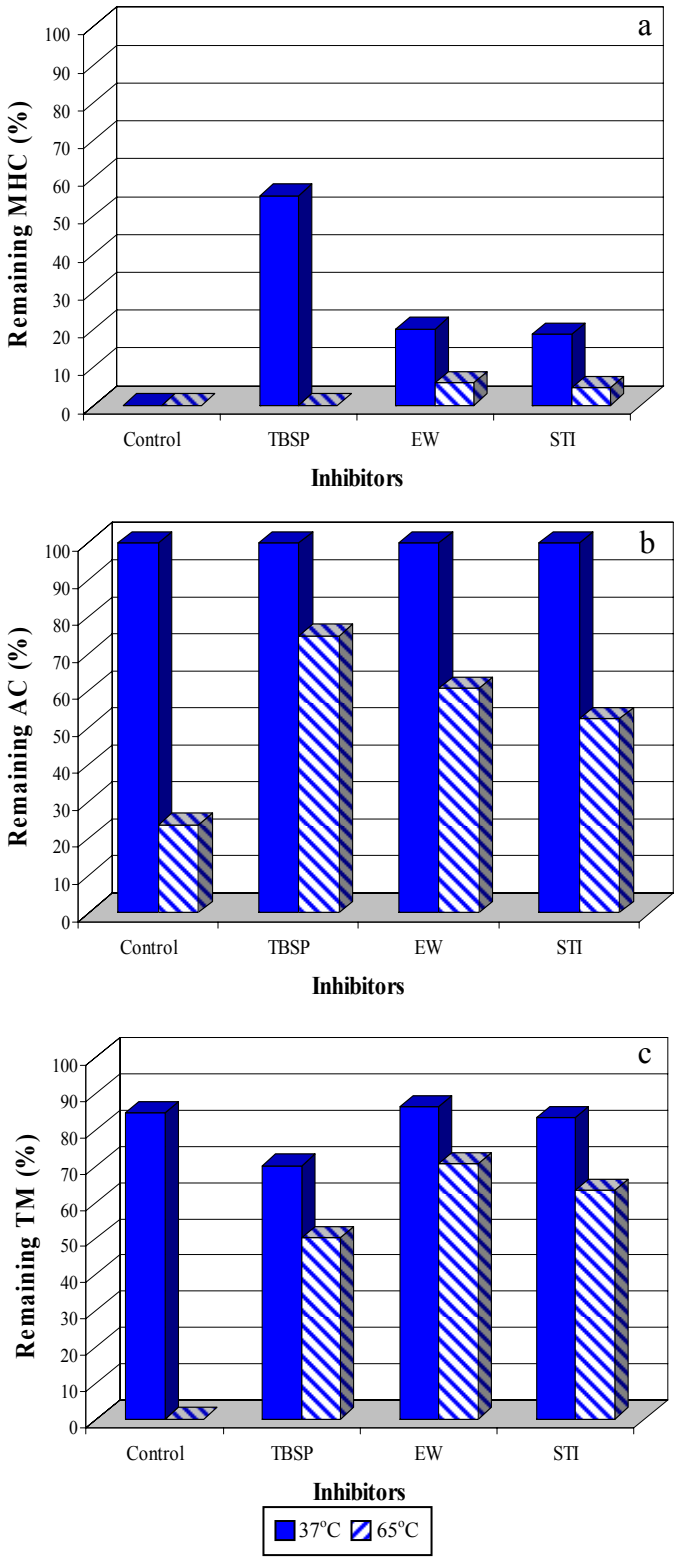

Figure 4.6 Densitogram of lizardfish surimi mixed with various inhibitors corresponded with Figure 4.5.

