CROSS-LINKING REACTION OF FISH MUSCLE PROTEINS CATALYZED BY THREADFIN BREAM AND MICROBIAL TRANSGLUTAMINASES

Bung-Orn Hemung

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การเร่งปฏิกิริยาเชื่อมข้ามของโปรตีนกล้ามเนื้อปลาโดยทรานกลูทามิเนสจากปลาทรายแดงและจากจุลินทรีย์

นางสาวบังอร เหมัง

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

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บังอร เหมัง : การเร่งปฏิกิริยาเชื่อมข้ามของโปรตีนกล้ามเนื้อปลาโดยทรานสกลูทามิเนสจากปลาทรายแดงและจากจุลินทรีย์ (CROSS-LINKING REACTION OF FISH MUSCLE PROTEINS CATALYZED BY THREADFIN BREAM AND MICROBIAL TRANSGLUTAMINASES) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. จิรวัฒน์ ยงสวัสดิกุล, 193 หน้า.

แคลเซียมไอออนเข้มข้น 10 - 100 มิลลิโมลาร์เหนี่ยวนำการเปิดตัวของมัยโอซินและแอคตินจากปลาทรายแดง (*Nemipterus* sp.) เมื่อบ่มที่ 25 และ 40 °ซ ทำให้ความเป็นไฮโดรโฟบิกที่พื้นผิวของโปรตีนเพิ่มขึ้น แคลเซียมไอออนมีผลให้ปริมาณแอลฟา-เฮลิกซ์ของมัยโอซินและแอคตินที่วิเคราะห์ด้วยเทคนิคเซอร์คูลาร์ไดโครอิซึม (Circular dichroism) ลดลง กิจกรรมแคลเซียมเอทีพีเอส (Ca-ATPase activity) ของมัยโอซินลดลงเมื่อแคลเซียมไอออนเข้มข้นมากกว่า 50 มิลลิโมลาร์ ซึ่งบ่งบอกการเปลี่ยนแปลงโครงร่างที่ส่วนหัวของมัยโอซิน (Globular head) ปริมาณหมู่ซัลฟ์ไฮดริลทั้งหมดของโปรตีนลดลงเมื่อความเข้มข้นแคลเซียมไอออนเพิ่มขึ้นจาก 10-100 มิลลิโมลาร์ แสดงให้เห็นว่าแคลเซียมไอออนส่งเสริมการสร้างพันธะไดซัลไฟด์ในระหว่างการบ่มอันตรกริยาไฮโดรโฟบิกและพันธะไดซัลไฟด์มีบทบาทสำคัญต่อการเกาะตัว (Aggregation) ของมัยโอซินและมีค่าเพิ่มขึ้นตามความเข้มข้นของแคลเซียมไอออน (10-100 มิลลิโมลาร์)

การทำบริสุทธิ์บางส่วนของเอนไซม์ทรานสกลูทามิเนสจากตับปลาทรายแดงด้วยหลักการการแลกเปลี่ยนไอออน การแยกตามขนาด และการแยกแบบจำเพาะ ทำให้ได้โปรตีนที่มีมวลโมเลกุลขนาดต่างๆคือ 95 63 และ 43 กิโลดาลตัน แต่พบโปรตีนเพียงหนึ่งแถบที่เรืองแสงฟลูออเรสเซนต์บนแผ่นเจลอะคริลาไมด์ (Acrylamide) เมื่อทำปฏิกิริยากับไดเมททิลเลเต็ด เคซีน (Dimethylated casein) และแดนซิลคาดาเวอรีน (Dansylcadaverine) เมื่อวิเคราะห์มวลโมเลกุลของแถบโปรตีนดังกล่าวด้วยเทคนิคอิเล็กโทรโฟรีซีสแบบสูญเสียสภาพดั้งเดิม (Denaturing electrophoresis) พบว่ามีขนาด 95 กิโลดาลตัน ทรานสกลูทามิเนสบริสุทธิ์บางส่วน (Partially purified transglutaminase) จากตับปลาทรายแดงต้องการแคลเซียมไอออนเข้มข้น 1 มิลลิโมลาร์ในการเร่งปฏิกิริยา กิจกรรมของ ทรานสกลูทามิเนสถูกยับยั้งด้วยสารเคมีที่ทำปฏิกิริยากับหมู่ซัลฟ์ไฮดริล สภาวะเหมาะสมต่อการทำงานคือที่อุณหภูมิ 50 °ซ และพีเอช 8.5-9.0 เอนไซม์ยังคงกิจกรรมได้ที่เกลือโซเดียมคลอไรด์เข้มข้น 0.6 โมลาร์และกิจกรรมลดลงเหลือ 75% ที่ความเข้มข้น 1.2 โมลาร์

การเปลี่ยนแปลงปริมาณแอลฟา-เฮลิกซ์ของแอคโตมัยโอซินธรรมชาติจากปลาแปซิฟิกไวทิง (Natural actomyosin from Pacific whiting) เกิดขึ้นที่ 31.8 และ 43.1 °ซ ขณะที่การเปลี่ยนแปลงของแอคโตมัยโอซินธรรมชาติจากปลาทรายแดงเกิดขึ้นที่ 35.0 และ 49.3 °ซ การเปลี่ยนแปลงค่าพลังงานแบบดูดความร้อน (Endothermic peak) ของแอคโตมัยโอซินธรรมชาติจาก

ปลาแปซิฟิกไวทิงที่วิเคราะห์ด้วยเทคนิค ดิฟเฟอเรนเชียล สแกนนิง แคลอรีเมทรี (Differential scanning calorimetry) เกิดขึ้นที่ 31.8, 42.1 และ 75.3 °ซ ในขณะที่แอคโตมัยโอซินธรรมชาติจากปลาทรายแดงเกิดการเปลี่ยนแปลงที่ 36.1 50.9 และ 78.4 °ซ แอคโตมัยโอซินธรรมชาติจากปลาแปซิฟิกไวทิงเปิดตัว (Unfold) อย่างมากที่ 25 °ซ ทำให้กิจกรรมแคลเซียมเอทีพีเอสลดลง ในทางตรงกันข้าม แอคโตมัยโอซินธรรมชาติจากปลาทรายแดงเปิดตัวเพียงเล็กน้อยทำให้การเชื่อมข้ามมัยโอซินเส้นหนัก (Myosin heavy chain) โดยทรานสกลูทามิเนสจากปลาทรายแดงและจากจุลินทรีย์เกิดได้น้อย ระดับการเปิดตัวของแอคโตมัยโอซินธรรมชาติจากปลาทั้งสองเพิ่มขึ้นเมื่อบ่มที่ 40°ซ ทรานสกลูตามิเนสจากจุลินทรีย์เร่งการเชื่อมข้ามมัยโอซินเส้นหนักจากปลาทั้งสองได้มากกว่าเอนไซม์จากปลาทรายแดง ทำให้มีประสิทธิภาพในการปรับปรุงเนื้อสัมผัสของเจลแอคโตมัยโอซินธรรมชาติได้มากกว่าเอนไซม์จากปลาทรายแดง การเปิดตัวของแอคโตมัยโอซินธรรมชาติมีอิทธิพลอย่างมากต่อการเชื่อมข้ามโปรตีนที่เร่งปฏิกิริยาโดยเอนไซม์จากปลาทรายแดง กิจกรรมแคลเซียมเอทีพีเอสของแอคโตมัยโอซินธรรมชาติลดลงเท่ากันในสภาวะที่มีและไม่มีเอนไซม์ แสดงให้เห็นว่าจุดเชื่อมข้ามที่เกิดขึ้นอาจไม่ได้อยู่ที่ส่วนหัวของมัยโอซิน

ทรานสกลูตามิเนสจากจุลินทรีย์มีเสถียรภาพต่อความร้อนสูงทำให้เร่งปฏิกิริยาการเชื่อมข้ามมัยโอซินเส้นหนักและการเชื่อมสารไบโอทินอะมิโดเพนทิลเอมีน (5-(Biotinamido) pentylamine, $BPNH_2$) เข้ากับกลูตามีนบนสายเปปไทด์ของมัยโอซินปลาทรายแดงได้มากกว่าทรานสกลูทามิเนสจากปลาทรายแดง การบ่งชี้ลำดับกรดอะมิโนของเปปไทด์ที่เชื่อมอยู่กับสารไบโอทินอะมิโดเพนทิลเอมีนด้วยเทคนิคแทนเดม แมสสเปคโทรเมทรี (Tandem mass spectrometry) โดยมีลำดับกรดอะมิโนของมัยโอซินเส้นหนักจากปลาแอมเบอร์แจคเป็นต้นแบบ พบว่าเปปไทด์เหล่านั้นอยู่บริเวณส่วนหางของมัยโอซิน กรดอะมิโนที่อยู่ปลายกลูทามินิลเอไมด์ (Glutaminylamide) ของกลูทามีนที่เกิดปฏิกิริยา (Reactive glutamine, Q*) ส่วนมากมีโซ่ข้างเป็นไฮโดรฟิลิก เช่น ไลซีน อาร์จีนีน และ กลูตามิก เปปไทด์ที่เชื่อมอยู่กับสารไบโอทินอะมิโดเพนทิลเอมีนด้วยการเร่งของเอนไซม์จากปลาทรายแดง ส่วนมากมีกรดอะมิโนไฮโดรโฟบิก เช่น ไกลซีน วาลีน ลิวซีน ไอโซลิวซิน อยู่ด้านแอลฟา-อะคริลาไมด์ (α-Acrylamide) ของกลูทามีนที่เกิดปฏิกิริยา ซึ่งกรดอะมิโนไฮโดรโฟบิกเหล่านั้นมักพบที่บริเวณไฮโดรโฟบิกภายในส่วนหางของมัยโอซิน กรดอะมิโนที่อยู่ด้านแอลฟา-อะคริลไมด์ของกลูทามีนที่เกิดปฏิกิริยาที่เร่งด้วยทรานสกลูทามิเนสจากจุลินทรีย์มีโซ่ข้างที่เป็นทั้งไฮโดรโบิกและไฮโดรฟิลิก ชี้ให้เห็นว่าทรานสกลูทามิเนสจากจุลินทรีย์มีความจำเพาะเจาะจงต่อสารตั้งต้นกลูทามีนน้อยกว่าทรานสกลูทามิเนสจากปลาทรายแดง

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2550 ลายมือชื่ออาจารย์ที่ปรึกษา_____

BUNG-ORN HEMUNG : CROSS-LINKING REACTION OF FISH MUSCLE PROTEINS CATALYZED BY THREADFIN BREAM AND MICROBIAL TRANSGLUTAMINASES. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D. 193 PP.

THREADFIN BREAM MYOSIN/FISH LIVER TRANSGLUTAMINASE/CROSS-LINKING/NATURAL ACTOMYOSIN/MASS SPECTROMETRY

Ca^{2+} at 10-100 mM induced the unfolding of threadfin bream (*Nemipterus* sp., TB) myosin and actin after incubation at 25 and 40 °C as evident by an increase of surface hydrophobicity. Circular dichroism spectra demonstrated that Ca^{2+} reduced the α-helical content of myosin and actin. Myosin Ca-ATPase activity decreased at Ca^{2+} > 50 mM, indicating conformational changes of myosin head. Total SH groups decreased with an increased Ca^{2+} concentration, suggesting that Ca^{2+} (10-100 mM) promoted the formation of disulfide bonds. Both hydrophobic interactions and disulfide linkages were possibly important in formation of myosin aggregates, and were promoted by addition of Ca^{2+} (10-100 mM).

Transglutaminase (TGase) from TB liver was partially purified by ion exchange, size exclusion and affinity chromatography. Three protein bands with molecular weight (Mw) of 95, 63, and 43 kilodalton (kDa) were observed on denaturing electrophoresis whereas only one distinct fluorescent band appeared on TGase activity staining on native-PAGE. Protein from the fluorescent band exhibited Mw of 95 kDa when it was eluted and analyzed on SDS-PAGE. Partially purified TB liver TGase (FTG) required Ca^{2+} up to 1 mM for full activation. TGase activity was markedly

inhibited by sulfhydryl reagents. Optimal conditions for catalytic activity were at 50 °C and pH of 8.5-9.0. TGase activity was not affected by NaCl up to 0.6 M and reduced to 75% at 1.2 M NaCl.

Changes in α-helical content revealed that transition temperatures of natural actomyosin (NAM) from Pacific whiting (PW) were at 31.8 and 43.1 °C, compared to at 35 and 49.3 °C of TB-NAM. Endothermic transitions of PW-NAM measured by differential scanning calorimetry were at 31.8, 42.1, and 75.3 °C, while those of TB were at 36.1, 50.9, and 78.4 °C. PW-NAM unfolded greatly after incubation at 25 °C and the cross-linking of PW-myosin heavy chain (MHC) catalyzed by FTG and microbial TGase (MTG) was observed. In contrast, TB-NAM slightly unfolded at 25 °C, resulting in less TB-MHC cross-linking. Unfolding of NAM from both species at 40 °C was greater than that at 25°C. MTG catalyzed MHC cross-linking to the greater extent than did FTG, resulting in higher textural improvement of NAM gels. Unfolding NAM played a much more critical role for FTG in catalyzing protein cross-linking than MTG. The decreases in Ca-ATPase activity of TB- and PW-NAM after incubation at 25 and 40 °C were not changed by either TGase, suggesting that the cross-linking sites might not be at myosin head.

MTG exhibited higher thermal stability, leading to its ability to catalyze TB-MHC cross-linking and 5-(biotinamido) pentylamine ($BPNH_2$) incorporation into TB-peptides to the greater extent than FTG. The amino acid sequences of $BPNH_2$-tagged peptides were identified by tandem mass spectrometry based on amberjack-MHC sequence. The identified sites of $BPNH_2$ modification catalyzed by both TGases were at the myosin rod and most $BPNH_2$-tagged peptides contained hydrophilic amino acids e.g. lysine, arginine, and glutamic acid at glutaminylamide site of reactive glutamine

(Q*). FTG tended to catalyze $BPNH_2$ incorporation of peptides that contain hydrophobic amino acids including glycine, valine, leucine, and isoleucine at the α-acrylamide site of Q*. Those hydrophobic amino acids located at the hydrophobic core of the intact myosin rod. Amino acids at α-acrylamide site of Q* for MTG were either hydrophobic or hydrophilic amino acids, suggesting broader glutamyl substrate specificity.

School of Food Technology Student's Signature_____

Academic Year 2007 Advisor's Signature_____

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Bung-Orn Hemung

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LIST OF ABBREVIATIONS

A	=	Alanine
A_{500}	=	Absorbance at 500 nm
A_{280}	=	Absorbance at 280 nm
ANS	=	8- Anilino-1-napthalenesulfonate
ATP	=	Adenosine 5'-triphosphate
BCA	=	Bicinchoninic acid
BME	=	β-Mercaptoethanol
C	=	Cysteine
CD	=	Circular dichroism
°C	=	Degree celsius
ΔCp	=	Molar excess heat capacity
CP	=	Cross-linked polymer
C-terminal	=	Carboxyl terminal
CSA	=	(1*S*)-(+)-10-Camphorsulfonic acid
D	=	Aspartic acid
Da	=	Dalton
DEAE	=	Diethylaminoethyl
DMC	=	*N,N'*-Dimethylated casein
DSC	=	Differential scanning calorimetry
DTT	=	Dithiothreitol
DTNB	=	5, 5'-Dithiobis (2-nitrobenzoic acid
E	=	Glutamic acid

LIST OF ABBREVIATIONS (Continued)

EDTA	=	Ethylenediaminetetraacetic acid
EF	=	Enhancing factor
EGTA	=	Ethylene glycol-bis (2-aminoethyl ether) *N*,*N*,*N'N*-tetraacetic acid
ESI-MS	=	Electrospray ionization mass spectrometry
F	=	Phenylalanine
FI	=	Fluorescence intensity
FI_b	=	Fluorescence intensity of blank with inactivated enzyme
FI_o	=	Fluorescence intensity of blank without enzyme
FI_s	=	Fluorescence intensity of sample
FTG	=	Fish transglutaminase
G	=	Glycine
G-actin	=	Globular actin
GTG	=	Guinea pig liver transglutaminase
g	=	Gram
×g	=	Relative centrifugal force
H	=	Histidine
ΔH_{cal}	=	Calorimetric enthalpy
HMM	=	Heavy meromyosin
h	=	Hour
I	=	Isoleucine
IAA	=	Iodoacetic acid
K	=	Lysine

LIST OF ABBREVIATIONS (Continued)

kDa	=	Kilodalton
L	=	Leucine
LC	=	Myosin light chain
LMM	=	Light meromyosin
M	=	Molar
MALDI-MS	=	Matrix assisted laser desorption/ionization-mass spectrometry
MDC	=	Monodansylcadaverine
MHC	=	Myosin heavy chain
MS	=	Mass spectrometry
MS/MS	=	Tandem mass spectrometry
MTG	=	Microbial transglutaminase
MWCO	=	Molecular weight cut-off
Mw	=	Molecular weight
mA	=	Milliampere
mg	=	Milligram
min	=	Minute
mL	=	Milliliter
mM	=	Millimolar
µL	=	Microliter
µm	=	Micrometer
N	=	Asparagine
NAM	=	Natural actomyosin
NCBI	=	The National Center for Biotechnology Information

LIST OF ABBREVIATIONS (Continued)

NEM	=	*N*'-Ethylmaleimide
N-termianl	=	Amino terminal
native-PAGE	=	Native polyacrylamide gel electrophoresis
nm	=	Nanometer
nmole	=	Nanomole
P	=	Proline
PBS	=	Phosphate buffer saline
Pi	=	Inorganic phosphate
PMSF	=	Phenylmethane sulfonyl fluoride
PMF	=	Peptide mass fingerprinting
PRODAN	=	6-Propionyl-2-dimethyl aminon aphthalene
PW	=	Pacific whiting
PW-NAM	=	Pacific whiting natural actomyosin
pI	=	Isoelectric point
%	=	Percent
Q	=	Glutamine
R	=	Arginine
rpm	=	Revolution per minute
S	=	Serine
S1	=	Subfragment 1
SDS	=	Sodium dodecyl sulfate
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S_o ANS	=	Surface hydrophobicity determined by ANS

LIST OF ABBREVIATIONS (Continued)

S_o PRODAN	=	Surface hydrophobicity determined by PRODAN
SH	=	Sulfhydryl
s	=	Second
[θ]	=	Molar mean ellipticity
$[\theta]_{222}$	=	Molar mean ellipticity at 222 nm
T	=	Threonine
TB	=	Threadfin bream
TB-NAM	=	Threadfin bream natural actomyosin
TCA	=	Trichloroacetic acid
Td	=	Denaturation temperature
TGase	=	Transglutaminase
T_m	=	Melting temperature
TLCK	=	Tosyl-L-lysine chloromethyl ketone
Tris	=	Tris (hydroxymethyl) aminomethane
U	=	Unit activity
UV	=	Ultraviolet
V	=	Valine
W	=	Tryptophan
Y	=	Tyrosine
Z	=	Charge

CHAPTER I

INTRODUCTION

1.1 Introduction

Transglutaminases (TGases) are the family of enzymes that catalyze the formation of isopeptide bond between peptide and protein – bound glutaminyl residues and primary amines or amino groups. They are classified as transferase enzymes with the common name of protein-glutamine γ-glutamyltransferase and the systematic name of (protein-glutamine: amine γ-glutamyltransferase (EC.2.3.2.13) (Folk, 1980). Ca^{2+}-dependent TGases are involved in numerous tissues with several biological functions, including blood clotting, cell matrix interactions, and regeneration of cells in tissue repairing. They have been found in several tissues, such as blood, muscle, keratinocyte, and liver tissue (Greenberg et al., 1991). Ca^{2+}-independent TGase was also isolated from bacteria, such as *Streptoverticillium sp.* (microbial transglutaminases, MTG) (Ando et al., 1989). TGases have been recently used to improve the functional, nutritional and textural properties of various food proteins (Lee and Park, 1998).

Surimi is a washed fish mince, mixed with cryoprotectants exhibiting high gel-forming ability. When surimi is mixed with salt and incubated at 0 - 40 °C for the periods of time before heating to 90 °C, gel strength is improved. Such phenomenon is called “setting” (Lanier, 2000). It was generally believed that Ca^{2+}-dependent TGase participates in protein polymerization by catalyzing the covalent bonds during setting.

It has been confirmed by the formation of high cross-linked polymer with a decrease in myosin heavy chain when endogenous TGase was activated in the presence of Ca^{2+}(Yongsawatdigul, Worratao, and Park, 2002).

It has been typically believed that Ca^{2+} improves gel-forming ability of fish proteins by activating endogenous TGase (Lee and Park 1998). Addition of Ca^{2+} has been reported to improve textural properties of Pacific whiting, threadfin bream, and Alaska pollock surimi (Lee and Park, 1998; Yongsawatdigul et al., 2002). However, Ca^{2+} is also known as a destabilizing salt in the Hofmeister series (Baldwin 1996). Binding of Ca^{2+} to proteins prevents the salt exclusion, resulting in a decrease of preferential hydration and destabilized structure (Arakawa and Timasheff, 1984). Ca^{2+} solubilized rabbit myofibrillar proteins by salting-in effect (Taylor and Etherington, 1991). Moreover, Ca^{2+} binds to β-lactoglobulin inducing partial unfolding of β-lactoglobulin, which led to an increased hydrophobicity during gelation (Jeyarajah and Allen, 1994). Therefore, Ca^{2+} could also have a direct effect on structure of muscle proteins, which could affect gelation during setting.

Myosin is the most abundant myofibrillar protein in muscle and constitutes of > 50% in vertebrate skeletal muscles. It consists of two myosin heavy chains (MHC) (200 kDa each) and four light chains (LC) (18-25 kDa each) (Harrington and Rogers, 1984). The N-terminal of each heavy chain is folded into a globular head, subfragment 1 (S1), which contains the ATP and actin - binding sites (Lowey, Slayter, Weeds, and Baker, 1969). The remainder participates in a rod like coiled coil helical structure. Thus, myosin heavy chain has properties of both fibrous and globular proteins (Kristinsson and Hultin, 2003). Myosin possesses a proteinase susceptible region called the “hinge region”. Limited proteolytic digestion of myosin at the hinge region

in low ionic strength yields two subfragments, S1 and myosin rod. S1 contained light chain and also remained ATP and actin binding ability (Margossian and Lowey, 1982). Actin constitutes > 30 % of myofibrillar protein with a globular monomer (G-actin) and polymerizes into fibrous actin (F-actin) in muscle system (Turoverov, Biktashev, Khaitlina, and Kuznetsova, 1999). In physiological conditions, myosin and actin form complex as called "actomyosin, AM". In addition, AM also associates to other proteins (troponin, tropomyosin, and α-actinin etc), generating the natural complex as called "natural actomyosin, NAM". NAM is also responsible for gelation during setting of fish proteins.

The stability of NAM has been reported to be closely related to habitat temperatures (Ogawa, Ehare, Tamiya, and Tsuchiya, 1993). Muscle proteins extracted from fish living in tropical habitat unfolded at high temperature and they can be set at about 40 °C (Klesk, Yongsawatdigul, Park, Viratchakul, and Virulhakul, 2000). In contrast, low setting temperatures at 4-25 °C are usually applied for muscle proteins from fish living at low water temperature, exhibiting low thermal stability (Togashi et al., 2002). Thermal unfolding of NAM was reported to be a pre-requisite for the reactions in setting (Ogawa, Kanamaru, Miyashita, Tamiya, and Tsuchiya, 1995). The reactivity of TGases toward NAM depended on the amount of reactive residues, which subsequently depend on conformational changes of NAM (Kishi, Nozawa, and Seki, 1991). The different thermal denaturation of NAM from fish living at different habitats would thus affect TGase reactivity since thermal denaturation governs the exposure of reactive groups for TGases. However, the effect of conformational changes of NAM on reactivity to cross-link by TGase has not been systematically elucidated, particularly pertaining to NAM extracted from fish with potentially different stability

and thermal denaturation. The cross-linking of those NAM should be investigated in order to evaluate their reactivity as substrates for TGases. This information would lead to understand setting phenomena of fish proteins from different habitats.

It was reported that threadfin bream surimi exhibited setting at both 25 and 40 °C. An increased gel strength of surimi corresponded with cross-linking of MHC catalyzed by endogenous TGase (Yongsawatdigul et al., 2002). However, knowledge of endogenous TGase in threadfin bream and its activity in cross-linking muscle proteins is still limited. Besides muscle, liver is another good source of tissue TGase and liver TGase also exhibit the Ca^{2+}-dependent property as fish muscle TGase. Yasueda, Kumazawa, and Motoki (1994) purified TGase from red sea bream liver and demonstrated that it could catalyze cross-linking of Alaska pollock myosin heavy chain (Yasueda et al., 1994). The gelation of myosin B from walleye pollock was promoted in the presence of purified TGase from walleye pollock liver (Kumazawa, Numazawa, Seguro, and Motoki, 1996). Purification and characterization of TGase from threadfin bream liver would lead to understanding its biochemical characteristics and cross-linking reactivity toward fish muscle proteins. Cross-linking activity of muscle proteins catalyzed by threadfin bream liver TGase would thus explain the setting phenomenon of fish protein gels, which was normally induced by muscle TGase. Beside tissue TGase, MTG was added to improve textural properties of surimi from various fish species (Jiang, Hsieh, Ho, and Chung, 2000). It exhibits completely different characteristics from Ca^{2+}-dependent TGases, including molecular weight, crystal structure, active site arrangement, and catalytic mechanism (Kashiwagi et al., 2002). Substrate requirements between these 2 types of TGases are different. The reactivity of MTG toward fish proteins during setting might be different from that of

fish TGase. However, comparison of the reactivity of fish TGase with MTG towards muscle proteins has rarely been documented. Understanding those catalytic reactions would lead to effectively improve textural properties of muscle proteins.

A decrease of protein substrate using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been used as a tool to monitor protein cross-linking catalyzed by TGases (Kumazawa, Numazawa, Seguro, and Motoki 1995). However, cross-linking sites within high molecular weight polymers cannot be characterized. Subsequently, the specificity of different TGases could not be obtained. Introduction of mass spectrometry (MS)-based analysis and the development of tandem mass spectrometry (MS/MS) procedures lead to the introduction of new proteomic strategy, which enable rapid identification of amino acid sequence of proteins or peptides (Piersma, Pijpekamp, Wijngaards, Gruppen, and Boumans 2002). Electrospray mass spectrometry (ESI-MS) with tandem mode was previously used to identify the susceptible sites of glutamyl substrate of TGase within gliadin peptides (Mamone et al., 2004). The labeling glutamyl substrates with affinity probe reduced the complexity after selective purification the glutamyl substrates by affinity chromatography (Rouppoo et al., 2003). Thus, modification of myosin peptides with affinity probe and followed by peptidomic approach could therefore facilitate identification of TGase-mediated modification of myosin peptides. Therefore, this approach would also be a tool for determining substrate specificity of fish TGase and MTG toward muscle protein from fish species.

1.2 Research objectives

The objectives of this research are:

(1) To study the effect of Ca^{2+} on conformational and physicochemical changes of threadfin bream myosin and actin in the setting conditions.

(2) To partially purify and characterize TGase from threadfin bream liver.

(3) To investigate catalytic reaction of threadfin bream liver TGase and MTG as affected by thermal stability of natural actomyosin.

(4) To identify glutamyl substrates for threadfin bream liver TGase and MTG on peptides derived from threadfin bream myosin.

1.3 Research hypothesis

Ca^{2+} induced conformational changes of myofibrillar proteins, promoting the gel- forming ability of fish proteins during setting. Threadfin bream liver TGase exhibits Ca^{2+}dependent characteristic as other tissue TGases and its catalytic reaction could represent the reaction catalyzed by endogenous TGase from fish muscle. Conformation of myofibrillar proteins affects ability of threadfin bream liver and microbial TGases to catalyze protein cross-linking. Those TGases have different glutamyl substrate specificity.

1.4 Expected results

The gained information leads to better understanding the effect of Ca^{2+}on protein conformation in the setting. Biochemical characteristics of threadfin bream liver TGase will be obtained. Catalytic activity of threadfin bream liver TGase and MTG toward proteins with potentially different stability will provide understanding of the effect of protein conformation on cross-linking catalyzed by TGase. Identification

of glutamyl substrate for both enzymes would also lead to localize the possible sites of cross-linking and glutamyl substrate specificity of these two TGases.

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CHAPTER II

LITERATURE REVIEWS

2.1 Gelation and setting of surimi

2.1.1 Gelation of surimi

Surimi is the stabilized myofibrillar protein from fish muscle. It is mechanically deboned fish flesh, which has been washed with water, and blended with cryoprotectants to provide a better frozen shelf life (Lanier, 1992). It is an intermediate product, which is used for a variety of foods from the traditional kamaboko products of Japanese to the recent shellfish substitutes (Kumazawa, Numazawa, Seguro, and Motoki, 1995). From the biochemical point of view, surimi can be considered as a concentrated myofibrillar protein. Mechanisms of thermal gelation of these proteins have also been studied (Roussel and Cheftel, 1990).

2.1.1.1 Myofibrillar proteins

Myosin is the most abundant myofibrillar protein and constitutes approximately 55- 60 % of vertebrate skeletal muscles. Its molecule consists of two heavy chains and four light chains (Harrington and Rogers, 1984). Myosin heavy chain (MHC) exhibits molecular weight of 200 kDa and the N-terminus of each heavy chain is folded into globular head or subfragment 1 (S1), containing ATP and actin- binding sites. The remainder participates in a coiled-coil helical structure exposing hydrophilic charges to the surface of molecule. Thus, MHC has a characteristic of both fibrous and globular structure (Kristinsson and Hultin, 2003). When myosin is digested by trypsin,

two major subfragments are formed, heavy meromyosin (HMM) and light meromyosin (LMM). HMM contains both myosin globular heads and a part of myosin rod, while LMM consists of the helical portion of myosin rod only (Lowey, Slayter, Weeds, and Baker, 1969). Further digestion of HMM results in the individual myosin head and the small portion of myosin rod, which are known as S-1 and S-2, respectively (Margossian and Lowey, 1982). Molecular weight of each light chain (LC) ranges from 16 – 27 kDa, depending on source of myosin (Asghar and Pearson, 1980).

Actin is a second major component and constitutes 15-30% of myofibrillar proteins. The monomer form of actin has a globular structure and is commonly referred to as G-actin, with molecular weight of about 43 kDa. In biological systems, G-actin is polymerized together into actin filaments called fibrous actin (F-actin) (Turoverov, Biktashev, Khaitlina, and Kuznetsova, 1999). Other minor proteins are tropomyosin, troponin complex, actinins, M-proteins, and C-proteins associated with either myosin or actin (Asghar and Pearson, 1980).

2.1.1.2 Thermal stability of fish muscle proteins

Myosin derived from fish muscle is known to be thermally less stable than their mammalian counterparts. Thermal stability of myosin is mainly related to their habitat temperature of fish (Ogawa, Ehara, Tamiya, and Tsuchiya, 1993). Howell, Matthews, and Donnelly (1991) compared thermal stability of tropical fish species including catfish, carp, Nile perch, red snapper, red mullet, and sea bream to that of cold-water species of trout and cod using differential scanning calorimetry (DSC). They also found that myofibrillar proteins from tropical fish exhibited higher onset temperature than did those proteins from fish living in cold-water habitat for 11 °C,

when monitored at low ionic strength and pH 7.0. These results were in agreement to the observation of Davies, Bardsley, and Ledward (1988). They also found that cod myosin exhibited lower melting transition than did snapper myosin for 10°C. However, denaturation temperature of myosin from tropical fish also decreased at high ionic strength of (0.05-1) and pH (6-8) (Howell et al., 1991). The helical structure of myosin was reported to be the region of instability within the myosin molecule (Goodno, Harris, and Swenson, 1976). Thermal stability of myosin rod from various species shifted in parallel with the working or habitat temperature of respective muscle (Rodgers, Karr, Biedermann, Ueno, and Harrington, 1987). Difference in thermal stability of myosin rod from carp reared at 10 and 30 °C was observed by DSC (Nakaya and Watabe, 1995). Temperature acclimation induced the changes in primary structure of LMM and substitution of charged amino acids at hydrophobic core of myosin rod from cold water fish might cause low thermal stability (Watabe et al., 1995). The stability of ATPase activity of fish myosin is strongly correlated with habitat temperature (Johnston, Frearson and Goldspink, 1973). Johnston and Walesby (1977) studied the inactivation rate of ATPase activity of myofibrillar protein from fish living in different habitat. They found that the half-life of inactivation was < 1.5 min for Antarctic species, while that of North Sea species (environmental temperature of 5-12 °C) was about 10 min. The highest stability was found in fish from Indian Ocean, exhibiting the half-life of inactivation around 60-100 min (Johnston and Walesby, 1977). The stability of fish protein has considerable implications for fish processing and storage.

2.1.1.3 Protein gelation

In surimi paste preparation, surimi (myofibrillar proteins) is

comminuted with salt and water. Addition of salt is needed to solubilize and destabilize the native structure of myofibrillar proteins prior to thermal denaturation (Park and Lanier, 1990). Thermal unfolding of these proteins, especially of myosin and natural actomyosin (NAM), is important for gelation. Ziegler and Acton (1984) demonstrated the mechanisms of gel formation by proteins from muscle tissue. Thermal denaturation of NAM begins at 30-35 °C and native tropomyosin and troponin dissociated from F-actin. Subsequently, helical structure of F-actin underwent a single chain at approximately 38 °C. LC subunits started to dissociate from the globular head of MHC when temperature reached at 40-45 °C and conformational changes of globular head also occurred. Actin-myosin complex started to dissociate from each other at 45-50 °C and helical regions of MHC then unfolded to the random coil structure. The denaturation of G-actin occurred at temperature greater than 70 °C. Thermal denaturation mechanism of NAM from carp was also proposed by Sano, Ohno, Otsuka-Fuchino, Matsumoto, and Tsuchiya (1994). The solubilized NAM began to unfold at temperature about 30 °C and aggregation started at this temperuature. Aggregation of proteins was formed extensively around 40-60 °C. Myosin molecule dissociated from F-actin at temperature greater than 40 °C. Taguchi, Ishizaka, Tanaka, Nagashima, and Ammano (1987) reported that myosin gelation was initiated by interaction of HMM at 30-40 °C followed by thermal unfolding as well as interaction of LMM at 50 °C. Gill and Conway (1989) also reported that the tail portion of myosin participated in aggregation at 40-50 °C. In contrast, Sano, Noguchi, Matsumoto, and Tsuchiya, (1990) reported that gel formation of carp myosin was started to form through the interaction of LMM at temperature about 30-45 °C. Chan, Gill, and Paulson (1993) suggested that myosin initially aggregated through the

interaction of S2 at 30-40 °C. Further aggregation was from interaction of LMM at 40-50 °C. The results of these studies indicated that the discrete regions of myosin subfragment play important role on forming gel network.

The role of actin in gelation of myofibrillar proteins was also observed as synergistic effect. The optimum weight ratio of myosin to actin which results in the highest gel enhancing effect was 15:1 (Samejima, Ishioroshi, and Yasui, 1981). The complex formed between F-actin and myosin played a role as cross-linker between rod and rod portions of myosin molecule, which consequently increase rigidity of the gel (Yasui, Ishioroshi, and Samejima, 1982).

2.1.2 Setting (Suwari)

Setting is a phenomenon explaining increased textural properties of surimi gels after pre-incubation at a certain temperature below 40 °C for a specific period of time prior to cooking (Lanier 2000). A gel with higher rigidity and elasticity is obtained when setting is followed by cooking (Roussel and Cheftel, 1990). This phenomenon involves gel network formation of muscle proteins triggered by protein unfolding.

Setting temperature can be varied, depending on fish species, and setting phenomenon is related to habitat temperature of fish species. The optimum temperature for setting among species may be determined by the heat stability of myosin (Morales, Ramirez, Vivanco, and Vazquez, 2001). Generally, setting can be carried out at low (0-4 °C), medium (25 °C), and high (40 °C) temperatures (Lanier, 1992). Low and medium temperatures are applied for setting of surimi from cold and temperate habitats. Setting of Alaska pollock surimi from Bering Sea performed well at 4-5 °C (Kim, Carpenter, Lanier, and Wichker, 1993). Park, Yongsawatdigul, and

Lin (1994) also reported that optimum setting temperature for Pacific whiting surimi was at 25 °C instead of 5 °C. High temperature at 40 °C has been applied for setting of surimi from tropical or warm water fish species such as Atlantic croaker, Mexican flounder, Northern kingfish, threadfin bream, bigeye snapper, barracuda, and bigeye croaker (Lee and Park, 1998; Morales et al., 2001; Yongsawatdigul, Worratao, and Park, 2002; Benjakul, Visessanguan, and Chantarasuwa, 2004). It has been documented that various types of protein–protein interactions contributed to textural changes during setting.

2.1.2.1 Protein-protein interactions in setting

Disulfide bond formation was found to participate in setting. Chan, Gill, Thompson, and Singer (1995) observed that disulfide bond of surimi from herring increased after setting at 10 °C for 24 h. Such increase resulted in polymerization of MHC during setting. Gel network formation of myosin was also initiated by disulfide formation between myosin head (Samejima et al., 1981). Disulfide bond formation was noticed during setting of herring myosin at 40 °C (Gill, Chan, Phonchareon, and Paulson, 1992).

The role of hydrophobic interactions on setting phenomenon was also suggested by Chan et al. (1995). They showed that the binding of 8- anilino-1-napthalenesulfonate (ANS), a hydrophobic probe, on myofibrillar proteins promoted fast gel setting at 35-40 °C in the presence of NaCl. Sano et al. (1994) suggested that hydrophobic interactions of carp NAM occurred extensively at 30 – 50 °C. Nowsad, Katoh, Kanoh, and Niwa (1996) reported that hydrophobic interactions play important role on gelation of fish proteins at high temperature. Thermal aggregation of cod

myosin was also formed by hydrophobic interactions of the rod portion (Chan et al., 1993). In addition, Gill and Conway (1989) reported that the tail region of cod myosin was involved in non-covalent interactions at about 40-50 °C. Thus, hydrophobic interactions would play a role in setting of fish proteins.

Isopeptide bond catalyzed by transglutaminases (TGases) has been reported to be the major force involving polymerization of myosin in the setting (Kamath, Lanier, Foegeding, and Hamann, 1992). TGase is an enzyme that catalyzes acyl transfer reaction between the γ-carboxyl amide group of glutamine residues within protein to the side chain of lysine, resulting in protein cross-linking. Tissue or endogenous TGases require Ca^{2+} for catalytic reaction (Folk, 1980). Cross-linking of MHC was also the Ca^{2+}-dependent reaction in the setting and such cross-links play a crucial effect on gel strengthening (Kumazawa et al., 1995). The effect of Ca^{2+} on setting at 25 °C (3 h) or 5 °C (20 h) in surimi from Pacific whiting and Alaska pollock has been reported (Lee and Park, 1998). Addition of Ca^{2+} significantly improved textural properties of surimi from threadfin bream after setting at either low or high temperatures but breaking force value of gels set at 40 °C was higher than at 25 °C (Yongsawatdigul et al., 2002). Tsukamasa et al. (1993) found that the isopeptide bond formation of sardine sol was found to be below 30 °C. They also demonstrated that an increase in gel strength was correlated to the amount of isopeptide bond. Endogenous TGase also played a role in setting of threadfin bream surimi (Yongsawatdigul et al., 2002). Setting phenomenon, gel strength, cross-linking of MHC, and the content of isopeptide bond increased with setting time (Kumazawa et al., 1995). Takeda and Seki (1996) reported that complete suppression of myosin cross-linking of walleye pollock surimi gel was associated with the inhibition of endogenous TGase. These results were

in agreement with the results from Kumazawa et al., (1995). They suggested that setting phenomenon was suppressed in the presence of TGase inhibitors, such as NH_4Cl and EDTA. Besides endogenous TGase, MTG has also been applied to improve textural properties of surimi gels (Sakamoto et al., 1995; Seguro, Kumazawa, Ohtsuka, Toiguchi, and Motoki, 1995). MTG has been isolated from microorganisms (*Streptoverticilium mobarens*), which produced a Ca^{2+}-independent TGase (Ando et al., 1989). MTG from *Streptoverticilium ladakanum* was also applied to surimi from threadfin bream and Alaska pollock (Tsai, Lin, and Jiang, 1996; Jiang, Hsieh, Ho, and Chung, 2000). MTG was also used to improve mechanical properties of surimi from silver carp (Ramirez, Santos, Morales, Morrissey, and Vazquez, 2000). Rate of MHC polymerization catalyzed by MTG was 3-fold higher than by endogenous TGase (Nakahara, Nozawa, and Seki, 1999).

2.1.2.2 Protein cross-linking catalyzed by TGase

NAM and myosin are also the major components responsible for setting and their molecules were aligned into three-dimensional network of gel. The changes in protein patterns of fish species, such as carp, Atlantic croaker, and Alaska pollock have been studied using SDS-PAGE (Wu, Lanier, and Hamann, 1985). The results revealed that oligomer and polymer of MHC could be formed after setting at 20 °C. Thus, MHC was the major component responsible for setting of fish proteins. Rate of setting is normally depended on myosin within fish species (Taguchi, Ishizaka, Tanaka, Nagashima, and Amano, 1987). Maruyama, Nozawa, Kimura, Satake, and Seki (1995) found that rate of dimerization of MHC from carp was slower than that from rainbow trout and atka mackerel. They also reported that glutamine residues within NAM from atka mackerel were more reactive than those from others

(Maruyama et al., 1995). Rate of MHC polymerization from fish species were walleye pollock > rainbow trout > chum salmon > atka mackerel > white croaker > carp (Araki and Seki, 1993). Kishi, Nozawa, and Seki (1991) observed that the extent of MDC incorporation appeared to be 2.4 folds higher in soluble NAM than that in insoluble NAM or myofibril at the identical conditions. They also suggested that salt soluble NAM exposed glutmaine residues to be more reactive for TGase catalyzed reaction. In addition, the reactivity of TGase on protein cross-linking was also varied by conformational changes of the protein substrates. Ogawa, Kanamaru, Miyashita, Tamiya, and Tsuchiya, (1995) suggested that unfolding of myosin rod is the pre-requisite for setting of fish proteins. Thus, type and conformation of proteins directly affect setting induced by TGase-catalyzed reaction.

Huang, Seguro, Motoki, and Tawada (1992) studied the cross-linking of contractile proteins from skeletal muscle by MTG. They found that myosin rod was quickly cross-linked, while S-1 was not. ATPase activity of myosin, HMM, and S-1 were not affected by the presence of MHC cross-linking (Huang et al., 1992). Incorporation of MDC and monobiotincadaverine into HMM from rabbit using guinea pig liver TGase were also performed by Kunioka and Ando (1992). They revealed that incorporation sites were at S2 near the HMM and LMM junction. In addition, dimerization sites on carp MHC catalyzed by endogenous TGase were also at S2 when the reaction was studied under the condition similar to setting. The results from these studies suggested that the rod portion of myosin participated in cross-linking rather than S-1. Although MHC is the preferred substrate for TGase, its cross-linking is also depended on myosin subfragments. Therefore, conformation of each myosin subfragment would also affect TGase catalyzed reaction and setting phenomenon.

2.2 Transglutaminases (TGases)

Transglutaminases (TGases) (protein-glutamine: amineγ-glutamyltransferase, EC 2.3.2.13) catalyze the formation of isopeptide bond between peptide, protein – bound glutamine and primary amines or ε-amino group of peptide bound lysine as shown in Figure 2.1. The term "transglutaminase" was first introduced by Clarke, Mycek, Neidle, and Waelsch (1957) to describe the transamidating activity observed in guinea-pig liver. TGases have been found in plant, invertebrates, amphibians, bird, fish, and microorganisms. They are the widely distributed enzymes that catalyze the post-translational modification of proteins by formation of isopeptide bonds through protein cross-linking or amine incorporation (Folk, 1980). The cross-linked products accumulate in several tissues including blood, skin, hair, and wound healing. In addition, these cross-linked products are highly resistant to mechanical challenge and proteolytic digestion.

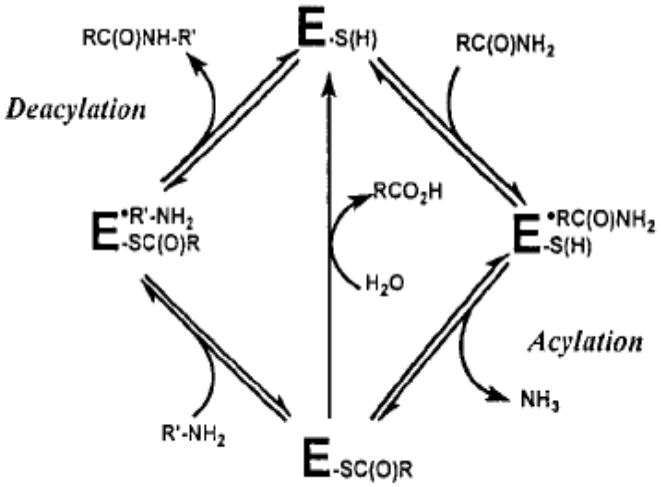

Figure 2.1 Reaction catalyzed by TGases in "ping-pong" mechanism.

From: Leblanc et al., (2001).