When TBSP was heated (90^{o}C for 15 min), the protein component in TBSP denatured resulting in the loss of proteinase inhibitory ability as evident by the result of thermal stability (Figure 4.4). Effect of raw (unheated) and heated TBSP addition were compared (Figure 4.7a,c). It was noticed that the improvement of lizardfish surimi was obviously increased by addition of raw TBSP. In contrast, textural properties of lizardfish surimi added heated TBSP were minimally affected. Addition of heated TBSP upto 0.8% did not significantly improved textural properties (p>0.05) compared with lizardfish surimi without TBSP (Figure 4.7c,d). Whereas, higher gel strength was found when added by heated TBSP at 1.6 % (p<0.05). These results implied that an increase in protein content of TBSP could not be a sole factor contributing to an increase in textural properties of lizardfish surimi.

Yongsawatdigul and Piyadhammaviboon, (2005) reported that textural properties of lizardfish (*Saurida* spp.) surimi were improved by addition of microbial transglutaminase (MTGase). Textural properties of lizardfish surimi improved when 0.1 unit of MTGase was added and pre-incubated at 25 and 40^{o}C. In an improvement of lizardfish surimi was also achieved by adding tilapia sarcoplasmic protein concentrate (SpC). Addition of 0.01% SpC and pre-incubation at 37^{o}C resulted in 91.6 and 26.7% increase in breaking force and deformation, respectively (Yongsawatdigul and Piyadhammaviboon, 2007). Those studies showed gel enhancing effects of endogenous TGase and MTGase. There were obvious evidences of cross-link polymer on SDS-PAGE. On the other hand, the cross-link protein was not found in this study although the addition of TBSP enhanced textural properties of lizardfish surimi. This might be contributed by the action of inhibitory activity of TBSP.

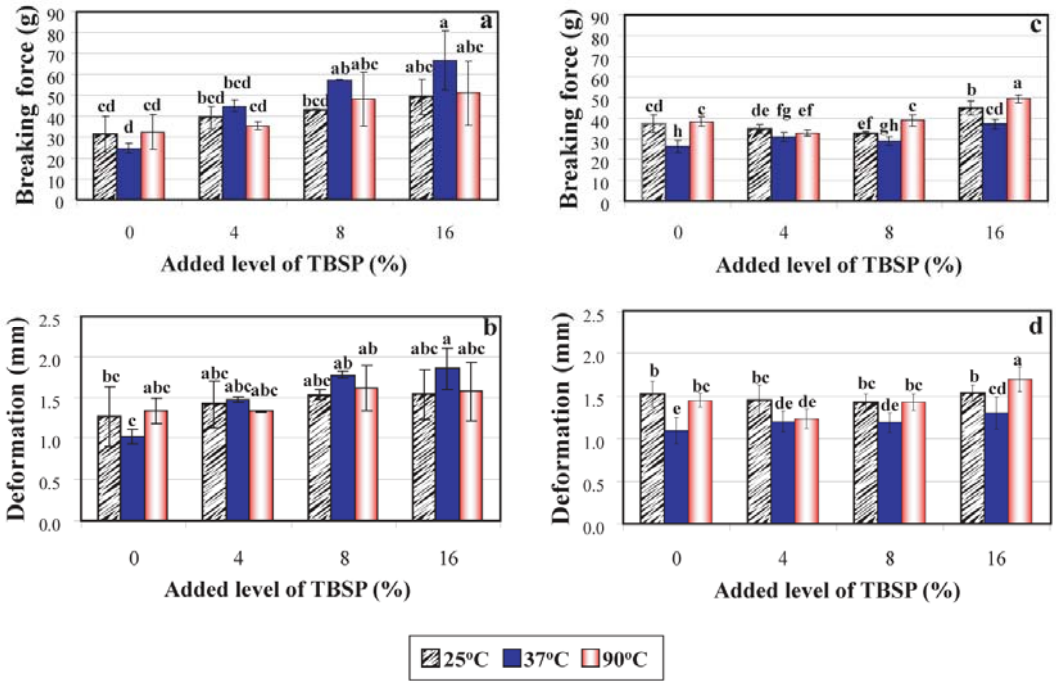

Figure 4.7 Textural properties of lizardfish surimi mixed with various concentrations of TBSP and subjected to various heating conditions: 25°C = pre-incubated at 25°C for 2 h, 37°C = pre-incubated at 37°C for 20 min followed by heating at 90°C for 10 min. 90°C = heated at 90°C for 10 min without pre-incubation. a,b = addition of TBSP. c,d = addition of heated TBSP (90°C for 15 min before used).