2.2.1 TGase isoforms

2.2.1.1Circulating zymogen factor XIII

It is converted into the active TGase Factor XIIIa (plasma TGase) by thrombin-dependent proteolysis. It is involved in stabilization of fibrin clots and in wound healing. Factor XIII consists of two α- and two β- subunits (Tamaki and Aoki, 1982). The a-subunit (Factor XIIIa) contains the active site of the enzyme. Factor XIIIa is the last factor involved in the blood coagulation cascade. Synonyms are fibrin stabilizing factor and Laki-Lorand factor. It catalyzes the polymerization of fibrin monomers into γ-γ-(fibrin) dimers and α-polymer during heamostasis (Chen and Doolittle, 1971). In addition, it catalyzes cross-linking of α-2 plasmin inhibitor to β-chain of fibrin as well as the cross-linking of factor V, fibronectin, lipoprotein, α-2-macro-globulin, plasmin-activator-inhibitor-2 (Bohn, 1972).

2.2.1.2 Keratinocyte TGase

Keratinocyte TGase (type 1 TGase) exists in both membrane-bound and soluble forms, which is activated by proteolysis (Chang and Chung, 1986). Keratinocyte TGase was first detected in keratinocytes and is involved in the formation of the envelope of stratum corneum during terminal differentiation (Hohl et al., 1991). The cDNA cloning of rat keratinocyte showed a TGase composing of 824 amino acids with the molecular weight of 90 kDa (Kim et al., 1991).

2.2.1.3 Hemocyte TGase

Hemocyte TGase is a part of the coagulating system of the vertebrate hemolymph. The hemocytes circulating in the hemolymph of vertebrates contain an intrinsic coagulation system. It converts the soluble coagulogen into an insoluble coagulin gel (Tokunaga et al., 1993).

2.2.1.4 Secretory TGase

Secretory TGase was originally identified in the secretion of the dorsal prostate and coagulating gland of rodents. The function of this TGase is to form an intravaginal coagulation plug directly after copulation (Steinhoff et al., 1994). The substrate used for this enzymatic reaction is SVS II, a monomer protein, originating from seminal vesicle secretion. The secretory TGase is a glycoprotein, which is synthesized and processed in the cytoplasmic compartment. Then, it is secreted by an alternative export mechanism via apical blebs. It was also identified in human prostates (Grant et al., 1994).

2.2.1.5 Ubiquitous type 2 tissue TGase

Ubiquitous type 2 tissue TGase (tTGase) is distributed in numerous vertebrate tissues. It functions as a cross-linker for components of the extracellular space as well as in the cytosolic compartment. In addition, there is some evidence that tissue TGase plays a role in cell-matrix interactions and modulates the regeneration of cells in tissue repair (Kojiama, Nara, and Rifkin, 1993). Furthermore, a role for tTGase in cell growth and in the complex processes of the programmed cell death has been suggested (Greenberg, Birchbichler, and Rice, 1991). tTGase has been found in several fish species including sardine, mackerels, red sea bream, carp eel, croaker, pollock, salmon, rainbow trout, and tilapia (An, Peters, and Seymour 1996; Araki and Seki, 1993; Yasueda, Kumazawa, and Motoki, 1994;Worratao and Yongsawatdigul, 2003) as well as in surimi of those fish species. However, purification and characterization of TGase from threadfin bream have not been thoroughly elucidated.

2.2.1.6 Microbial TGase

Microbial TGase (MTG) has been isolated from culture medium of S*treptoverticillium* sp. S8112, which has been identified as a variant of S*treptoverticillium mobaraense* (Ando et al., 1989). It is the first TGase obtained from non-mammalian source. Thus far, few TGases has been identified from other microorganisms, such as *Streptoverticillium ladakanum* (Tsai et al., 1996). MTG was also found in *Bacillus subtilis* and in *Physarum polycephalum* as intracellular forms (Klein, Gozman, and Koehn, 1992). It is secreted from the cytoplasm as a zymogen and is activated by proteolytic processing. In contrast to many TGases, MTG activity is Ca^{2+}-independent enzyme.

2.2.2 Molecular Structure of TGases

2.2.2.1 Factor XIIIa

Factor XIIIa circulates in blood as a tetramer (Mw 320 kDa) and consists of two catalytic "a" subunits (Mw 75 kDa each) and two non-catalytic "b" subunits (Mw 80 kDa each). The "b" subunits of Factor XIIIa contain 641 amino acids with Mw of 80 kDa. Amino acid sequence of "a" subunit consists of 730-731 residues with Mw of 83 kDa after deducing from cDNA cloning (Ichinose, Bottenus, and Davie, 1990). There are nine cysteine residues in the protein, at least four of them in the free sulfhydryl form. No disulfide bonding has been found. The molecule has six potential sites for *N*-glycosylation but carbohydrate residue was not detected. By computer analysis, three potential Ca^{2+} binding sites were identified. In native state, TGases folded into three-dimensional structure. The X-ray crystallographic technique is introduced to solve electronic structure of each TGase. The three-dimensional structure of Factor XIIIa subunit composes of activation peptide (1-37) and four sequential domains namely β-sandwich (38-184), core (185-515), barrel 1(516-628),

and barrel 2 (629-730) from the N-terminus of polypeptide (Figure 2.2a) (Noguchi et al., 2001). In the dimer form, Factor XIIIa forms alignment of the long edges of two subunits, with the dimer interface formed by the sandwich and core domains. The resulting overall shape of the Factor XIIIa dimer is that of a puckered hexagon with the six β-sheet domains at the periphery and the large α-helical and β-sheet containing catalytic core domain at the center (Kurochkin et al., 1995). The activation peptide of one monomer crosses to another subunit of a homodimer.

2.2.2.2 Tissue TGase

Tissue TGase (tTGase) from guinea pig liver exists as monomer and consists of 690 amino acid residues with Mw of 77 kDa (Ikura et al., 1988). It contains 16-18 sulfhydryl groups without disulfide bond formation. The tTGase in human consists of 692 amino acids and exists as two monomers stabilized by salt bridge interaction (Kim, Gormann, Park, Chung, Steinert, 1993). The tTGase from red sea bream liver contains 695 amino acid residues. This amino acid sequence shared 43.3% overall similarity with tissue-type TGase from guinea pig liver (Yasueda et al., 1995). The N-terminal sequence of red sea bream TGase showed homology with the sequence of chicken erythrocyte TGase. The sequence also contains two relatively highly acidic regions which could act as Ca^{2+} binding site (Aspartic acid, D, 140 – D 159) and (Glutamic acid, E, 181 - E 193) (Yasueda et al., 1995). The three-dimensional structure of activated tTGase from human consists of four folded domains similar in structure to human Factor XIIIa. However, the arrangement of the two monomers of human tTGase is different from human Factor XIIIa. In human tTGase, three strands of the β-pleated sheet of barrel 2 domain of one monomer forms intermolecular contacts with four strands of the β-sandwich domain of the second monomer. The 2

Ca^{2+} binding sites located at the loop containing residues 462-471 and another site was at the catalytic core (Ahvazi, Kim, Kee, Nemes, and Steinert, 2002). The overall structures of tTGase from red sea bream liver and human Factor XIIIa resemble each other, although the former lacks an activation peptide at the N-terminus. In red sea bream liver TGase, the β-sandwich contains 6-34, the core domain contains 135-461, barrel 1 contains 472-583, and barrel 2 contains 584-684 (Noguchi et al., 2001).

2.2.2.3 MTG

MTG from *Streptoverticilium* sp. Strain s-8112 consists of 331 amino acid residues in a single peptide chain with Mw of 37.8 kDa (Kanaji et al., 1993). The amino acid sequence of MTG revealed that there is no overall structural relationship of MTG to other types of TGase except in the regions around the reactive cysteine (Cys) residue. Moreover, the homologous sequences with the Ca^{2+} binding sites of other TGases were not detected. MTG forms a single and compact domain, which is totally different from other TGases. MTG also adopts disk-like shape and has a deep cleft at the edge of the disk (Kashiwagi et al., 2002). The structure of MTG belongs to the α+β folding class, containing 11 α-helices and 8 β-strands. The α-helices and β-sheets are concentrated at amino and carboxyl ends, respectively of polypeptide. The secondary structures are arranged such that β-sheet is surrounded by α-helices, which are clustered into three regions. The first cluster of α-helices exists on the left side of the front view of MTG (Figure 2.2d) comprising of the α1-α3 and the Cys-64 located on the loop between α2 and α3 helices. The second cluster comprises of the α4, α5, and α10. The third one composes of α6, α7, α8, and α9.

2.2.3 Active site structures of TGases

2.2.3.1 tTGase and Factor XIIIa

Active site structure of red sea bream TGase and human Factor XIIIa showed the Cys- His -Asp catalytic triad reminiscent of the cysteine proteases, consisting of Cys-272, His-332, and Asp-355 in red sea bream liver (Noguchi et al., 2001) and Cys-314, His-373, and Asp-396 in human Factor XIIIa (Muszbek, Yee, and Hevessy, 1999). The catalytic triad was similar to that of cysteine proteases such as papain. The data from crystal structure showed that the catalytic Cys of tTGase from red sea bream liver and human Factor XIIIa locate between tyrosine (Y) and another Cys residue as shown in Figure 2.3. Moreover, the O atom in the phenolic side chain of Y residue forms a hydrogen bond with the S atom in the sulfhydryl group of C residue, which suppresses activity of these TGases. Then, the Y residue must be removed to activate the enzymes (Chica, Gagnon, Keillor, and Pelletier, 2004). The mechanism for activation of both TGases was proposed to be as follows. 1) Ca^{2+} binds to its site in enzyme molecule. 2) An acyl donor is introduced to the active site. 3) The Y covering the catalytic Cys residue is removed by conformational changes. 4) An acyl – enzyme intermediate is formed between acyl donor and Cys residue. The Ca^{2+}–activated proteolytic cleavage by thrombin is necessary for step 1 and 2 for human Factor XIIIa, but only Ca^{2+} binding is sufficient for activation of red sea bream liver TGase and human tTGase. Binding of three Ca^{2+} ions opens a channel, which removes the 2 tryptophan (W) residues that control substrate accessibility to the active site of human tTGase (Ahvazi et al., 2002).

2.2.3.2 MTG

The active site loops of MTG are tightly associated with many polar and hydrophobic interactions. The arrangement of secondary structure around the active sites of MTG and red sea bream TGase are very similar. The active cysteine,

Cys-64 in MTG and Cys-272 in red sea bream TGase exist near the N terminus of the α-helices. However, the catalytic Cys-64 residue of MTG is sufficiently exposed to solvent and promptly reacts with substrate without Ca^{2+} binding, leading to Ca^{2+}-independent reaction.

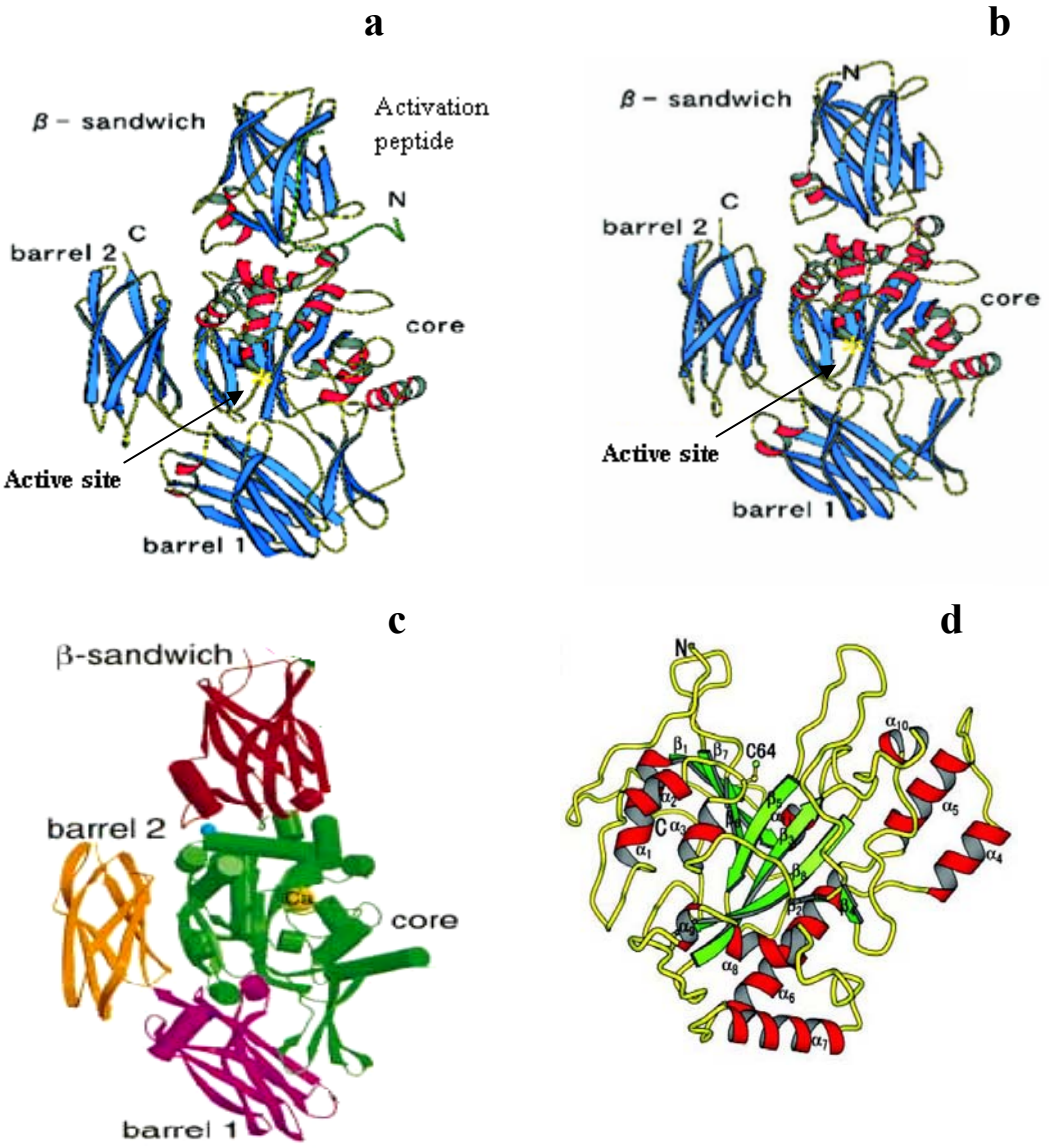

Figure 2.2 Crystal structure of human Factor XIIIa (a), red sea bream liver tTGase (b), human tTGase (c), and MTG (d).

Modified from Noguchi et al. (2001) (a and b), Ahvazi et al.2002 (c), and Kashiwagi et al. (2002) (d).

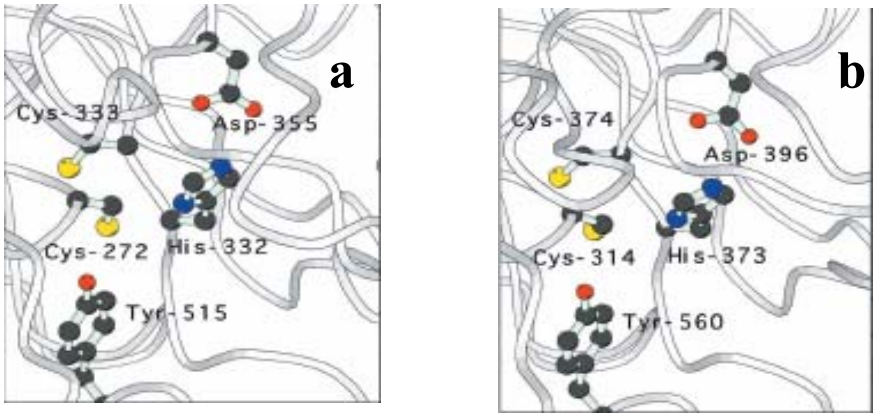

Figure 2.3 Active sites of red sea bream liver TGase **(a)** and human Factor XIIIa **(b)** From Noguchi et al. (2001).

The flexibility of the right side wall of the active site cleft may decrease the steric hindrance between the enzyme and substrates. Moreover, MTG did not contain the catalytic triad "Cys-His-Asp" as in Factor XIIIa and red sea bream liver TGase. In MTG, Asp-255 and His-274 ocupy the positions corresponding to those of His-332 and Asp-355 in red sea bream, respectively. The three residues, Cys-64, Asp-255, and His-274 of MTG superimpose well on the catalytic triad of red sea bream TGase (Cys-272, His-332, and Asp-355). The structure and structural comparison between active site of MTG and FTG are shown in Figure 2.4. Based on the mechanism of catalysis, the negatively charged of Asp-255 at active site of MTG plays advantageous for the acyl donor specificity. That is, the positively charged acyl acceptors are more attracted than the neutral species such as water molecules by the electrostatic interaction with Asp-255. Thus, the deamidation activity of MTG is weaker than that of FTG (Ohtsuka, Umezawa, Nio, and Kubota, 2001).

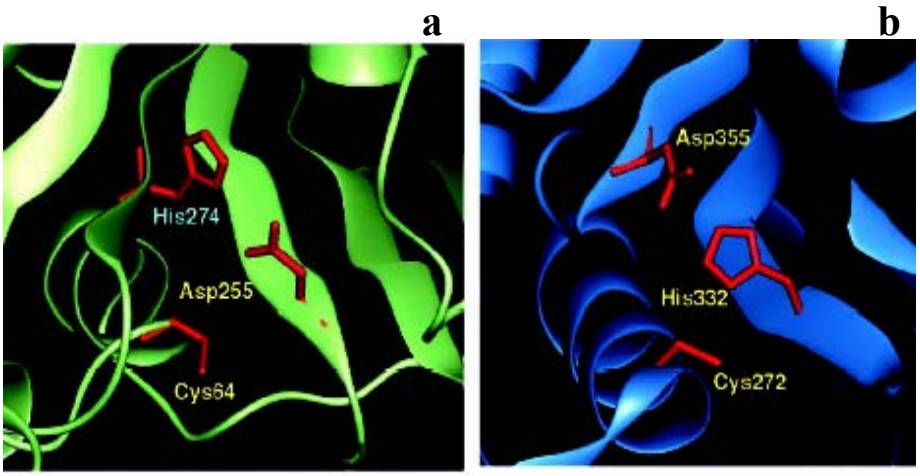

Figure 2.4 Structure around active sites of MTG **(a)** and red sea bream liver TGase **(b)**

From: Kashiwagi et al. (2002).

2.2.4 TGase activity measurement

2.2.4.1 Fluorometric assay

This assay is based on the catalytic function of TGase that incorporates amine into peptide bound-glutamine. In most assays, the fluorescent-labeled primary amine derivatives, such as monodansylcadaverine (MDC), are incorporated into dimethylated casein (Takagi, Saito, Kikuchi, and Inada, 1986). The covalent attachment of the amines gives a blue shift and an increase in the quantum yield of emission by the dansyl group.

2.2.4.2 Colorimetric assay

The peptide-bound glutamyl hydroxamate formation from N-carbobenzoxy (CBZ)-L-glutaminylglycine in the presence of hydroxylamine is the principle of this assay. The formation of peptide bound hydroxamate can be monitored at 525 nm.

The fluorometric and colorimetric methods are suitable to detect TGase activity during purifications due to the rapid measurement and straightforward procedure. Usually, the most sensitive and accurate method of TGase activity assay is fluorometric measurement, while the more reproducible data is obtained by the colorimetric analysis (Wilhelm, Meinhardt, and Seitz, 1996).

2.2.4.3 Fluorometric and electrophoretic assay

A fluorescent amine (MDC) is incorporated into protein substrates and then separated by electrophoresis. The activity staining of TGases on polyacrylamide or agarose gels have been described. After electrophoretic separation of the enzyme in native condition, the gel is reacted with MDC and dimethylated casein (DMC) in the presence of Ca^{2+}. The incorporation of dansyl groups is visualized using an UV lamp after protein is fixed with ethanol-acetic acid (Lorand, Siefring, Tong, Lorand, and Gray, 1979). MDC staining method is suitable for detection of multiple forms of TGases because the electrophoretic mobility of the enzyme is dependent on charge density.

2.2.5 Substrate specificities of TGases

Folk and Cole (1965) reported that the free glutamine (Q) cannot be catalyzed by guinea pig liver TGase (GTG) and the C- and N- terminal side of Gln must be blocked. The Gln must be located at least in the third position from the N-terminus and at least the second position from the C- terminus. Ohtsuka, Ota, Nio, and Motoki (2000) suggested that TGases did not only recognize the L-glutamine but also D-glutamine. The factors governing specificity of TGase were proposed by Coussons and Price (1992). They reported that the minimal requirements for modification of Gln side chain in the proteins are: (i) The Q must be in the satisfied accessibility, either by

being in a highly flexible region of polypeptide chain or by being clearly exposed to solvent in a more open structure. (ii) The feature surrounding Gln must not be discouraged enzyme-substrate interactions. Ohtsuka, Sawa, Kawabata, Nio, and Motoki (2000) reported that substitution of C-terminal side of Q with phenylalanine (F) had a strong activation effect for GTG and FTG, while activity suppression was found in MTG. In addition, substitution of that site by leucine (L) showed the similar results. Effect of amino acids surrounding reactive Q on TGase activity was investigated by Ohtsuka et al. (2000). Glycine (G) and alanine (A) were used to represent amino acids with small hydrophobic side chain, valine (V) and F as the bulky hydrophobic side chain, serine (S) and glutamic acid (E) as the small hydrophilic side chains, and Y and arginine (N) as bulky hydrophilic side chains. They found that the replacement of large hydrophobic amino acid at the N-terminal side of Q accelerated the reactivity of MTG, while that of the C-terminal side accelerated the reactivity of GTG. These results suggested that enzyme-substrate interactions on both sides are important for optimum catalysis. Folk et al. (1983) also described that the N-terminal side of Q interacts with enzyme and that the C-terminal side arranges the carboxylamide in the direction of the active center of the enzyme to form the stable acyl-enzyme. Thus, amino acid at both sides of Q play important role for enzyme substrate interaction.

Ohtsuka et al. (2000) reported that TGases showed broader substrate specificity toward primary amines and they had no stereo specificity for lysine molecules. TGase could use D-lysine as an acyl acceptor in the TGase-catalyzed reaction but the reactivity toward L-lysine was higher than that of D-lysine. TGases required the primary amines with more than four carbon atoms and did not recognize the α-amino

groups of amino acids. It speculated that the space around the amine-binding site is narrow and sensitive to the steric hindrance and amine containing negatively charged moieties. Grootjans, Groenen, and Jong (1995) reported that G or D residues before the reactive lysine has the strongest adverse effects on substrate reactivity for TGase. The proline (P), H, and W residues are less favorable. The V, N, and W residues had an enhancing effect on the reactivity. The preferential feature of glutamyl substrates for guinea pig liver TGase on gliadin peptides was reported to be the concensus sequence Q-X-P (Mamone et al., 2004), while other peptides or proteins showed different preferred sequences (Ruoppolo et al., 2003). This concensus sequence was also catalyzed slowly by MTG (Piersma, Pijpekamp, Wijngaards, Gruppen, and Boumans, 2002). However, the concensus sequence and reactive Q on myosin molecules for tissue-TGase and MTG have not been investigated.

2.3 Mass spectrometry for protein analysis

Mass spectrometry (MS) is a technique for separating and identifying molecules based on mass and it has become an important tool for proteomics, the analysis of the whole range of proteins expressed in a cell. Mass spectrometry is used to identify proteins and to determine their amino acid sequence (Aebersold and Goodlett, 2001).The use of mass spectrometry has greatly aided proteomics since protein sequencing is not simple and straightforward as DNA sequencing. It can also be used to determine if a protein has been modified by the post translational or chemical modifications, such as modification by phosphate groups and sugars.

2.3.1 Mass spectrometers

Mass spectrometer requires charged and gaseous molecules for analysis. This made it difficult to apply MS for protein analysis since large biomolecules with high polarity are not easily transferred into the gas phase. Electrospray ionization (ESI) (Fenn, Mann, Meng, Wong and Whitehouse, 1989) and matrix assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp, 1988) are the ionization techniques that can successfully be used to transfer biomolecules into gas phase. These methods were developed in 1980 and were the basis of increasingly powerful instrumentation of mass spectrometers that have been now become available (Mann, Hendrickson, and Pandey, 2001). This situation made polypeptides and proteins accessible to mass-spectrometric analysis.

2.3.1.1 MALDI-time of flight spectrometer

The ionization process of MALDI is accomplished through the utilization of a matrix compound capable of absorbing pulsed laser beam. Sample is mixed with an excessive amount of matrix before allowing co-crystallization on the sample probe. When the probe is hit by a pulsed laser beam at high vacuum, energy is absorbed by the matrix, which is partially vaporized and carries intact sample into gas phase. During expansion of the MALDI plume, protons are exchanged between analytes and matrix, resulting in formation of charged analytes (Karas, Gluckmann, and Schafer, 2000). Hillenkamp, Karas, Beavis, and Chait (1991) reported that α-cyano-4-hydroxycinnamic acid was suitable for analysis of peptide and proteins smaller than 10,000 Da, whereas sinapinic acid was used as a matrix for high mass proteins. The generated ions from MALDI are typically analyzed by time-of-flight (TOF) mass analyzer. Ions are accelerated in equal energies and fly along the tube with

different velocities, depending on their masses. The mass-to-charge ratio (m/z) of those ions is deduced from their flight time through a tube of specified length (Weickhardt, Moritz, and Grotemeyer, 1996). MALDI is also referred to as a soft ionization method, providing the advantage that large molecules are desorbed into gas phase without undergoing degradation **(**Karas and Hillenkamp, 1988**).** Chen and Hagerman (2004) applied MALDI-TOF to characterize non-covalent complexes between bovine serum albumin (BSA) and β-1, 2, 3, 4, 6-penta-O-galloyl-D-glucopyranose (PGG). The peaks were assigned to BSA-PGG_1 to BSA-PGG_4 and dialysis of these complexes removed PGG from BSA, confirming non-covalent interactions (Chen and Hagerman, 2004). The direct energy transfer required a small sample to be analyzed providing high sensitivity from femtomole to the low picomole. In 1994, Vorm, Roepstorff, and Mann have demonstrated that low attomole detection limit of peptide could be determined by MALDI-TOF. The MALDI process generates a broad spatial and kinetic energy distribution, resulting in low resolution. However, improvement of resolution by applying the pulsed extraction process to MALDI-TOF was reported by Brown and Lennon (1995). They found that mass resolution of cytochrome *c* (12,361.1 Da) was higher than 1000 fwhm. Whittal and Li (1995) also demonstrated that mass resolution in the range of 3000-6000 fwhm can be obtained for detection of peptides with masses up to 3000 Da when an electrostatic mirror called a refectron is applied. MALDI-TOF has the advantage that it could analyze sample in the complex mixture. Jin and Manabe (2005) applied MALDI-TOF to determine mass of the protein mixture containing cytochrome *c*, α-lactalbumin, myoglobin, β-lactoglobulin, trypsinogen, and carbonic anhydrase without purification process.

2.3.1.2 ESI-quadrupole mass spectrometer

ESI has been developed for use in biological samples by Fenn et al. (1989). This ionization technique is atmospheric pressure ionization (API), which produces the gaseous ionized molecules from a liquid solution by creating a fine spray of highly charged droplets in the presence of a strong electric field. The mechanism of the formation of gas-phase ions from the charged droplets is based on the charge residue model (CRM) **(**Whitehouse, Dreyer, Yamashita, and Fenn, 1985). The droplets shrink in size due to the solvent evaporation and the charge density on their surface increases until it reaches the instability limit. Then, the repulsive forces exceed the droplet surface tension to cause breakdown the droplets. The sequence of solvent evaporation and fission of droplets is repeated until the small droplet containing only one solute molecule is obtained. Another mechanism is the ion desorption model (IDM). The sequence of solvent evaporation and droplet fission are repeated. However, the electric field generating by surface charged density is sufficient to overcome the cohesive forces leading to direct desorption of ion. Since ESI is applicable to ionize peptides presenting in the liquid phase, it can be interfaced to high performance liquid chromatography (HPLC) and capillary electrophoresis (Devis, Stahl, Hefta, and Lee, 1995). ESI is considered to be the ionization technique for the low range of mass analyzer, quadrupole mass analyzer, since ESI generates multiply charged ions (Chowdhury, Katta, and Chait, 1990). The multiply charged ion allowed the large ion to be detected under low m/z range of quadrupole mass analyzer. In addition, quadrupole mass analyzer is also tolerant for relatively high pressure of separation instrument such as HPLC. Separation process of ion by quadrupole mass analyzer relies on electric field generating by four metal rods (quadrupoles) (Leary and

Schmidt, 1996). Quadrupole is also considered to be the ions filter, which controls the trajectories of the ion by a set of time-dependent force. This force is generated by applying direct current (DC) and radiofrequency (RF) potentials to a set of electrodes**.** Oscillation processes allow the selected ion (specific m/z) to pass through the quadrupole, while others are eliminated. The m/z of particular ions is determined by correlating the field applied to the quadrupoles with the ions reaching the detector**.** The ability to generate multiply charged ions of proteins allows ESI-MS to observe the large molecules with the relative small mass range and provide direct measurement of large biomolecules. Chen et al. (1995) measured individual T4 DNA ions (Mw = 1.1×10^8 Da) by ESI-MS. Martin, Shabanowitz, Hunt, and Marto (2000) reported that the detection limit of nano HPLC-ESI Fourier transform ion cyclotron resonance was also subfemtomole and the 400 amol of tryptic peptides derived from standard proteins could be analyzed by this instrument.

2.3.2 Tandem mass spectrometry and peptide sequencing

Tandem mass spectrometry (MS/MS) is the technique for determining ion mass usingmass spectrometer, which is coupled with several mass analyzers. These mass analyzers are designed to select, collide, and separate ions facilitating the structural determination (Canas, Lopez-Ferrer, Ramos-Fernandez, Camafeita, and Calvo, 2006). In tandem mode, peptide ion is selected in the first mass spectrometer and is then dissociated by collision with an inert gas, such as argon or nitrogen. The resulting fragments are separated in the second part of the tandem mass spectrometer. Several bonds along the backbone can be broken by the collisions. The most common types are the b- and the y- ions, which denote fragmentation at amide bond with charge retention on the N- or C- terminal, respectively, as shown in Figure 2.4. The sequence

of peptides determined from fragmentation of peptides in MS/MS is called *de novo* peptide sequencing. Tryptic peptides most likely contain Arg or Lys residues at C-terminus and may also be doubly charged or triply charged (Canas et al., 2001). These ions are fragmented easily providing structural information at lower activation energy. Thus, *de novo* sequencing is typically performed with tryptic peptides. It is possible to interpret the fragmentation ions from the low mass end through the highest mass ion (Mann et al., 2001). For example, the y- ion series of tryptic peptides starts with masses of y_1 = 147 (Lys) or 175 (Arg). To calculate y1 ion, 19 Da (H_3O^+) is added to residue mass of Lys or Arg. The mass residue of the following amino acids is added to y_1 to obtain y_2 ion. This process is continued until the mass of full sequence is reached. For b-ions, 1 Da of (H^+) is added to the first residue to obtain b_1 ion. To calculate the masses for the b_2, b_3, and following fragment ions, the process is continued by the addition of the residue mass for the second, third, and following amino acid residues, respectively, until the final residue is included. A complete set of b- and y- ions confirms the entire peptide sequence. However, not all fragment ions are present at detectable levels and thus it is often possible to interpret only part of the sequence with confidence. Normally, MS/MS spectrum includes immonium ions, which are derived from double cleavage of the peptide backbone, N- and C- terminal to the amino acid residues. These immonium ions can indicate or confirm the presence of individual amino acids.

Most peptide sequencing experiments have been performed on ESI-triple quadrupoles (TQ) consisting of 3 sections (2 sections of mass analyzer separated by a central fragmentation cell). The third quadrupole can be replaced by other mass analyzers, such as TOF (QqTOF) or linear ion trap (QqLIT). The resolution and

sensitivity of either QqTOF or QqLIT were higher than normal TQ (Morris et al., 1996).

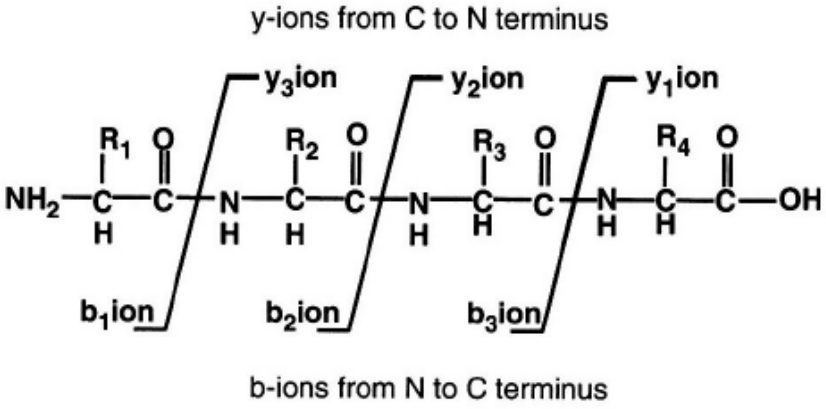

Figure 2.5 Principal ion series generated upon fragmentation of peptides by MS/MS. Cleavage at the peptide bonds produces two ions containing either the C-terminus (y-ions) or the N-terminus (b ions).

From: Canas et al., (2006)

In 1995, Brown and Lennon observed the fragmentation at amide bond between peptide residues of oxidized B chain from bovine serum insulin under MALDI-TOF. Some peptide ions decay because of the low energy from departed ionization process and voltage has also been applied to correct for the low kinetic energy, as so called “post source decay”. Significant amount of structural information can be obtained from this technique. The sequence specific fragments were found from both N- and C-terminal, providing the sequence information of peptide. Brown and Lennon (1995) also demonstrated the potential *de novo* sequencing of cytochrome c by

this method. MALDI-TOF high resolution tandem mass spectrometer was developed and it could generate MS/MS spectra of several peptides in a single acquisition. The *de novo* sequencing of synthetic peptide was observed at 10 fmol level (Medzihradszky et al., 2000). The results from these studies suggested that MALDI-TOF with post source decay is also capable of the *de novo* peptide sequencing with high sensitivity.

2.3.3 Protein identification by database searching

The accumulation of an exhaustive list of nucleotide and protein databases allows the rapid characterization of proteins via mass spectrometry. The general approach is applicable to those proteins whose sequences are already known, and is based on the hypothesis that the primary structure of a protein is the direct product of genome transcription (Yates, Eng, McCormack, and Schieltz, 1995). The primary sequence of protein can be deduced from the knowledge of the organism's genome. The partial or complete amino acid sequence of the target protein can be used to search for determining a protein of identical or homologous sequence within protein and DNA databases (Henzel, Billeci, Stults, and Wong, 1993).

2.3.3.1 Protein identification by peptide mass fingerprinting

Protein identification by peptide mass fingerprinting (PMF) is based on the fact that the pattern of accurate masses of peptides derived from specific proteolysis constitutes the unique mass fingerprint for specific protein (Fabris et al., 1995). A mass fingerprint is obtained from a protein which is enzymatically degraded by a sequence specific proteinase, such as trypsin. Mass profile of peptides is commonly performed using MALDI-TOF which shows higher sensitivity than ESI-MS without complication of multiple signals derived from each constituent (Jensen, Podtelejnikov,

and Mann, 1996). The set of mass is then compared to the theoretically expected tryptic peptide masses for each entry in database. The proteins can be ranked according to the number of peptide matches and more sophisticated scoring algorithms take the mass accuracy and percentage of the protein sequence covered into account and attempt to calculate a level of confidence for the match (Pappin, Hojrup, and Bleasby, 1993). Mass accuracy is the important parameter for protein identification and an increase in mass accuracy would therefore ensure the obtained results (Perkins, Papin, Creasy, Creasy, and Cottrell, 1999). The development of delayed extraction of TOF instrument yielded substantial improvements in mass accuracy (30 ppm) and this technique reduced the data acquisition and analysis time, providing high throughput analysis (Jensen et al., 1996). Park and Russel (2001) identified individual proteins including rabbit phosphorylase, bovine serum albumin, chicken egg avalbumin, rabbit aldolase, horse myoglobin, and chicken lysozyme from complex mixtures by peptide mass fingerprinting using high resolution MALDI-TOF. This indicated the capability of MALDI-TOF to identify proteins in mixture without separation. Bensalem et al. (2007) successfully identified membrane proteins, mitochondrial uncoupling protein 1 (UCP1). The results indicated the high sensitivity of MALDI-TOF to detect such a low content of membrane protein. However, the pitfall and limitation of mass mapping still exist. Protein modification could affect accuracy of the search since masses of modified peptides were different from those without modifications (Poutanen, Salusjarvi, Rouhonen, Penttila, and Kalkkinen, 2001).

2.3.3.2 Protein identification by peptide fragmentation data

Database can be searched by tandem mass spectrometric data because tandem mass spectra contain structural information related to the sequence of peptide,

rather than only mass (Eng, McCormack, and Yates, 1994). The protein is cleaved into smaller segments. Those peptides are fragmented to generate the pattern of fragment ions. The fragmentation pattern containing b- and y- series ions are considered to be un-interpreted product ions and these ions are matched with the patterns of fragment ions that are calculated from the database sequences using an algorithm, such as SEQUEST or MASCOT **(**Perkins et al., 1999). Carrera, Canas, Pineiro, Vazquez, and Gallardo (2007) introduced this search to identify the species-specific peptides from nucleoside diphosphate kinase B (NDK B) from 11 fish species belonging to the family Merlucciidae. By searching the MS/MS data on SEQUEST program, tryptic peptides from NDK B showed 108 amino acid sequences whereas only 17 sequences of those were assigned by PMF. The study also indicated that protein identification by MS/MS search is a definitive method. MS/MS data also required lesser peptides for positive identification of prostanoid inducible proteins than did by PMF (Person et al., 2003).

2.3.4 Application of MS techniques in food protein research

Proteomic strategy and mass spectrometric techniques, such as the *de novo* sequencing, protein and peptide identification, have been introduced to use as a tool to investigate, identify and characterize food proteins in several research purposes.

Mass spectrometry was used for molecular weight (Mw) determination of proteins. Mw of rice prolamin was determined to be 15 kDa using MALDI-TOF (Losso, Bansode, Hiba, and Bawadi, 2003). Wang, Geil, Kolling, and Padua (2003) used MALDI-TOF to measure Mw of 3 fractions of commercial zein and reported to be 26.8, 24.1, and 23.4 kDa, corresponding to the deduced amino acid sequences of zein encoded genes. In contrast, zein fractions showed only 2 bands on SDS-PAGE

with Mw of 22 and 24 kDa. This method was also used to detect Mw of α-zein at 24.5 kDa and was used to monitor the purity of extracted zein (Cabra et al., 2005). These studies clearly demonstrated that MALDI-TOF method is superior to SDS-PAGE in having higher resolution and mass accuracy (Wang et al., 2003).

Peptide identification by mass spectrometry was also applied to identify the angiotensin I-conversting enzyme (ACE) inhibitory peptides from protein hydrolysates derived from soy protein isolate, caseinate, wheat protein, and milk whey proteins using soybean protease (protease D3), which is produced during germination of soybean (Kodera and Nio, 2006). The fractionated peptides from HPLC separation were analyzed by LC-MS/MS in order to obtain mass profile and amino acid sequence. The MS/MS spectra were searched to characterize the amino acid of those peptides from NCBI database through SEQUEST analysis software. The sequence of those peptides showed either similarity or homology to those reported to be ACE inhibitory peptides. The obtained sequences were used as candidate peptides to screen for the most active peptide and further peptide synthesis. LC-MS/MS was also used as a tool to monitor that those peptides were synthesized correctly. LC-MS/MS could determine the primary sequence of abundant seed albumin in cacao (Kochhar, Gartenmann, and Juillerat, 2000). The sequence was obtained by alignment of tryptic peptide sequences. Those sequences were derived from MS/MS spectra searching against Swiss-Prot database via PeptIdent software available at the www.expasy.org. The obtained sequences revealed that several varieties of cacao contain 2 variants of albumin.

Proteomics analysis was used to investigate the changes of proteins during postmortem stage of porcine muscle as related to tenderness development (Lametsch et al., 2003). The changes of 103 protein spots of the slaughter and 72 h postmortem

sample were observed on 2-demensional polyacrylamide gel electrophoresis (2D-PAGE). Tryptic peptides from each spot were analyzed by MALDI-TOF in order to identify the corresponding protein using PMF technique. The results clearly showed that postmortem degradation of myosin heavy chain (MHC) and actin is related to meat tenderness (Lametsch et al., 2003). In 2006, Morzel, Chambon, Lefevre, Paboeuf, and Laville applied MALDI-TOF and PMF to study the proteome of muscle proteins from rainbow trout after pre-slaughtering with different treatments. Proteins involved in energy-producing pathways and structural proteins were different between the rested and intensely active fish. Changes in desmin and MHC suggested the effect of pre-slaughter treatment on post-mortem muscle integrity. Proteomic and tandem mass spectrometry were applied to study the carbonyl derivatives of amino acids such as P, N, K, and T residues within muscle proteins from rainbow trout during frozen storage. Carbonyls can be formed from all reactive oxygen species (ROS), having potential to act as marker of muscle oxidation (Kjaersgard, Norrelykke, Baron, and Jessen, 2006). Several carbonylated proteins were identified by submitting LC-MS/MS spectra of tryptic peptide from each spot on 2D-PAGE. Those identified proteins were adenylate kinase, pyruvate kinase, actin, creatine kinase, tropomyosin, LC, and MHC. These proteins may be used as markers of protein oxidation and also provide mechanisms of muscle oxidation (Kjaersgard et al., 2006). Proteomic analysis of hen egg white was performed by Guerin-Dubiard et al. (2006) and 69 protein spots were identified. The well known proteins, such as ovalbumin, ovostransferrin, and ovoinhibitor, were identified by PMF using MALDI-TOF analysis, while LC-MS/MS was also performed to confirm the mass fingerprint for an unambiguous identification of the remaining 55 protein spots. Two functional protein families were firstly discovered in egg white, i.e.,

Tenp, a protein with strong homology with a bacterial permeability-increasing protein family (BPI), and VMO-1, an outer layer vitelline membrane protein (Dubiard et al., 2006). LC-MS/MS was also used to identify tryptic peptides from low density lipoprotein (LDL) from hen egg yolk after separation on SDS-PAGE (Jolivet, Boulard, Beaumal, Chardot, and Anton 2006). MS/MS spectra of each protein band were used to search for hypothetical proteins using SEQUEST algorithm against the NCBI database. Based on the search, an apovitellenin I in either monomer or dimer and 7 fragments of apolipoprotein B were also identified.

Detection and characterization of undesirable compounds and allergen in food products can be achieved using mass spectrometry. Kwon (2004) applied nano HPLC-MS/MS to generate profile of soluble proteins in Sauvignon Blanc wine. They identified 1, 5, 12, and 2 proteins from fungi, grapes, yeast, and bacteria, respectively. Tryptic peptides derived from digestion of the peanut allergen, Ara h 1, were also analyzed by LC-MS/MS (Shefcheck and Musser, 2004). Four specific sequences were used as biomarker sequences since they were not identified in any other known proteins. These results were used to develop the method for detecting allergic proteins in ice cream model. In addition, peanut protein in dark chocolate was also successfully identified using these biomarkers and the detection limit of LC-MS/MS methods was also determined (Shefcheck, Callahan, and Musser, 2006). These studies indicated that specific peptide identification by mass spectrometry can be a feasible approach for food allergen detection. For milk allergens, the most abundant casein is also considered to be the most allergenic. Marker peptides were analyzed from tryptic peptides derived from casein using nano-electrospray-quadruple TOF hybrid mass spectrometer (Weber, Raymond, Ben-Rejeb, and Lau, 2006). MS/MS spectra of all

tryptic peptides were identified using MASCOT search against peptide sequences in NCBI database. Two peptides, YLGYLEQLLR (m/z 634.2) and FFVAPFPEVFGK (m/z 692.8) derived from αS1-casein were found as markers and their MS/MS spectra were also used as fingerprint to increase the confidence in identification of these markers in the food matrix. Aluko, Reaney, McIntosh, Quellet, and Katepa-Mupondwa (2004) reported that calcium-soluble protein (CSP) isolated from yellow mustard seed meal exhibited higher solubility and emulsion formation ability in the presence of $CaCl_2$ compared to similar isolate from brown mustard or soybean seed meal. This protein may be used as an additive or emulsifier in calcium-rich food products. When CSP was characterized by MALDI-TOF and PMF using MS-Fit program of the ProteinProspector software, napins, the major allergenic protein, with the small peptide of 5 kDa were identified. Thus, the presence of allergenic peptide should be concerned in the application of CSP. MALDI-TOF was also used as a tool for detecting the allergenic proteins from commercial soy and sunflower lecithins (Martin-Hernandez, Benet, and Marvin-Guy, 2005). Those proteins were identified by PMF of tryptic peptide masses generated by MALDI-TOF. The results were confirmed by searching for proteins in NCBI using MS/MS spectra generated by ESI-MS. Those identified proteins belonged to 11S and 7S globulin fractions, which have been implicated as the allergens in soy proteins. These studies indicated that MS is a valuable tool to detect the allergens in food products.