The highest TCA-soluble oligopeptide content was found in surimi gels without TBSP addition (Figure 4.8). This corresponded to textural properties observed in Figure 4.7. TCA-soluble oligopeptide content decreased with an increased addition of TBSP. TBSP exhibited about 40-50% inhibition at 0.4-1.6% addition. These results indicated that TBSP inhibited proteolysis of lizardfish surimi, which was confirmed by SDS-PAGE pattern (Figure 4.9).

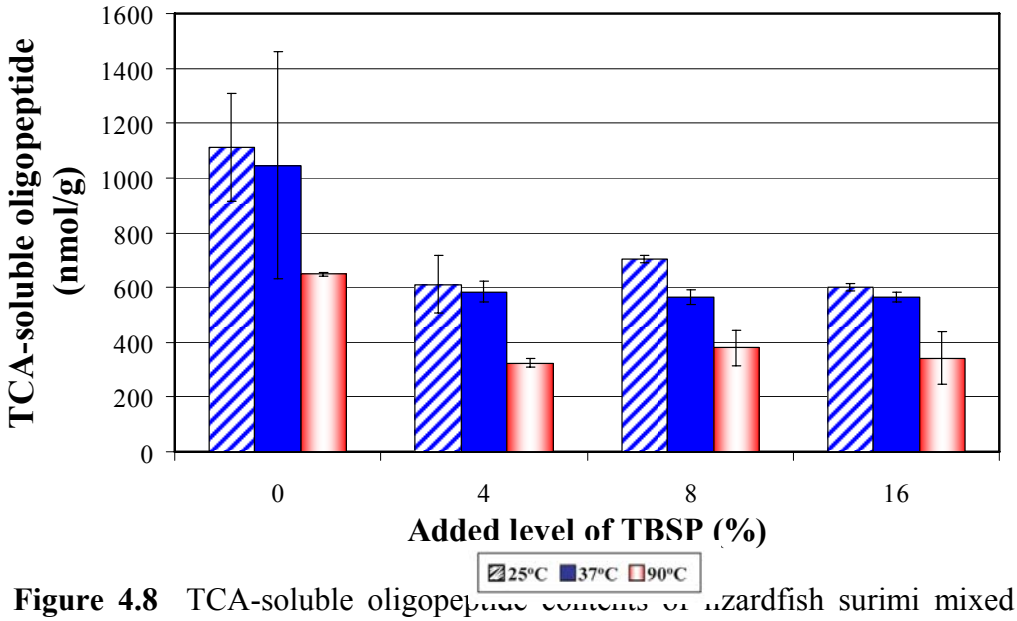

Figure 4.8 TCA-soluble oligopeptide contents of lizardfish surimi mixed with TBSP at various concentrations. 25, 37 and 90°C are the same as described in Figure 4.7.