Food protein modifications, such as glycosylation, oxidation, carbonylation, and covalent cross-linking, were investigated by mass spectrometry in food model and food stuff. Cross-linking in the Maillard reaction between cyclotene, a common flavor compound, and a protein model, ribonuclease (rNase), was demonstrated on ESI-MS

spectra by Gerrard, Fayle, and Sutton (1999). They found 3 molecules of cyclotene attaching into Schiff-base adduct by covalent cross-linking with Lys residues within rNase. ESI-MS was used to rapidly characterize 14 glycosylated forms of α-lactalbumin generated by heat treatment (Slangen and Visseer, 1999). Czerwenka, Maier, Pittner, and Linder (2006) used LC-MS/MS to demonstrate that lactosylation of α-lactalbumin and β-lactoglobulin resulted in the physicochemical changes e.g. charged state and hydrophobicity. The degree of glycosylation was also used as an indicator of extension of heat treatment. This was evident by glycosylation of milk products increased upon increasing in heat treatment (Czerwenka et al., 2006). Monaci and Hengel (2007) found that the structural changes of β-lactoglobulin in solution or milk sample resulted in higher degree of protonation during ionization process of ESI-MS, compared to those without heating (Monaci and Hengel, 2007). Modifications of α-lactalbumin and β-lactoglobulin, through the Maillard reaction in the presence of lactose were monitored by MALDI-TOF (Meltretter, Seeber, Humeny, Becker, and Pischetsrieder, 2007). The modification sites as well as further modifications were identified after digestion of those modified proteins with endoproteinase AspN prior to MALDI-TOF analysis. The mass difference of peptides derived from control and modified proteins revealed that modifications were lactulosyllysine, N-carboxymethyllysine, oxidation of lysine to aminoadipic semialdehyde, oxidation of Met to Met sulfoxide, cyclization of N-terminal Glu to a pyrrolidone, and oxidation of Cys to Typ (Meltretter et al. 2007). The results of these studies suggested that mass spectrometry can be used as a tool to monitor the modifications of nutritional proteins.

2.3.5 Characterization of TGases and cross-linking reaction by MS

Molecular weight of MTG from *Streptoveritcillium* sp. strain s-8112 was

measured to be 37869 ± 8.8 Da using ESI-MS (Kanaji et al., 1993). This value agreed with that deduced from the composition of 331 amino acids of the enzyme, suggesting the high accuracy of Mw determination by MS technique.

MALDI-TOF was used to study the conformational changes upon activation of Factor XIIIa using hydrogen/deuterium (H/D) exchange experiment (Turner and Maurer, 2002). The results showed that deuteration of the catalytic core (220-230) increased upon activation and the degree of deuteration depended on activation method. The barrel1 domain showed a decrease in H/D exchange upon activation by Ca^{2+} exposure but such exchange was not observed when the enzyme is activated by thrombin. The results also suggested the individual contribution of Ca^{2+} and thrombin to activate the enzyme. This research indicated that MS technique is the alternative method for observing isotope exchange, taking advantage over nuclear magnetic resonance (NMR) technique. NMR technique is not suitable to study the extremely large proteins because it generates many overlapping resonances that are difficult to interpret (Hoofnagle, Resing, and Ahn, 2003).

The ε-(γ-glutamyl)lysine isopeptide detection and identification can be achieved by either MALDI-TOF or LC-MS/MS. Kim and Yim (1999) observed the ε-(γ-glutamyl)lysine isopeptide at the m/z of 275.3 of MALDI-TOF spectra after exhaustive proteolytic digestion of cross-linked peptides. Moreover, mass spectra of digested cross-linking reaction also revealed that the presence of isopeptide cross-linking affected digestion ability of proteinases (Kim and Yim, 1999). Identification of ε-(γ-glutamyl)lysine isopeptide catalyzed by MTG in leguminous proteins using ESI-MS/MS was also reported (Schafer, Schott, Brandl, Neidhart, and Carle, 2005). Ion mass of ε-(γ-glutamyl)lysine isopeptide at m/z of 275 was identified by MS/MS

spectra, showing the specific fragments at m/z of 147 and 130. These masses are assigned to be the protonated lysine and deaminated lysine fragments, respectively. These fragmentation ions differentiated ε-(γ-glutamyl)lysine isopeptide from other dipeptide isomers, (H-Glu-Lys-OH) and (H-Lys(retro-Glu-H)-OH. These isomers most likely yield fragmentation masses at m/z of 240 and 128 by elimination of water from (H-Glu-Lys-OH) and ammonia from (H-Lys (retro-Glu-H)-OH, respectively. Due to the specific identification, additional chromatographic identification could be omitted (Schafer et al., 2005). Subsequently, quantitative analysis can be performed based on the peak area of specific retention time corresponding to ε-(γ-glutamyl)lysine isopeptide.

The extent of TGase mediated protein modification was analyzed by MS. MALDI-TOF was introduced to detect fluorescein-labeled proteins catalyzed by TGase. The mass of fluorescein labeled sample exhibited higher value than that value of unlabeled protein and the mass difference corresponded to the fluorescein mass (Taki, Shiota, and Taira, 2004). The different potential of β–lactoglobulin (BLG) isoforms to be modified with 6-aminohexanoic acid catalyzed by the action of MTG was demonstrated on mass spectra generated by MALDI-TOF (Nieuwenhuizen, Dekker, Groneveld, Koster, and Jong, 2004). MALDI-TOF/MS was also applied to quantify in vitro TGase-mediated hydroxylation of Gln residues in a Gln-rich protein, dB1 (Piersma et al., 2002). The results showed that approximately 70% of the 64 Gln residues of dB1 were modified after prolonged incubation with TGase and hydroxylamine. The incorporation of dansylcadaverine and dansyl-PGGQQIV peptide into tryptic peptides derived from staphylococcal fibronectin-binding protein (FnbA) by the action of human Factor XIIIa can also be detected by MALDI-TOF (Anderson

et al., 2004). The acyl donors, (Q-103, 105,783, and 830), and acyl acceptors, (K-157, 503, 620, 762), were identified by N- terminal amino acid sequencing of those peptides. These sites located in the solvent exposed surface or N- and C- terminal of the FnbA molecules, suggesting the effect of solvent accessibility on cross-linking reaction of Factor XIIIa. The probability of staphylococcal colonization and establishment of infection to human wound via cross-linking of FnbA to human extra-cellular matrix proteins were also discussed (Anderson et al., 2004). In addition, the results from these studies also suggested that MALDI-TOF is a suitable technique for determining the overall extent of TGase mediated protein modification whereas localization of modification sites might not be achieved.

ESI-MS has been a choice for localization of modification at the amino acid level. The modified dB1 was proteolytically digested and the peptides obtained were subjected to ESI-MS/MS analysis. The un-interpreted MS/MS spectra of non-modified peptides were used for searching to identify peptide by SEQUEST algorithm. Modification sites were obtained from the mass difference between modified and non-modified peptides. The results showed that non-modified Gln residues are flanked C-terminally by a Pro residue (Piersma et al., 2002). Mass spectrometry was also used as a tool to characterize the cross-linking between human tissueTGase (TG2) molecule and two immunodominant gliadin peptides via ε-(γ-glutamyl)lysine isopeptide (Fleckenstein et al., 2004). The acyl acceptor sites were identified using ESI-MS/MS analysis. The fragmentation patterns revealed that six K residues within TG2 molecule (K-444, 562, 590, 600, 649, and 677) could be an acyl acceptor for the reaction and these residues were cross-linked to Q-65 and 72 within gliadin. In addition, these K residues are remote from the Cys-277 at the active site. Thus, the transfer reaction of

acyl to amine might be the intermolecular reaction. RP-HPLC interfaced with ESI-MS/MS was also introduced to predict the susceptible glutamine to GTG TGase within gliadin peptides using MDC as an acyl acceptor (Mamone et al., 2004). The MDC-tagged peptides were selected by detecting the fluorescent peaks after separation by HPLC. MDC-tagged peptides were analyzed by ESI-MS/MS to obtain amino acid sequences and MDC modification sites from fragmentation patterns. The modified Gln residues revealed that MDC modification sites shared the consensus feature of Q-X-P, where X can be any amino acid. This consensus sequence would possibly be the recognition feature of TGase substrates, inferring the substrate specificity of tTGase. Deamination sites on peptide or recombinant proteins catalyzed by TGases were also investigated based on LC-MS/MS (Mazzeo et al., 2003). The results from these studies suggested that LC-ESI-MS is the powerful technique for identification and localization the protein modifications mediated by TGases. The kinetic analysis of the dual MDC incorporation into FibN peptide, EAQQIVM, was detected by LC-ESI-MS. The results revealed that the first MDC modification site retarded the rate of MDC modification at another site (Sato, Yamada, Shimba, and Takahara, 2000). However, acyl donor sites for either tissue TGase or MTG on fish muscle proteins determined by MS have not been documented.

Orru, Caputo, Amato, Ruoppolo, and Esposito (2003) used MALDI-TOF spectra and peptide mass fingerprinting (PMF) to identify acyl-acceptor and acyl-donor substrates for TGase in human intestinal cell line. For acyl donor identification, an affinity probe, 5-(biotinamido) pentylamine ($BPNH_2$), was used as an acyl acceptor for TGase. This probe was labeled into Gln residues within proteins in cell homogenates. The $BPNH_2$ tagged proteins were selectively purified by avidin affinity

column before separating on SDS-PAGE. Protein bands on SDS-PAGE were in-gel digested with trypsin and the mass profile of digested peptides were analyzed by MALDI-TOF. Those mass values were submitted to perform PMF by searching for redundant proteins within several web available search programs (Mascot at www.matrixscience.com, MS-Fit at prospector.ucsf.edu, and ProFound at prowl.rockefeller.edu). Fatty acid synthase was identified as an acyl donor with the highest score. For acyl acceptor identification, a biotinylated glutamine containing peptide (A25 peptide) was used instead of $BPNH_2$. Myosin heavy chain was also identified as an acyl acceptor at the top scoring (Orru et al., 2003). PMF was also reported to be the efficient tool for detection protein cross-linking and identification the modification site. Emanuelsson, Boros, Hjernoe, Boelens, and Hojrup (2005) applied PMF to detect the cross-linking of hexapeptides (GQDPVR and GNDPVK) to the small heat shock proteins (sHsps) catalyzed by guinea pig liver TGase using data from MALDI-TOF. Based on post-data acquisition improvement of analytical software, the results can be used to search for the novel TGase-susceptible sites within sHsps (Q31/Q27 in Hsp20 and HspB2) (Emanuelsson et al., 2005).

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CHAPTER III

Ca^{2+} AFFECTED PHYSICOCHEMICAL AND CONFORMATIONAL CHANGES OF THREADFIN BREAM MYOSIN AND ACTIN IN A SETTING MODEL

3.1 Abstract

The effect of Ca^{2+} on conformational and physicochemical changes of threadfin bream (TB) myosin and actin during incubation at 25 and 40 °C was investigated. Ca^{2+} ion at 10-100 mM induced the unfolding of myosin and actin as observed by an increase of surface hydrophobicity (S_o ANS). Circular dichroism spectra demonstrated that Ca^{2+} reduced the α-helical content of myosin and actin incubated at studied conditions. Myosin Ca-ATPase activity decreased at Ca^{2+} > 50 mM, indicating conformational changes of myosin head. Total SH groups decreased with an increased Ca^{2+} concentration, suggesting that Ca^{2+} promoted the formation of disulfide bonds particularly at 40 °C. Both hydrophobic interactions and disulfide linkages were involved in formation of myosin aggregates, and were enhanced by addition of 10-100 mM Ca^{2+}. Ca^{2+} induced conformational changes of TB myosin and actin incubated at 40 °C to a greater extent than at 25 °C.

Key words: Threadfin bream, myosin, actin, calcium, setting

3.2 Introduction

When fish muscle proteins are ground with 2- 4 % salt and pre- incubated at 4-40 °C for a period of time prior to heating, an increase in gel elasticity is observed. Such phenomenon is known as "setting" or "suwari" in Japanese (Lanier 2000). It has been generally accepted that setting is mainly attributed from the activity of endogenous transglutaminase (TGase), the Ca^{2+}-dependent enzyme (Kumazawa, Numazawa, Seguro, and Motoki, 1995). The enzyme catalyzes an acyl transfer reaction between γ-carboxy amide groups of glutamyl residues in proteins as the acyl donor and variety of primary amines as the acyl acceptor (Folk 1980). The formation of ε-(γ-glutamyl) lysyl isopeptide bonds between glutamine (acyl donors) and lysine (acyl acceptor) resulted in a covalent cross-linking of muscle proteins.

Addition of Ca^{2+} has been reported to improve textural properties of Pacific whiting, threadfin bream, and Alaska pollock surimi (Lee and Park 1998; Yongsawatdigul, Worratao, and Park, 2002). Gel enhancing effect is more evident when sample is subjected to setting. It has been typically believed that Ca^{2+} improves gel-forming ability of fish proteins by activating fish endogenous TGase (Lanier 2000). However, Ca^{2+} is also known as a destabilizing salt in the Hofmeister series (Baldwin, 1996). Binding of Ca^{2+} to proteins prevents the salt exclusion, resulting in a decrease of preferential hydration and destabilized structure (Arakawa and Timasheff, 1984). The effects of Ca^{2+} on structural changes of various proteins have been reported. Ca^{2+} solubilized rabbit myofibrillar proteins by salting-in effect (Taylor and Etherington, 1991). Tertiary and secondary structure of α-crystallin decreased in the presence of Ca^{2+} (Valle, Escribano, Perez, and Garriga, 2002). Moreover, binding of Ca^{2+} to β-lactoglobulin induced partial unfolding of β-lactoglobulin, which led to an

increased hydrophobicity during gelation (Jeyarajah and Allen, 1994). Therefore, Ca^{2+} could also have a direct effect on structure of muscle proteins, which could affect gelation during setting. The role of Ca^{2+} on such conformation changes of fish protein has not been thoroughly investigated.

Several studies have investigated the gelation of fish proteins during setting. Ogawa, Kanamaru, Miyashita, Tamiya, and Tsuchiya (1995) found that the unfolding of actomyosin as measured by a decrease in α-helicity was a pre-requisite to initiate setting of actomyosin. Hydrophobic interactions were also responsible for aggregate formation of cod and herring myosin during setting at 40 °C (Gill, Chan, Phonchareon, and Paulson, 1992). In addition, formation of disulfide bonds was noticed during setting of herring myofibrillar proteins (Chan, Gill, Thompson, and Singer, 1995). These studies suggested that other bondings, besides ε-(γ-glutamyl) lysyl isopeptide bonds, were involved in setting. However, the effect of Ca^{2+} on hydrophobic interactions and disulfide linkages of fish myosin and actin during setting have not been elucidated.

Threadfin bream (*Nemipterus* sp.*)* is the second largest resource used for surimi production, after Alaska pollock. Thailand is one of the major threadfin bream surimi producers in the world with an approximate annual production of over 80,000 metric tons. Despite its large production quantity and value, scientific information related to setting phenomenon is still limited. Understanding the role of Ca^{2+} ion on conformational changes of myosin and actin would be critical knowledge for improving textural properties of surimi gels from threadfin bream and other warm water species. Therefore, our objectives were to investigate the effects of $CaCl_2$ on

physicochemical and conformational changes of threadfin bream myosin and actin during incubated at 25 and 40 °C, typical setting temperatures of fish proteins.

3.3 Materials and methods

3.3.1 Fish sample

Threadfin breams (TB) (*Nemipterus bleekeri*) were caught off the Gulf of Thailand at Rayong province. Fish were immediately transported to Suranaree University of Technology in polystyrene boxes packed with ice. Fish were manually eviscerated upon arrival and kept on ice. Myosin preparation was carried out 24 h after catch.

3.3.2 Myosin preparation

Myosin was purified according to the method of Martone, Busconi, Folco, trucco, and Sanchez (1986) with slight modifications. All steps were performed at 0-4 °C to minimize proteolysis and protein denaturation. TB mince was added with 10 volumes of buffer A (0.10 M KCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.02 % NaN_3 and 20 mm Tris-HCl, pH7.5) and homogenized at 15,000 rpm for 2 min in a homogenizer (AM-8, Nihonseiki Kaisha, Ltd., Tokyo, Japan). The homogenate was stirred for 10 min and centrifuged at 1,000 × g (Sorvall RC-5C Plus, Dupont, Del., USA) for 10 min. The pellet was collected and washed with the same buffer twice. The washed pellet was subsequently extracted with 5 volumes of buffer B (0.45 mM KCl, 5 mM β-mercaptoethanol (BME), 0.2 M Mg $(CH_3COO)_2$, 1 mM ethylene glycol bis (2-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), and 20 mM Tris-maleate, pH, 6.8). Adenosine 5'-triphosphate (ATP) was added to a final concentration of 15 mM. The mixture was kept for 1 h with stirring on ice and then centrifuged at

10,000×g for 15 min. Pellets were collected for actin preparation. Twenty five volumes of 1 mM $NaHCO_3$ was slowly added to the supernatant and kept on ice for 30 min. The precipitate was recovered by centrifugation at 12,000×g for 15 min. The pellet was resuspended with 5 volumes of buffer C (0.50 M KCl, 5 mM BME, and 20 mM Tris-HCl, pH7.5) and homogenized for 30 s. The solution was kept on ice for 10 min and $MgCl_2$ was added to a final concentration of 10 mM. Myosin was obtained by ammonium precipitation at 40-50 % saturation. The myosin pellet was kept at -40 °C and used throughout the study. The purity of extracted myosin was estimated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with densitometric analysis (Lab works Version 4.0, UVB Ltd., New York, USA). Before use, myosin pellet was dissolved in 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0 and dialyzed against 100 volumes of the same solution. Dialyzed myosin was clarified by centrifugation at 10,000 × g for 15 min and used as myosin solution. Protein concentrations were determined by Lowry, Rosebrough, Farr, and Randall (1951).

3.3.3 Actin preparation

Actin pellet was added with buffer D (0.80 M KCl, 5 mM BME, 20 mM Tris-HCl, pH 7.5) and stirred for 30 min before centrifugation at 10,000×g for 15 min. The pellet was collected and added with 5 volumes of 2 mM $NaHCO_3$. The mixture was stirred on ice for 1 h and centrifuged at 75,000×g for 1 h. The supernatant was used as actin preparation. Actin was dialyzed against 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0. Dialyzed actin solution was concentrated by ultrafiltration using membrane with molecular weight-cut-off 10,000 Da (Viva flow 50, Vivascience Sartorius AG, Goettingen, Germany). Purity of actin was estimated using SDS-PAGE and densitometric analysis.

3.3.4 Turbidity measurement

Myosin (3 mg/mL) and actin (1.5 mg/mL) solutions were solubilized in 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0 containing 0, 10, 30, 50 and 100 mM $CaCl_2$. Turbidity at 25 and 40 °C were monitored at 350 nm using UV/VIS 916 spectrophotometer (GBC Scientific Equipment, Ltd., Victoria, Australia) equipped with temperature-controlled unit. Turbidity changes at 25 and 40 °C were monitored at each time interval for 4 and 2 h, respectively.

3.3.5 Aggregation of TB myosin and actin

Myosin (3.2 mg/mL) and actin (1 mg/mL) solutions containing 0-100 mM $CaCl_2$ were incubated at 25 and 40 °C for 4 and 2 h, respectively. Subsequently, samples were centrifuged at 78,000×g for 1 h (XL-100 Ultracentrifuge, Beckman instruments, Inc., California, USA) to precipitate large aggregates. Protein concentrations in supernatants were determined by dye binding method due to interference of $CaCl_2$ with Lowry method (Bradford, 1986). Bovine serum albumin was used as a standard. Remaining proteins (%) was expressed as a ratio of protein remained in supernatant at any $CaCl_2$ concentrations to that of sample without $CaCl_2$ at 4 °C.

3.3.6 Surface hydrophobicity (S_o)

Changes of S_o were monitored using 8- anilino-1-napthalenesulfonate (ANS) according to the method of Li-Chan, Nakai, and Wood (1985) with slight modifications. Myosin and actin were diluted to various protein concentrations: 0, 1.25, 0.25, 0.5 and 1 mg/mL in the presence of 0-100 mM $CaCl_2$ and incubated at 25 and 40 °C for 4 and 2 h, respectively. To 2.0 mL of diluted myosin and actin, 10 μL of 10 mM ANS dissolved in 20 mM Tris-maleate (pH 7.0) was added. Fluorescence

intensity (FI) was measured using a RF-1501 spectrofluorophotometer (Shimadzu, Kyoto, Japan) at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. Blanks were prepared as the same mixture without protein solution. The regression slope between FI and protein concentrations (%) was calculated as S_o ANS.

3.3.7 Total sulfhydryl groups (SH)

Total SH groups of myosin and actin were determined using 5, 5'-dithiobis (2-nitrobenzoic acid), (DTNB). Myosin (3 mg/mL) and actin (1 mg/mL) solutions containing 0, 10, 30, 50 and 100 mM $CaCl_2$ were incubated at 25 and 40 °C for 4 and 2 h, respectively. Then, 1.5 mL of 0.2 M Tris-HCl (pH 6.8) containing 8 M urea, 2 % SDS and 10 mM EDTA was added. Subsequently, 0.2 mL of 0.1% DTNB solution were added to all samples before further incubated at 40 °C for 15 min and absorbance at 412 nm was measured. Total SH content was calculated using molar extinction of 13600 M^{-1} cm^{-1} for myosin. Molar extinction of actin of 12508 M^{-1} cm^{-1} was obtained using standard L-cysteine. Blanks were performed without protein solution.

3.3.8 Ca-ATPase activity

Ca-ATPase activity of myosin was estimated using the method described by MacDonald and Laneir (1994). The reaction was carried out at 1.5 mg of myosin, 17 mM Tris-maleate, pH 7.0 at 0-200 mM $CaCl_2$ concentrations. The mixtures were incubated at 25 °C for 5 min. ATP was added to final concentration of 0.67 mM and samples were incubated for 10 min. To stop the reaction, chilled TCA was added to final concentration of 5 % and samples were centrifuged at 3,000×g for 10 min. The supernatant was collected for inorganic phosphate (Pi) determination using KH_2PO_4 as a standard. Ca-ATPase activity was expressed as μmole of Pi / mg protein/min at 25 °C.

3.3.9 Circular dichroism (CD)

The effect of $CaCl_2$ on secondary structural changes of myosin and actin were analyzed using CD measurement. Myosin and actin were dissolved in 0.6 M NaCl, 20 mM Tris-HCl, pH 7.0 due to strong UV absorption of Tris-maleate buffer. Myosin and actin solutions (0.25 mg/mL) containing $CaCl_2$ (0-100 mM) were incubated at 25 and 40 °C for 4 and 2 h, respectively. All incubated samples and samples at 4 °C were scanned at far UV (195-280 nm) using a JASCO PS-150J spectropolarimeter (Jasco spectroscopic Co, Ltd., Tokyo, Japan). The instrument was calibrated using (1*S*)-(+)-10-camphorsulfonic acid (CSA). The circular quartz cuvette (0.02 cm path length) was equipped with temperature-controlled unit. All samples were scanned with step resolution of 1 nm, speed 50 nm/min, accumulation for 3 times, respond for 2 s, 2 nm of band width and sensitivity of 50 mdeg. Molar mean ellipticity [θ] and α-Helical content from [θ] at 222 nm was calculated according to Ogawa, Ehare, Tamiya, and Tsuchiya (1993).

3.4 Results and discussion

3.4.1 Effect of $CaCl_2$ on aggregation of myosin and actin

Purity of myosin was about 90-91%. Four contaminat bands with Mw of 43, 37, 34, and 27 kDa were observed in myosin (Figure 3.1). The 43 and 37 kDa bands were possibly actin and tropomyosin, respectively. TB actin showed molecular weight of 43 kDa and exhibited high purity (97-98%). Ca-ATPase activity of purified myosin in the presence of 3.3 mM $CaCl_2$ was 0.220 μmole Pi/mg protein/min.

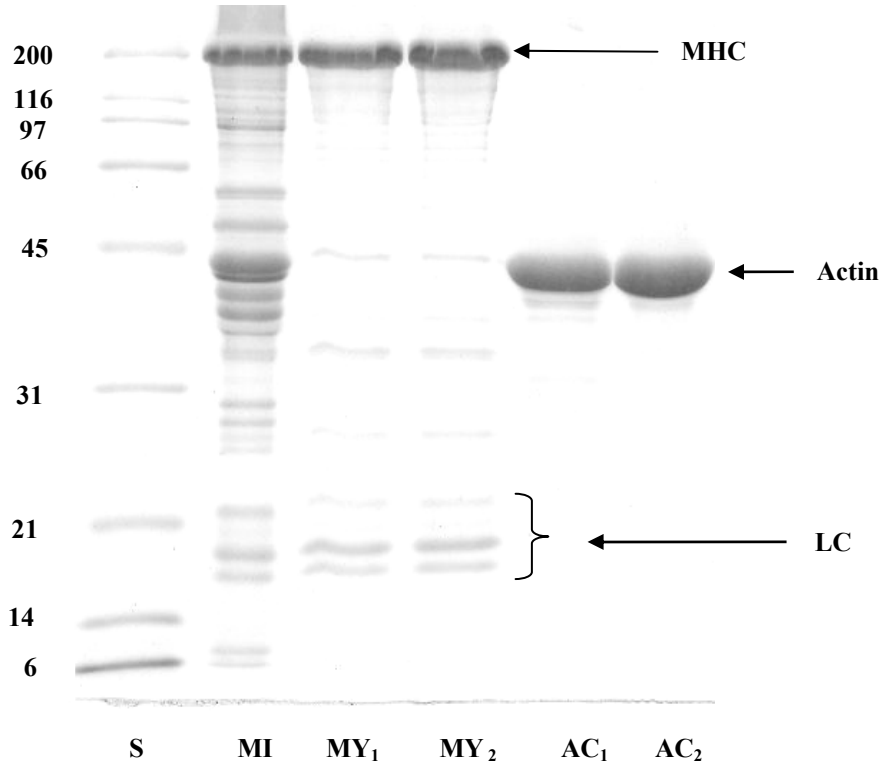

Figure 3.1 SDS-PAGE patterns of TB myosin and actin. S = molecular weight standard, MI = TB mince, MY_1 and MY_2 = myosin preparation lot 1 and lot 2, respectively. AC_1 and AC_2 = actin preparation lot 1 and lot 2, respectively. MHC = myosin heavy chain, LC = myosin light chains.

solution incubated at 25 °C for 4 h, while 100 mM $CaCl_2$ increased turbidity of myosin at 25 °C (Figure 3.2a). Gill et al. (1992) demonstrated that an increase in turbidity of heated fish myosin solution was the direct result of formation of myosin aggregates. Therefore, aggregation of TB myosin was enhanced at 25 °C in the presence of 100 mM $CaCl_2$. Moreover, aggregation of TB myosin occurred to a greater extent at 40 °C than at 25 °C (Figure 3.2b). Turbidity of actin solution incubated at 25 °C sharply increased with $CaCl_2$ concentration, especially at 50 and 100 mM $CaCl_2$ (Figure 3.2c). Aggregation of actin dramatically increased when incubated in the

presence of 10 mM $CaCl_2$ at 40 °C. However, a further increase of $CaCl_2$ from 30 to 100 mM did not increase actin aggregation (Figure 3.2d). Actin appeared to aggregate to a greater extent than myosin even at lower protein concentration. Large protein aggregates tend to precipitate under high centrifugal force. TB myosin incubated at 25 °C did not form large aggregates that could be precipitated under centrifugation at any studied levels of $CaCl_2$ (Figure 3.3a). In contrast, precipitation of myosin was observed when incubated at 40 °C and the extent of aggregation appeared to increase with $CaCl_2$ (10-100 mM). Based on turbidity results, TB myosin appeared to form soluble aggregate at 25 °C., while large aggregates were formed at 40° C. Since denaturation temperature (Td) of TB actomyosin was about 35 °C (Yongsawatdigul and Park, 2003), TB myosin subjected to 40 °C could unfold and re-associate to form aggregates. Addition of 10-100 mM $CaCl_2$ further promoted myosin aggregation. These myosin aggregates were unlikely to be resulted from the catalytic reaction of endogenous TGase because the enzyme was mainly removed during extensive washing and precipitation steps of myosin purification. The self-cross-linking ability of purified myosin was also tested and the cross-linked product was not found (data not shown).

Actin readily precipitated even at 4 °C without $CaCl_2$ (Figure 3.3b). The extent of actin aggregation also increased with temperature. Similar to myosin, actin aggregation was also enhanced by $CaCl_2$. The extent of aggregation monitored by ultracentrifugation corresponded with changes of turbidity. Moreover, aggregation of actin was completely attained when incubated in 10-100 mM $CaCl_2$ at 40 °C for 2 h (Figure 3.3b). These results indicated that Ca^{2+} induced aggregation of TB myosin and actin when incubated at 40 °C to a greater extent than at 25 °C.

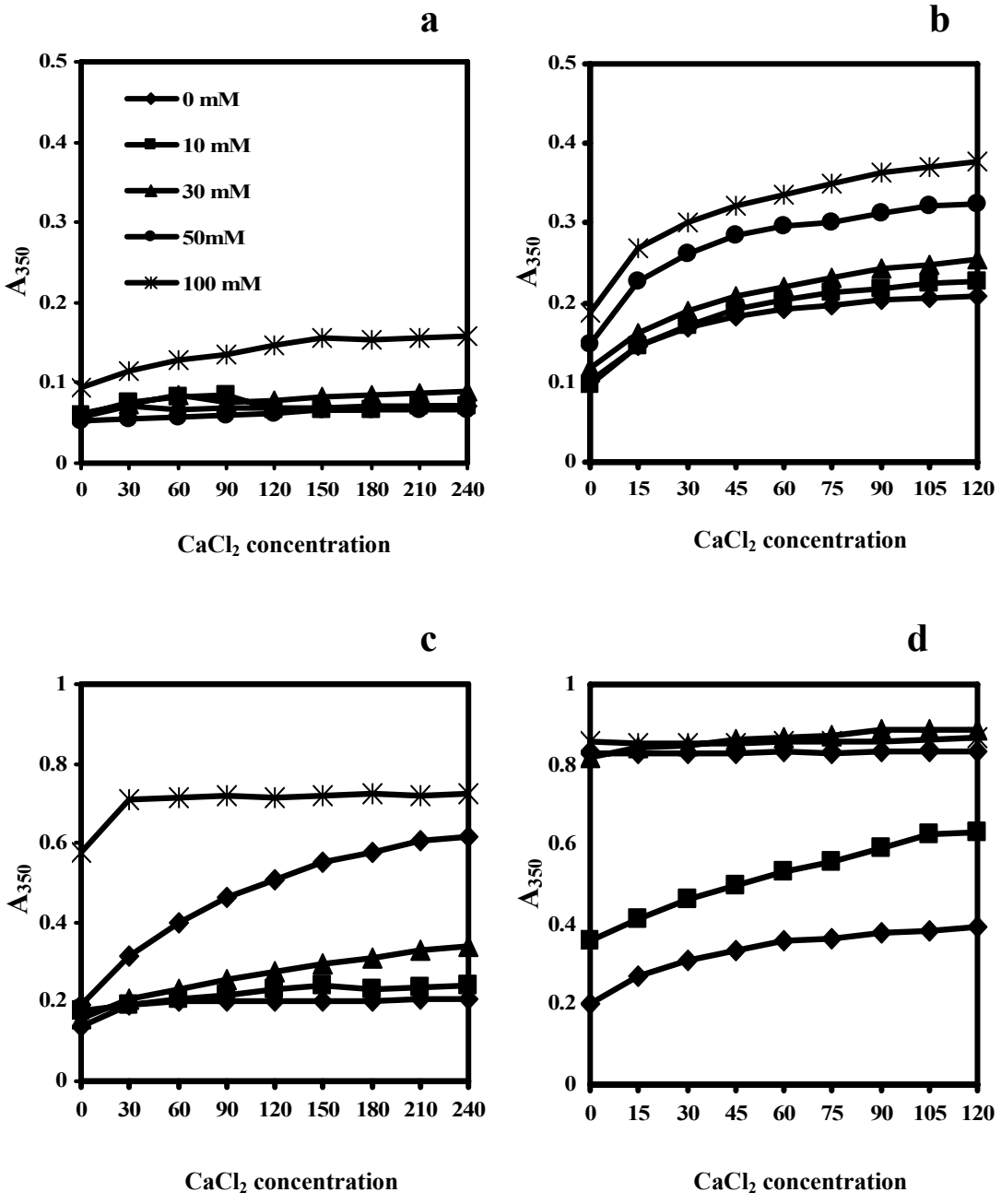

Figure 3.2 Effect of $CaCl_2$ on turbidity of TB myosin at 25 °C (**a**), myosin at 40 °C (**b**), actin at 25 °C (**c**), actin at 40 °C (**d**) in 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0.

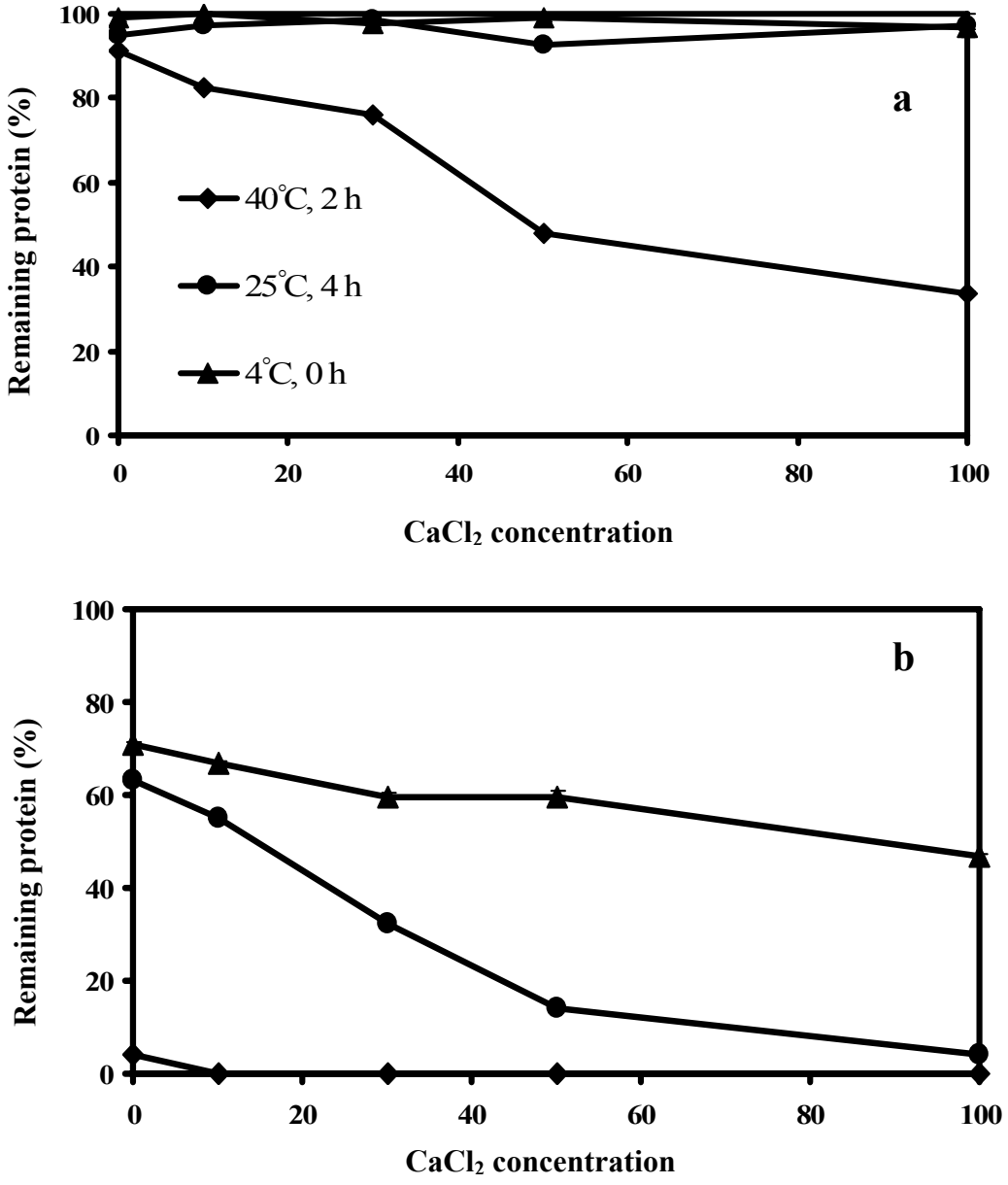

Figure 3.3 $CaCl_2$ induced a decrease of remaining proteins of TB myosin (**a**), and actin (**b**) after centrifugation at 78,000 × g for 1 h.

3.4.2 Effect of $CaCl_2$ on surface hydrophobicity (S_o ANS) of myosin and actin

S_o ANS of myosin slightly increased with $CaCl_2$ concentration at all studied

temperatures (Figure 3.4a), indicating that Ca^{2+} promoted the unfolding of myosin. It was noted that changes in S_o ANS of myosin incubated at 25 °C for 4 h were similar to those incubated at 40 °C, but higher than those at 4 °C (Figure 3.4a). These results indicated that incubation of myosin from tropical fish at 25 °C for long period (4 h) could induce the unfolding to the similar extent as those incubated at high temperature (40 °C) for shorter period (2 h). The partial unfolded molecules of myosin at 40 °C could subsequently re-associate together via hydrophobic interactions resulting in large aggregate formation at this temperature (Figure 3.4a). Lanier (2000) suggested that hydrophobic interactions participated in gelation during setting. Thus, Ca^{2+} ion induced the unfolding of myosin, which could in turn enhance hydrophobic interactions among myosin molecules during setting.

Although S_o ANS values of TB myosin incubated at 25 °C for 4 h at all studied $CaCl_2$ concentrations were similar to those incubated at 40 °C for 2 h, TB myosin only form soluble aggregates at 25 °C (Figure 3.3a). Incubation of myosin at 25 °C was far below Td of tropical fish myosin, which has been reported to be 37- 43 °C (Sano, Noguchi, Matsumoto, and Tsuchiya, 1990). Limited unfolding of myosin would restrict intermolecular entanglement via any interactions, resulting in formation of soluble aggregates, rather than large aggregates, when incubated at 25 °C.

S_o ANS of actin also increased with $CaCl_2$ concentration and exhibited higher values than those of myosin at all temperature studied (4, 25, and 40 °C) (Figure 3.4b). This may be partly due to the inactivation of actin by EGTA used in actin extraction (Turoverov, Biktashev, Khaitlina, and Kuznetsova, 1999). The inactivated actin tended to expose hydrophobic clusters on the surface and showed high affinity to hydrophobic probes (Lehrer, 1972). Moreover, existence of large hydrophobic groups on the surface

led to self-association of actin monomers (Mazhul et al., 2003). Thus, the greater extent of aggregation and exposure of surface hydrophobicity was observed in actin. Transformation of native G-actin to inactivated form resulted in partially unfolded structure, which was more prone to denaturation. Our study showed that Ca^{2+} induced more open structure of inactivated actin, leading to the aggregate formation via hydrophobic interactions.

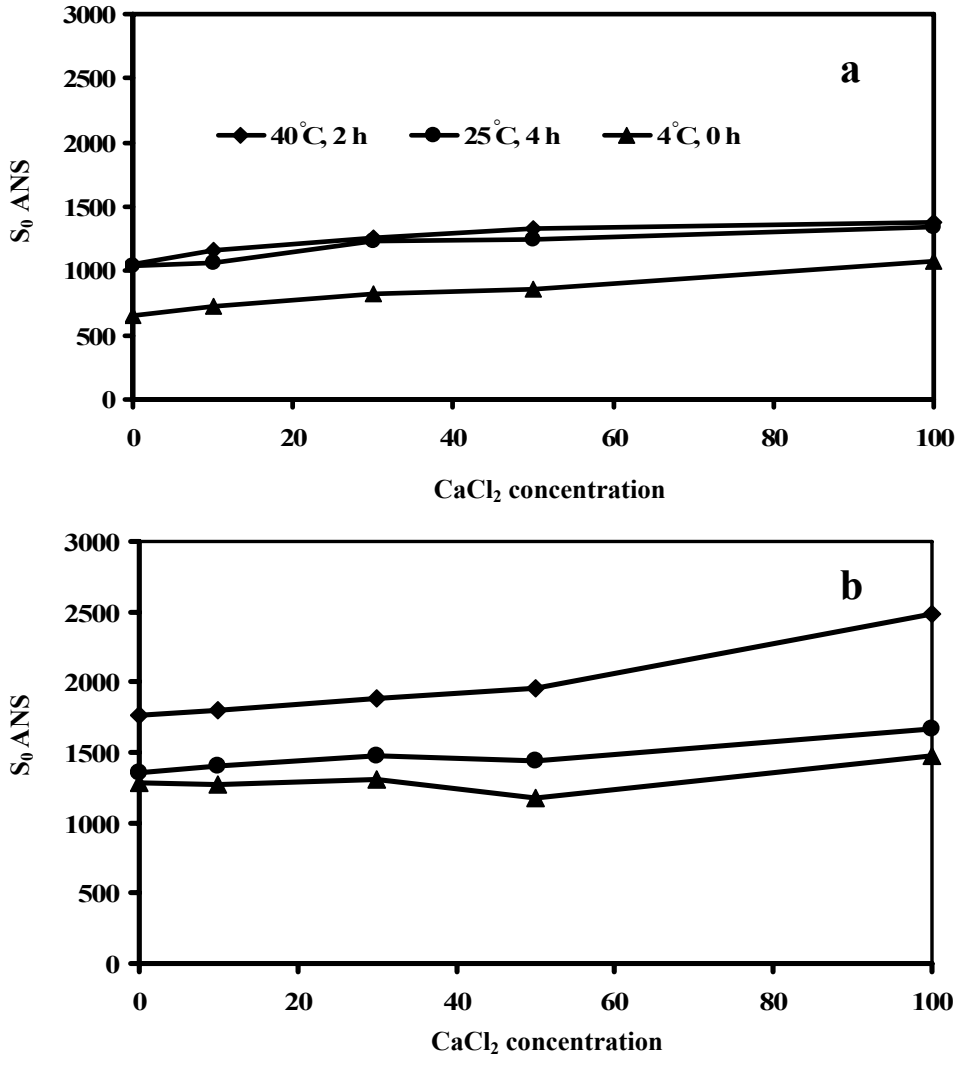

Figure 3.4 Effect of $CaCl_2$ on the changes in S_o ANS of TB myosin (**a**), and actin (**b**) in 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0.

3.4.3 Effect of $CaCl_2$ on total SH groups of myosin and actin

Total SH groups of myosin and actin in the absence of $CaCl_2$ were 6×10^{-5} and 5×10^{-5} mole /g protein, respectively, at 4 °C. When myosin was incubated at 40 °C for 2 h, total SH groups decreased to ≈ 4.8 x 10^{-5} mole /g protein as a result of thermal denaturation. In the presence of Ca^{2+} ion, SH groups of myosin incubated at all studied conditions continuously decreased as $CaCl_2$ concentration increased (Figure 3.5a**).** A marked decrease in SH group was observed when incubated at 40 °C for 2 h. The similar trend was also observed in actin (Figure 3.5b).

These results suggested that Ca^{2+} induced the formation of disulfide linkages of both myosin and actin when incubated at 40 °C. The unfolding of myosin and actin induced by $CaCl_2$ resulted in an exposure of free SH groups, which subsequently underwent disulfide interchanges. Similar effect of $CaCl_2$ on the formation of disulfide linkages and hydrophobic interactions were also found in α-crystallin molecules (Valle, Escribano, Perez, and Garriga, 2002).

It should be noted that the extent of disulfide bond formation of myosin at 40 °C was higher than that at 25 °C (Figure 3.5a). It was, therefore, speculated that disulfide bond might be partly responsible for aggregation of myosin set at 40 °C. Due to the lower extent of disulfide bond formation at 25 °C than at 40 °C whereas S_o ANS values of both samples were comparable (Figure 3.4a), TB myosin only formed soluble aggregates at 25 °C. Thus, disulfide bonds were also responsible for the formation of soluble aggregates of myosin after incubation at 25 °C for 2 h. Addition of $CaCl_2$ to fish protein paste induced hydrophobic interactions and disulfide linkages of myosin and actin at 40 °C to a greater extent than at 25 °C. Besides ε-(γ-glutamyl)

lysyl isopeptide bonds catalyzed by Ca^{2+}- dependent endogenous TGase, hydrophobic interactions and disulfide linkages could be involved during setting of fish protein.