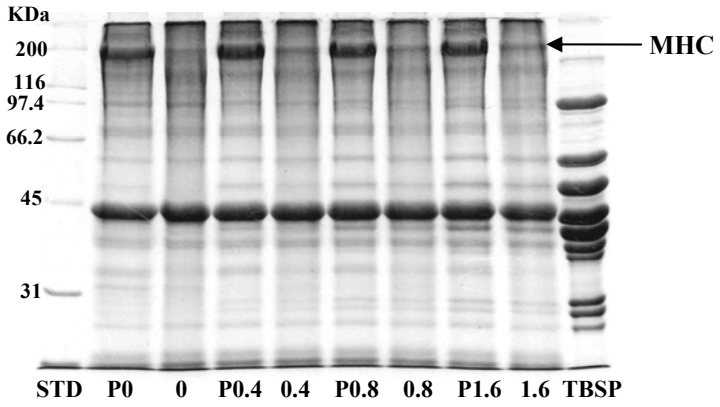

Figure 4.9 SDS-PAGE (10% polyacrylamide gel) pattern of lizardfish surimi added TBSP and pre-incubated at 37°C for 20 min followed by heating at 90°C for 10 min. STD =standard markers, P=raw paste, numbers indicate addition level of TBSP in percentage of total weight, MHC= myosin heavy chain

In the absence of TBSP, severe degradation of MHC was found as a significant loss of MHC was noticed (Figure 4.9). Retention of MHC appeared to increase with added level of TBSP up to 1.6%. These results suggested that TBSP reduced degradation of MHC at 37^{o}C and could be used to reduce proteolysis of lizardfish surimi. The main function was perhaps its proteinase inhibitory activity.

4.5 Conclusions

TBSP exhibited inhibitory activity toward trypsin. MW of trypsin inhibitor found in TBSP were 37, 41 and 95 kDa, suggesting the presence of trypsin inhibitors. Addition of TBSP and pre-incubated at 37^{o}C for 20 min decreased degradation of MHC on lizardfish surimi. TBSP showed a greater inhibitory activity on lizardfish surimi when compared with EW and STI. Addition of concentrated TBSP can enhance gel strength of lizardfish surimi, particularly when setting at 37^{o}C.

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CHAPTER V

SUMMARY

Sarcoplasmic proteins from threadfin bream (TBSP) contained TGase activity with optimum temperature at 37°C and pH 7.5. TBSP TGase is a Ca^{2+}-dependent TGase. Activity staining of TGase revealed 2 distinct fluorescent bands with molecular weight of 78 and 189 kDa. Molecular weight (MW) of 2 fluorescent bands were 66 kDa based on SDS-PAGE, indicating the presence of isozyme. Concentrated TBSP induced protein cross-linking of BSA when incubated at 25°C for 6 h. In addition to gel strength of lizardfish surimi were enhanced when adding of 0.1% $CaCl_2$ and 1.6% TBSP with pre-incubation at 37°C.

TBSP exhibited inhibitory activity toward trypsin. Trypsin activity staining of non reducing SDS-PAGE showed 3 remaining protein bands with MW of 37, 41 and 95 kDa, demonstrating the presence of trypsin inhibitors. Incubation at 55°C for 15 min, inhibitory activity was diminished and inhibitory activity was completely lost when incubating at 60 and 65°C for 15 min, suggesting that the application of TBSP as proteinase inhibitor was limited at temperature not above 55°C. Addition at 0.4% TBSP and pre-incubated at 37°C for 20 min in lizardfish surimi increased both of breaking force and deformation. Addition of concentrated TBSP can enhance gel strength of lizardfish surimi, particularly when set at 37°C. Therefore, both of TGase activity and proteinase inhibitory activity contained in TBSP involved in enhancing lizardfish surimi gel.

BIOGRAPHY

Penprabha Piyadhammaviboon was born in August 17, 1979 in Maung District, Phichit, Thailand. She studied at Phichit Pittayakom School and graduated in 1997. She received a Bachelors Degree in Food Technology from Suranaree University of Technology, Nakhon Ratchasima in 2001. In 2003, she continued her Master Degree in School of Food Technology at Institute of Agricultural Technology, Suranaree University of Technology. She received the TRF-MAG Scholarship from Thailand Research Fund (in 2005-2006). While she was studying in Master Degree, she also worked as teaching assistant school of food technology, SUT, in Food Chemistry and Food Analysis classes.

During her graduate study, she had a chance to participate in Institute of Food Technologists (IFT) Annual Meeting 2007 at Chicago, IL, USA. with a travel grant from Suranaree University of Technology.