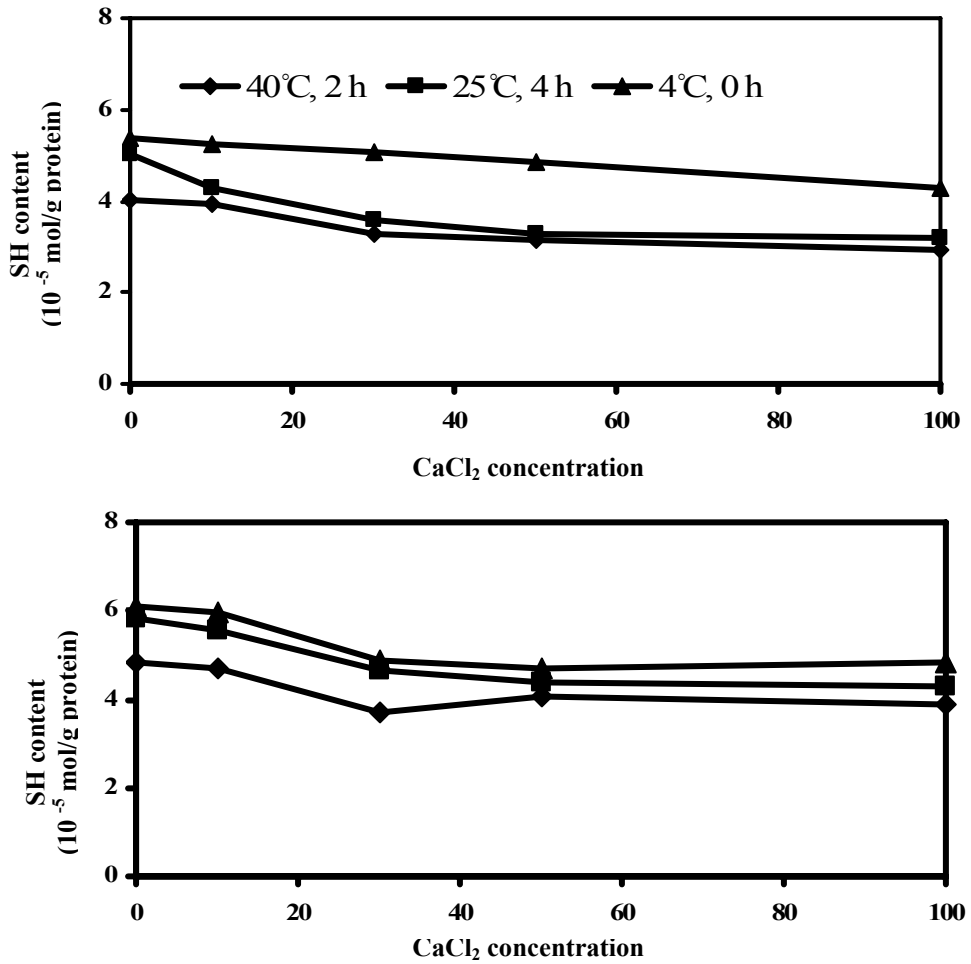

Figure 3.5 Effect of $CaCl_2$ on total SH content of TB myosin (a) and actin (b) in 0.6 M NaCl, 20 mM Tris-maleate, pH 7.0.

When Ca^{2+} ion was not added, setting phenomenon at 25 °C was not observed in surimi made from tropical fish (Kamath, Lanier, Foegeding, and Hamann, 1992; Klesk, Yongsawatdigul, Park, Viratchakul, and Virulhakul, 2000). The existing explanation was that tropical fish exhibited higher thermal stability that limited the exposure of reactive groups on myosin molecule for TGase catalytic reaction.

However, Yongsawatdigul et al. (2002) reported the setting of TB surimi at 25 °C when 0.1% $CaCl_2$ (≈ 10 mM) was added. Our study revealed that addition of Ca^{2+} ion (≥10 mM) increased more exposure of hydrophobic amino groups and more disulfide linkages of myosin and actin, which subsequently contributed to setting phenomenon of TB at 25 °C.

4.4 Effect of $CaCl_2$ on Ca-ATPase activity of myosin

Ca-ATPase activity of myosin slightly increased and reached the maximum at 30-50 mM $CaCl_2$ (Table 3.1). Further increase of $CaCl_2$ concentration dramatically reduced Ca-ATPase activity. Ca-ATPase activity at 200 mM $CaCl_2$ was about 36 % of that at 50 mM $CaCl_2$. High level of $CaCl_2$ (> 50 mM) induced conformational changes of globular head of myosin, resulting in a decrease of Ca-ATPase activity. The exposure of hydrophobic and changes of total SH groups at $CaCl_2$ < 50 mM was likely to occur at myosin rod, while both globular and rod portions underwent such changes at high $CaCl_2$ concentration (> 50 mM).

Binding of Ca^{2+} to anionic sites on protein structure can induce the unfolding. These binding interactions prevent salt exclusion from protein structure and decrease preferential hydration of salts, resulting in salting-in and destabilization of protein structure (Arakawa and Timasheff, 1984). Myosin contained negative charges at pH 7 because pI of myosin is around 4.8-6.2 (Stefansson and Hultin, 1994). Thus, the ionic interactions between Ca^{2+} and negatively charged myosin might be responsible for disturbance of native myosin molecules. Ca^{2+} also induced aggregation of β-lactoglobulin (theoretical net charge, Z = -8) by selective binding to carboxylated anions (Simons and others 2002). For myosin, most negative charges are located at myosin rod (Z = - 34 to -52) and followed by myosin light chains (Z = -6 to -27), while

globular head myosin has positive charges (Z = 6 to 16) (Bechet and Albis, 1989). Therefore, Ca^{2+} was more likely to bind to myosin rod than the globular head. For this reason, the rod portion was more susceptible to conformational changes induced by Ca^{2+}.

Table 3.1 Ca-ATPase activity of TB myosin determined at various $CaCl_2$ concentrations at 25 °C

$CaCl_2$ concentrations (mM)	**Ca-ATPase activity[a] (µmol Pi/mg protein/min)**
0	0
3.3	0.274 ± 0.025
10	0.297 ± 0.013
30	0.313 ± 0.023
50	0.299 ± 0.010
100	0.229 ± 0.011
150	0.185 ± 0.020
200	0.106 ± 0.019

[a] Mean and standard deviation values were from 2 measurements.

4.5 Effect of $CaCl_2$ on CD spectra of myosin and actin

CD spectra in far UV region of myosin showed predominant α-helix structure (Figure3.6a). $CaCl_2$ promoted the loss of secondary structure of both myosin and actin even at 4 °C (Figure 3.6a and b). The helical content of myosin at 4 °C without $CaCl_2$ was 71.2% and decreased to 51.4% in the presence of 100 mM $CaCl_2$ (Figure 3.7a).

The helical content of myosin incubated at 25 °C for 4 h was slightly decreased with increasing $CaCl_2$ concentration. In contrast, $CaCl_2$ markedly decreased helical content of myosin incubated at 40 °C for 2 h (Figure 3.7a). Both thermal energy and $CaCl_2$ synergistically contributed to unfolding of myosin at 40 °C, leading to considerable loss of helicalstructure.

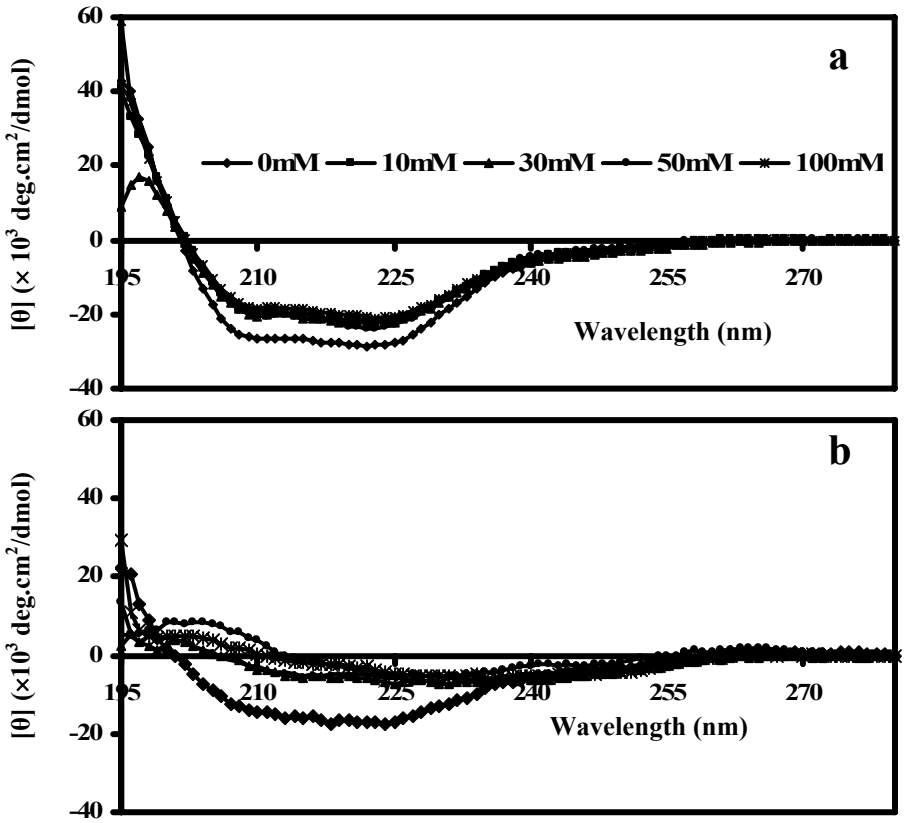

Figure 3.6 Effect of $CaCl_2$ on CD spectra of TB myosin **(a)** and actin **(b)** in 0.6 M NaCl, 20 mM Tris-HCl, pH 7.0 at 4 °C.

Ogawa et al. (1995) reported that loss of helical structure of fish actomyosin was a pre-requisite to initiate setting. Therefore, addition of $CaCl_2$ accompanied by incubating at 40 °C enhanced myosin unfolding, which subsequently resulted in a higher degree of hydrophobic interactions and formation of disulfide linkages. Low

helical content (28.73 %) was observed in actin at 4 °C (Figure 3.7b). Nagy and Goaszewska (1972) reported that actin contained 30% α-helix structure, 10% of β-sheet and the remaining residues did not appear to contribute to the optical activity.

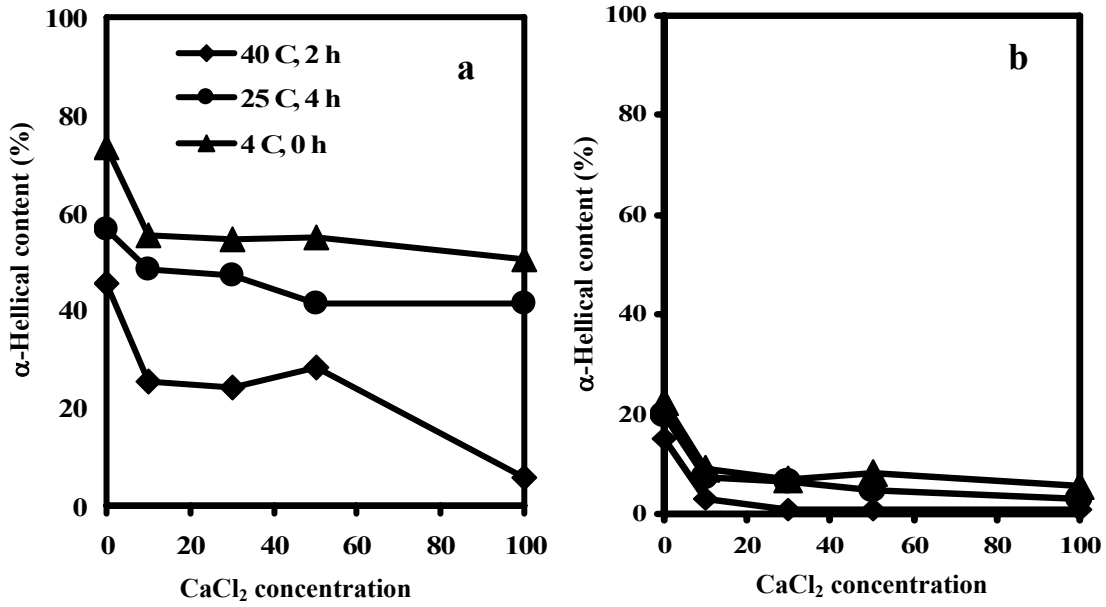

Figure 3.7 Effect of $CaCl_2$ on the changes in α-helical contents of TB myosin **(a)**, and actin **(b)**.

α-Helical content of actin decreased when incubated at 25 °C in the presence of 10 mM $CaCl_2$. However, further increase of $CaCl_2$ from 30 to 100 mM did not further decrease helical content of actin. Moreover, α-helical structure of actin incubated at 40 °C for 2 h was destroyed completely at10 mM $CaCl_2$. These results suggested that $CaCl_2$ at ≥10 mM also induced the changes of secondary structure of actin.

3.5 Conclusions

Ca^{2+} induced conformational changes of TB myosin and actin, leading to unfolding and exposure of hydrophobic amino acids. The unfolded molecules subsequently aggregated via hydrophobic interactions and disulfide linkages when incubated at either 25 or 40 °C. Such interactions could be important in gel-forming of TB during setting. Thus, $CaCl_2$ not only enhanced gelling properties of TB myosin through activating endogenous TGase but also directly induced conformational changes of myosin and actin, promoting hydrophobic interactions and disulfide linkages of "set" gel.

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CHAPTER IV

PARTIAL PURIFICATION AND CHARACTERIZATION OF TRANSGLUTAMINASE FROM THREADFIN BREAM LIVER

4.1 Abstract

Transglutaminase (TGase) from threadfin bream (TB, *Nemipterus* sp.) liver was partially purified using ion exchange, size exclusion and affinity chromatography. Three protein bands with molecular weight (Mw) of 95, 63 and 43 kilodalton (kDa) on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were observed. Only one distinct fluorescent band appeared on TGase activity staining using native-PAGE. Protein from the fluorescent band exhibited Mw of 95 kDa when it was eluted and analyzed on SDS-PAGE. The enzyme required Ca^{2+} up to 1 mM for full activation. TGase activity was markedly inhibited by sulfhydryl blocking reagents. Dithiothreitol had no effect on activity. TGase activity was unaffected by NaCl up to 0.6 M and reduced to 75% at 1.2 M NaCl. Optimal conditions of this TGase were at 50 °C and pH of about 8.5-9.0. The enzyme catalyzed cross-linking of TB myosin heavy chains.

Key words: Threadfin bream, fish liver transglutaminase, purification, protein cross-linking, myosin heavy chain

4.2 Introduction

Transglutaminases (TGases, EC 2.3.2.13) catalyze the protein cross-linking and amine incorporation by an acyl transfer reaction (Folk, 1980). The γ-carboxyamide group of glutamine residues in protein serve as an acyl donor and the amino group of primary amines or ε-amino group of lysine residues serve as an acyl acceptor. The reaction results in the formation of ε- (γ-glutamyl) lysine isopeptide bond, providing the cross-linked proteins or peptides (Folk and Finlayson, 1977). The enzymes can be found in various living tissues, such as microorganisms, vertebrates, invertebrates, and plants (Nozawa, Cho, and Seki, 2001; Greenberg, Birckbichler, and Rice, 1991). Formation of isopeptide bonds by the action of TGases improves functional properties of food proteins (Seki et al., 1990). It is generally believed that TGase plays an important role in cross-linking of fish muscle proteins pre-incubated at 25-40 °C, resulting in increased gel strength (Yongsawatdigul, Worratao, and Park, 2002).

TGases have been studied and isolated from several fish species. Biochemical characteristics of the enzyme showed the Ca^{2+}-dependent characteristic. Nozowa, Mamegoshi, and Seki (1997) characterized the partially purified TGases from various fish (carp, rainbow trout and atka mackerel) and found the different effect of salt on activity when compared with marine invertebrates (scallop, botan shrimp and squid). TGase from freshwater fish muscle was also purified from carp (Seki et al., 1990) as well as tropical tilapia (Warratao and Yongsawatdigul, 2005). Threadfin bream is the major resource for surimi production in Asia, especially Thailand and India. Pre-incubation of threadfin bream paste at 40 °C for 0.5-1 h, so called “setting”, was contributed from the catalytic activity of endogenous TGase in muscle (Yongsawadigul et al., 2002).

Besides muscle, liver is another good source of tissue TGase. Yasueda, Kumazawa, and Motoki (1994) purified TGase from red sea bream liver and demonstrated that it could catalyze cross-linking of Alaska pollack myosin heavy chain. The gelation of myosin B from walleye pollack was promoted in the presence of purified TGase from walleye pollack liver (Kumazawa, Nakanishi, Yasueda, and Motoki, 1996). In addition, the cloned liver TGase from red sea bream showed the similar catalytic activity to the wild type (Yaseuda et al., 1995). Thus, protein cross-linking activity of fish liver TGase could also be used to improve the textural properties of protein gels. Understanding biochemical characteristics of threadfin bream liver TGase is critical for application in food, especially for improving textural properties of food protein gel. In addition, a simple purification protocol could practically lead to a more efficient utilization of TB liver, which is currently discarded from surimi plant or being processed to a low value fish meal. Therefore, the objectives of this research were to partially purify TB liver TGase and to characterize its cross-linking abilities.

4.3 Materials and methods

4.3.1 Materials

Threadfin bream, TB (*Nemipterus* sp.) were purchased and transported from Rayong province to Suranaree University of Technology laboratory in a polystyrene box filled with ice within 10 h after catch. Fish were cleaned, vacuum-packed, and kept at- 40 °C throughout the study. Frozen TB was thawed and manually eviscerated. Fish liver was carefully collected using a scalpel and stored at – 40 °C for further TGase purification.

Monodansylcadaverine (MDC), *N,N'*-dimethylated casein (DMC), monoiodoacetic acid (IAA), *N'*-ethylmaleimide (NEM) and tosyl-L-lysine chloromethyl ketone (TLCK) were purchased from Sigma Chemicals (St Louis, MO). Ethylenediaminetetraacetic acid (EDTA) was purchased from Fluka BioChemica (Buchs, Switzerland). Dithiothreitol (DTT) was purchased from Promega (Madison, WI). Other chemicals and reagents used were of analytical grade.

4.3.2 Preparation of crude TGase

TB liver was homogenized with 3 volumes of extraction buffer (50 mM Tris-Cl (pH 7.5), 10 mM NaCl, 2 mM EDTA, 2 mM DTT, and 10 μM TLCK). EDTA and DTT were added to prevent metal-catalyzed oxidation of cysteine residue at the active site of TGase. Additionally, proteolytic activity during purification was minimized by the addition of TLCK. All steps were carried out at 4°C. The homogenate was centrifuged at 20,000 ×g (Sorvall RC 28S, Dupont Co., Wilmington, DE) for 30 min. The supernatant was collected and filtered through three layers of cheesecloth. Subsequently, the filtrate was centrifuged at 45,000×g for 1 h. The supernatant was used as crude TGase. Protein concentration of crude TGase was determined using the dye binding method (Bradford, 1976). Bovine serum albumin was used as a standard.

4.3.3 Determination of TGase activity

TGase activity was measured based on the incorporation of MDC into DMC according to the method of Takagi, Saito, Kikuchi, and Inada (1986) with slight modifications. The reaction mixture contained 1 mg/ml DMC, 15 μM MDC, 70 mM Tris-Cl (pH 7.5), 5 mM $CaCl_2$, 3 mM DTT and 100 μL of crude enzyme. The reaction was performed at 37°C for 10 min. EDTA was added to contain final concentration of 20 mM in order to stop the reaction. The fluorescence intensity of sample (FI_S) of the

reaction was measured at excitation and emission wavelength of 350 and 480 nm, respectively (RF-1501, Shimadzu, Kyoto, Japan). Blanks with inactivated enzyme (FI_b) were prepared with the similar fashion except that MDC was added after adding EDTA and the fluorescence intensity was immediately measured. Blanks without enzyme (FI_0) were also carried out by substituting crude enzyme with deionized water and fluorescence intensity was measured without incubation. Enhancing factor (EF) as described by Takagi et al. (1986) was determined to be 1.17 in this study. One unit of TGase was defined as the amount of enzyme that catalyzed 1 nmole of incorporated MDC into DMC within 1 min at 37 °C according to the equation:

$$TGase \quad activity \quad (U) = \frac{(FI_s - FI_b) \times 15{,}000 nmol \times 2ml}{10\,\text{min} \times 1.17 \times FI_0 \times 1000ml}$$

1 U = nmole of incorporated MDC/min.

4.3.4 Partial purification of TGase

4.3.4.1 DEAE-Sephacel chromatography

Crude extract was loaded onto a DEAE-Sephacel (GE Healthcare, Bio-sciences AB, Uppsala, Sweden) column (2.5×27 cm) equilibrated with 50 mM Tris-Cl (pH 8.0) containing 2 mM DTT, 2 mM EDTA, 10 μM TLCK (buffer A) and washed with the same buffer for 3 bed volumes at a flow rate of 1 ml/min. The bound proteins were eluted with linear gradient of 0-1 M NaCl in buffer A using BioLogic system equipped with a fraction collector (Bio-Rad Laboratories, Hercules, CA, USA). Fractions of 7 mL were collected for protein determination at 280 nm and for TGase activity. Fractions containing TGase activity were pooled and concentrated to 2 mL

using ultrafiltration with molecular weight cut-off (MWCO) 10 kDa (Vivaspin, Vivascience, Sartorius, Gottingen, Germany).

4.3.4.2 Hydroxyapatite chromatography

Diafiltration of the concentrated solution was carried out against buffer B (10 mM potassium phosphate (pH 7.2) containing 1 mM DTT and 1 mM EDTA) using ultrafiltration membrane with MWCO 10 kDa (Vivaspin, Vivascience, Sartorius, Gottingen, Germany). Subsequently, the sample was loaded onto the 5-mL hydroxyapatite column (CHT-II ceramic hydroxyapatite, Bio-Rad Laboratories, Hercules, CA, USA.) equilibrated with buffer B. After washing with buffer B for 5 bed volumes at a flow rate of 0.5 ml/min, the bound components were eluted using a linear gradient of 10 to 400 mM potassium phosphate, pH 7.2. Fractions of 5 mL were collected and determined for TGase activity. Fractions containing TGase were pooled and concentrated to 2 mL using ulfrafiltration membrane with MWCO 10 kDa (Vivaspin, Vivascience, Sartorius, Gottingen, Germany).

4.3.4.2 Sephacryl S-200 chromatography

The concentrated sample obtained from hydroxyapatite column was applied onto a Sephacryl S-200 (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) column (1.5×80 cm) equilibrated with buffer A. A constant flow rate of 0.5 ml/min was maintained and 5 mL fractions were collected for 1.5 bed volumes. Absorbance at 280 nm and TGase activity were determined. The active fractions were pooled and concentrated to 2 mL using ulfrafiltration membrane with MWCO 10 kDa (Vivaspin, Vivascience, Sartorius, Gottingen,Germany).

4.3.4.4 Hi-trap heparin chromatography

The concentrated sample from Sephacryl S-200 was loaded onto a 5

mL heparin column (Hi-trap heparin, GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) equilibrated with buffer A. Unbound components were washed with the same buffer for 50 mL at the flow rate of 1 mL/min. Bound proteins were eluted using a linear gradient of buffer A containing 0 - 1 M NaCl at the same flow rate. The active fractions were pooled and exchanged buffer against 50 mM Tris-Cl (pH 8.0) containing 1 mM DTT and 1 mM EDTA using ulfrafiltration membrane with MWCO 10 kDa (Vivaspin, Vivascience, Sartorius, Gottingen, Germany). Purity of TGase at each step was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% acrylamide stained with silver nitrate.

4.3.5 Activity staining

The fluorescent activity staining of TGase was conducted according to the method described by Stenterg and Stenflo (1979) with slight modifications. The purified enzyme was loaded onto the native gel containing 4 and 7.5 % acrylamide for stacking and separating gel, respectively. The gel was electrophoresed at constant voltage at 95 V at 0 - 4°C until complete separation and washed with cold water for 10 min. The washed gel was immersed in a substrate solution containing 5 mg/ml DMC, 2 mM MDC, 70 mM Tris-Cl (pH 7.5), 5 mM $CaCl_2$ and 5 mM DTT and incubated at 37°C for 2h. Subsequently, the gel was transferred to 10 % TCA at room temperature and incubated for 2h with gentle shaking. Finally, 7.5% acetic acid was added to fix the protein on polyacrylamide gel and the fluorescent pattern of MDC-bound proteins was detected under ultraviolet illumination (Universal Hood S.N. 75S, Bio-Rad Laboratories, Segrate, Milan, Italy). The gel was also stained with Coomassie brilliant blue R-250 to obtain protein patterns.

4.3.6 Molecular weight (Mw) determination

The protein band showing fluorescence under UV illumination was selectively cut using a clean razor blade and ground into smaller pieces. The cut samples were then filled into the Nanosep® MF centrifugal devices (Pall Life Science, Ann Arbor, MI) equipped with 0.2-μm membrane. The elution buffer (500 μL) containing 0.15 M NaCl, 0.25 M Tris-Cl (pH 7.5) and 0.1% SDS was added and thoroughly mixed. The mixtures were then centrifuged at 13,000×g until the filtrate was obtained. The protein extraction was repeated once and filtrates were collected and concentrated using Nanosep® centrifugal devices (Pall Life Science, Ann Arbor, MI) with 10-kDa-MWCO membrane. The concentrated samples were analyzed using 10-15% SDS-polyacrylamide gradient gel (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden). The wide range molecular markers (Sigma Chemicals, St. Louis, MO, USA.) were applied to estimate Mw of the enzyme.

4.3.7 Biochemical characteristics of partially purified TGase

4.3.7.1 Optimal Temperature

The effect of temperature on catalytic activity was conducted using the reaction mixture as described previously. Samples were pre-incubated for 5 min and assayed at various temperatures; 4, 20, 30, 40, 50, 60 and 65°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.

4.3.7.2 Optimal pH

The optimal pH was performed in various buffers: 100 mM sodium acetate (pH 6-7), 70 mM Tris-Cl (pH 7.5-9.0), and 50 mM glycine (pH 10-11). All reactions were carried out at 1 mg/ml DMC, 15 μM MDC, 5 mM $CaCl_2$ and 3 mM DTT at 37°C for 10 min.

4.3.7.3 Effect of $CaCl_2$

The effect of $CaCl_2$ was tested at 0-5 mM in the presence of 1 mg/ml DMC, 15 μM MDC, 70 mM Tris-Cl (pH 7.5) and 3 mM DTT and incubated at 37°C for 10 min.

4.3.7.4 Effect of DTT

The effect of DTT was measured at 0-10 mM in the presence of 1 mg/ml DMC, 15 μM MDC, 70 mM Tris-Cl (pH 7.5) and 5 mM $CaCl_2$ and incubated at 37°C for 10 min.

4.3.7.5 Effect of NaCl

The effect of NaCl was studied at 0-1.2 M NaCl in the presence of 1 mg/ml DMC, 15 μM MDC, 70 mM Tris-Cl (pH 7.5), 5 mM $CaCl_2$ and 3 mM DTT at incubated at 37C for 10 min.

4.3.7.6 Effect of ions

To investigate if other divalent ions were able to activate TGase, the assays were prepared by substituting $CaCl_2$ with either $SrCl_2$, $MgCl_2$ or $BaCl_2$ at 10 mM. The inhibitory effect of other metal ions, namely $MnCl_2$, $FeCl_2$, $CoCl_2$ and $CuCl_2$, were studied in the presence of $CaCl_2$. Concentration of all ions studied was 10 mM and the reaction contained 1 mg/ml DMC, 15 μM MDC, 70 mM Tris-Cl (pH 7.5) and 3 mM DTT.

4.3.7.7 Effect of inhibitors

The effect of EDTA, IAA, $(NH_4)_2SO_4$, and NEM on TGase activity was investigated at final concentration of 10 mM. The relative activity was calculated as the percentage of remaining activity in the presence of 1 mg/ml DMC, 15 μM MDC,

70 mM Tris-Cl (pH 7.5) and 5 mM $CaCl_2$. Activity of the sample without inhibitor was considered to be 100 %.

4.3.8 Cross-linking of TB myosin catalyzed by partially purified TGase

The cross-linking reaction was conducted at a ratio of partially purified TGase to myosin of 2.5 U: 1 mg in 50 mM Tris-Cl (pH 8.0) containing 5 mM DTT. The effect of $CaCl_2$ on myosin cross-linking was studied at 0, 5, 30 and 50 mM $CaCl_2$. When the reaction time of 1 h at 40 °C was attained, the equal volume of 10% SDS was added to stop the reaction. Subsequently, all samples were boiled for 10 min and centrifuged at 10,000 × g for 10 min. The extent of myosin cross-linking was analyzed using 10-15% SDS-polyacrylamide gradient gel (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden).

4.4 Results and discussion

4.4.1 Partial purification of TGase

Chromatographic profiles of TB liver TGase at various purification steps are shown in Figure 4.1. Purification fold increased after anion exchange chromatography (Table 4.1). Although Ca-hydroxyapatite removed protein contaminants, a decrease in total TGase activity was observed (Table 4.1). Ca-hydroxyapatite resin was successfully applied to purify TGase from squid gill (Nozawa, Cho, and Seki, 2001). Protein separation on hydroxyapatite is based on the competition for calcium ion on the resin surface. Bound proteins are selectively eluted by higher concentration of phosphate buffer. Since phosphate is able to chelate Ca^{2+}, higher concentration of phosphate might reduce available Ca^{2+} required for full activation of TGase. This would explain why TGase activity decreased after Ca-hydroxyapatite chromatography.

The results showed that Sephacryl S-200 effectively removed other protein contaminants as purification fold markedly increased (Table 4.1). In affinity chromatography, the sulfated glycosaminoglycan of heparin specifically binds to coagulating proteins, namely Factor IX, X and thrombin (Josic, Bal, and Schwinn, 1993). Heparin has been used to purify TGase from various aquatic species (Yasueda et al., 1994; Kumazawa et al., 1997; Warratao and Yongsawatdigul, 2005). It appeared to be effective for TB liver TGase (Table 4.1). Despite of several purification steps, TB liver TGase still contained 3 protein bands with molecular weight (Mw) of 95, 63, and 43 kDa under denaturing condition (Figure 2.2). SDS-PAGE without BME showed similar pattern (data not shown), suggesting that these 3 proteins were not composed of subunits linked by disulfide bonds. The fluorescent band observed on native-PAGE was resulted from MDC incorporation into DMC which was catalyzed by TGase (Figure 4.3a). Therefore, it also indicated the location of the enzyme. It should be noted that a single protein band was visualized on the native-PAGE (Figure 4.3b). When the protein was extracted from the native-PAGE and separated on the SDS-PAGE, a single band with Mw of 95 kDa was evident (Figure 4.3c). Thus, it was likely that Mw of TB liver TGase was 95 kDa. Native-PAGE separates protein based on charge density, rather than molecular mass. It could be presumed that protein with Mw of 43 kDa (Figure 4.2) possessed less charge density than the enzyme (95-kDa protein), and became less mobile under native-PAGE condition. However, 43-kDa protein was negatively charged in the presence of SDS and was separated from 95-kDa protein under SDS-PAGE due to differences in mass. Mw of TGase from scallop muscle and squid gill was reported to be 95 and 94 kDa, respectively (Nozowa, Cho, and Seki, 2001), which was similar to the estimated mass of TB liver TGase.

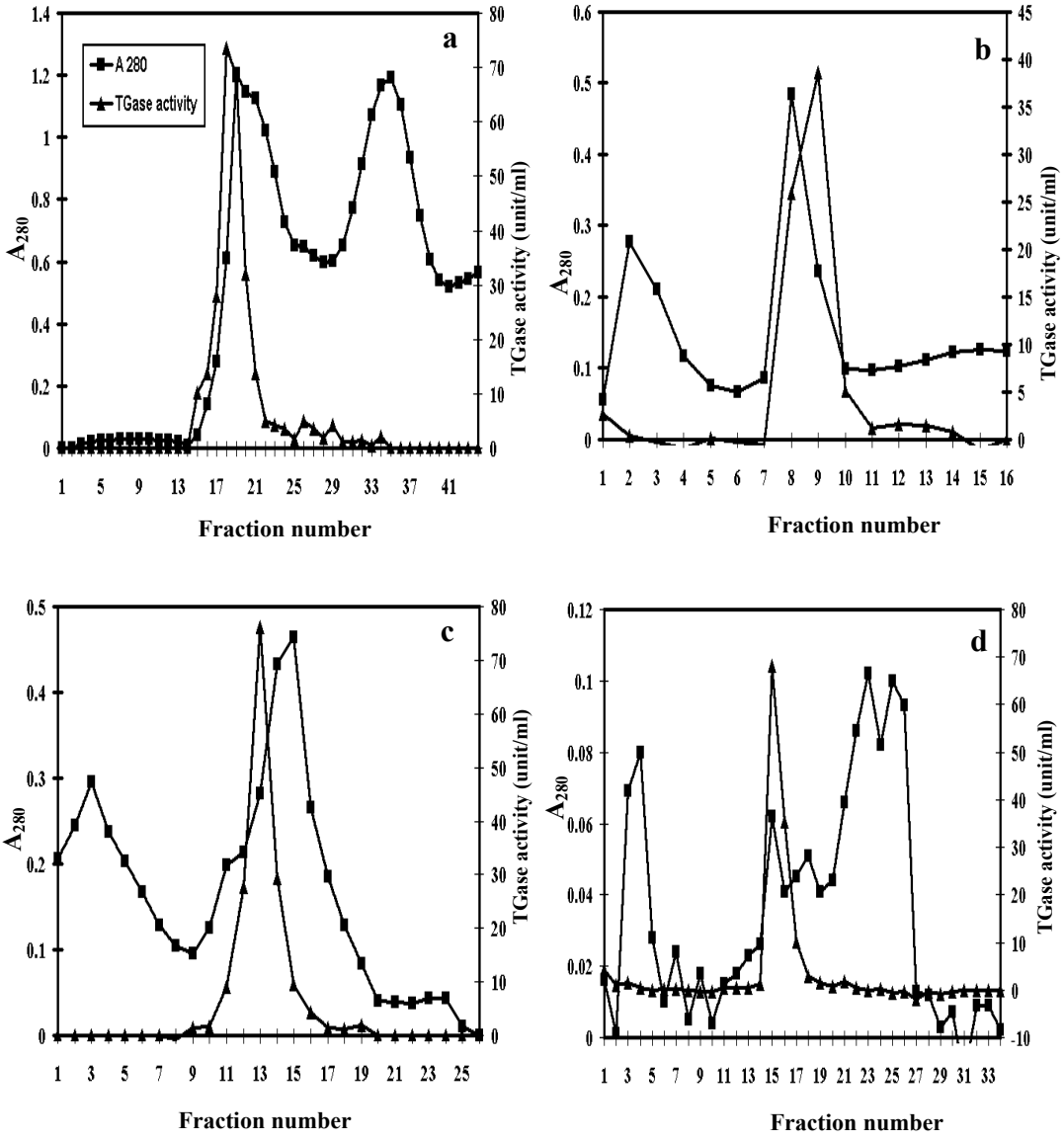

Figure 4.1 Chromatographic profile TB liver TGase purification on DEAE-Sephacel (**a**), hydroxyapatie (**b**), Sephacryl S-200 (**c**), and heparin (**d**).

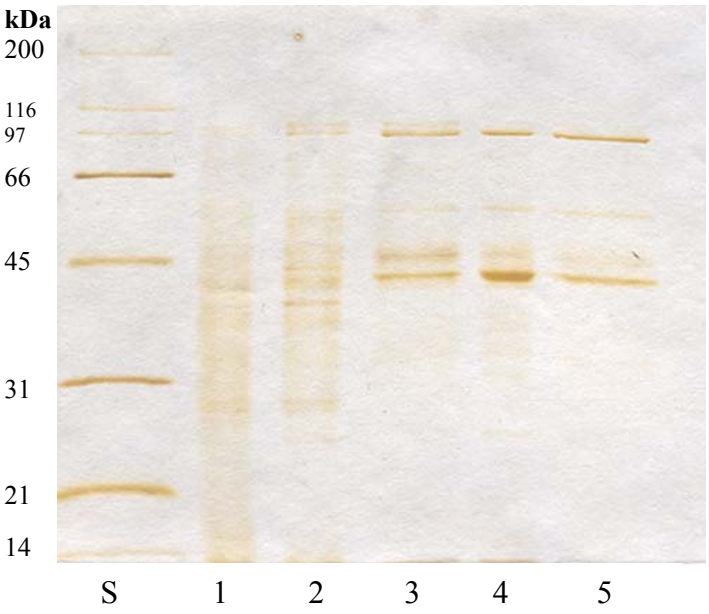

Figure 4.2 Protein patterns of partially purified TB liver TGase**.** S = Standard markers, 1 = Crude, 2 = DEAE Sephacel, 3 = Hydroxyapatite, 4 = S-200, 5 = Heparin.

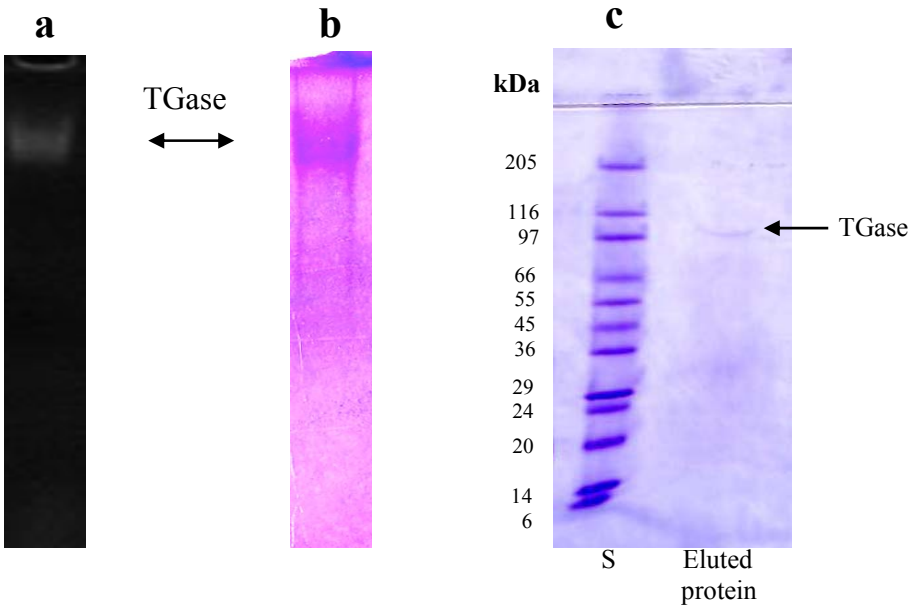

Figure 4.3 Activity (**a**) and protein (**b**) staining of TB liver TGase on native-PAGE and SDS-PAGE of protein extracted from native-PAGE **(c)**. S = Standard marker

Table 4.1 Purification table of TB liver TGase

Steps	Total protein (mg)	Total activity (U[a])	Specific activity (U/mg)	Fold	Yield (%)
Crude	134.70	6122.14	45.45	1.00	100.00
DEAE-Sephacel	20.16	1940.96	96.27	2.12	31.70
Hydroxyapatite	3.10	614.00	198.06	4.35	10.03
Sephacryl-200	0.23	451.00	1960.87	43.14	7.37
Hi-trap heparin	0.08	313.60	3920.35	86.28	5.12

[a]1 U = 1 n mole of incorporated MDC into DMC /min at 37°C.

4.4.2 Biochemical characteristics of partially purified TGase

4.4.2.1 Effect of $Ca^{2+}$and ions

The enzyme showed no activity in the absence of Ca^{2+} and activity increased with Ca^{2+} concentration and reached the maximum at 1-5 mM Ca^{2+} (Figure 4.4). These results indicated that partially purified TGase is Ca^{2+}-dependent. TGases from various sources require different Ca^{2+} concentrations for full activation. TGase from limulus hemocyte and guinea pig liver required 8 and 10 mM Ca^{2+}, respectively (Tokunaga et al., 1993). Red sea bream liver TGase needed only 0.5 mM of Ca^{2+} for full activation (Yasueda et al., 1994). Ahvazi, Kim, Kee, Nemes, and Steinert (2002) reported that 3 molecules of Ca^{2+} were required to activate human TGase 3, which is the cytosolic TGase expressed in stratified squamous epithelia. They also suggested that Ca^{2+} participated in maintaining appropriate conformation of TGase by enhancing coordination at the active site. It should be noted that Sr^{2+} activated TB liver TGase to a greater extent than did Ca^{2+} (Table 4.2).

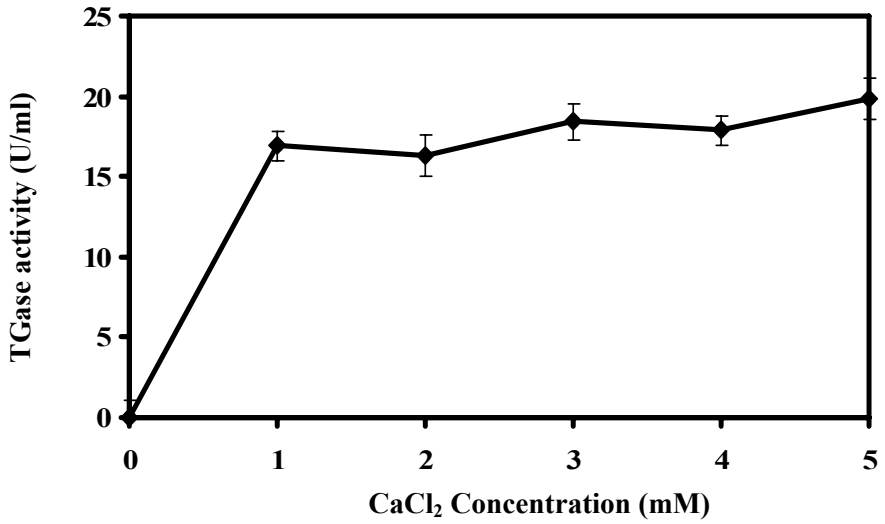

Figure 4.4 Effect of $CaCl_2$ on activity of TB liver TGase

Table 4.2 Effect of ions on activity of TB liver TGase

Ions	Remaining activity (%)
Control (Ca^{2+})	100.00 ± 1.30
*Mg^{2+}	35.75 ± 1.23
*Ba^{2+}	54.79 ± 1.40
*Sr^{2+}	159.80 ± 1.32
Mn^{2+}	76.77 ± 1.10
Cu^{2+}	0
Co^{2+}	0
Fe^{2+}	0

* The reactions were carried out without Ca^{2+}

The similar finding on the effect of Sr^{2+} was also reported in red sea bream liver and walleye pollack liver TGases (Yasueda et al., 1994; Kumazawa et al., 1996). In addition, replacement of Ca^{2+} by other ions (Er^{3+}, Sm^{3+}, $Tb^{3+}$and Lu^{3+}) could

recover the activity of human TGase 3 (Ahvazi et al., 2002). Thus, other appropriate ions could be used to activate the enzyme even though TGase showed a Ca^{2+}-dependent characteristic. Other divalent ions, Mg^{2+} and Ba^{2+}, could not replace Ca^{2+} for TGase activation (Table 4.2). Both Mg^{2+} and Ba^{2+} might not be able to induce a proper TGase conformation, resulting in reduced activity.

TGase activity was moderately inhibited by Mn^{2+} and completely inactivated by Cu^{2+}, Co^{2+} and Fe^{2+} (Table 4.2). Since these assays were conducted in the presence of Ca^{2+}, a decrease in TGase activity would indicate an apparent inhibitory effect of these ions. These results were in agreement with other TGases previously reported (Jiang and Lee, 1992). Those ions had strong affinity towards sulfhydryl group, suggesting that TB liver TGase could have a thiol group at the active site.

4.4.2.2 Effect of inhibitors

Calcium chelator, EDTA, inhibited TGase activity, confirming the Ca^{2+}-dependent characteristic. The enzyme was also completely inhibited by sulfhydryl alkylating agents, namely NEM and IAA (Table 4.3). These results strongly supported the assumption that TB liver TGase possesses thiol group at the active site. $(NH_4)_2SO_4$ is the well-known inhibitor, acting by product inhibition and drastically reduced activity of TB liver TGase. Basically, TGase reacts with γ-carboxyamide groups of glutamine and releases ammonia. The excess of ammonium ions prevent further progress of reaction involving in the formation of covalent bonds (Takagi et al., 1986).

4.4.2.3 Effect of DTT

DTT at concentration up to 10 mM did not affect TGase activity (Figure 4.5). The similar results were found in crude and purified TGase from tropical

tilapia (Worratao and Yongsawatdigul, 2005). It was speculated that the cysteine (Cys) at the active site was less susceptible to disulfide bridge formation. The crystal structure of red sea bream liver TGase indicated that disulfide bridges between the catalytic Cys-272 and Cys-333 was hampered by hydrogen bonding of Cys 272 and tyrosine (Tyr)-515 (Noguchi et al., 2001). Therefore, it is less likely for Cys-272 at the active site to undergo oxidation. This explains why DTT is not absolutely needed to maintain activity of the enzyme. However, DTT was needed to protect thiol group of the enzyme from oxidation when the covering Tyr was removed (Greenberg, Enghild, Mary, Dobson, and Achyuthan, 1998).

Table 4.3 Effect of inhibitors on activity of TB liver TGase

Inhibitors	Remaining activity (%)
Control (without inhibitor)	100.00 ± 1.37
IAA	1.74 ± 1.97
NEM	6.75 ± 1.54
EDTA	0.09 ± 1.19
$(NH_4)_2SO_4$	17.22 ± 1.59

4.4.2.4 Effect of NaCl

NaCl slightly reduced activity of partially purified TB liver TGase (Figure 4.6). The remaining activity of 75 % was found at 1.2 M NaCl. Nozawa et al. (1997) reported that 0.5 M NaCl had no effect on TGase activity from carp, rainbow trout and atka mackerel, while the enhancing effect of NaCl was found in marine

invertebrate, such as scallop, botan shrimp and squid TGases. NaCl also activated TGase from hemocyte and Japanese oyster (Kumazawa et al., 1997; Nozawa, Mori, Mimura, and Seki, 2005). TGase is secreted extracellularly when the muscle of marine invertebrate is injured in order to participate in wound healing process. Therefore, the enzyme from these sources is normally exposed to high NaCl content of the environment during its catalytic reaction. Unlike the enzyme from invertebrate, activity of TGases from marine fish was not activated by NaCl, but retained at higher NaCl concentration.

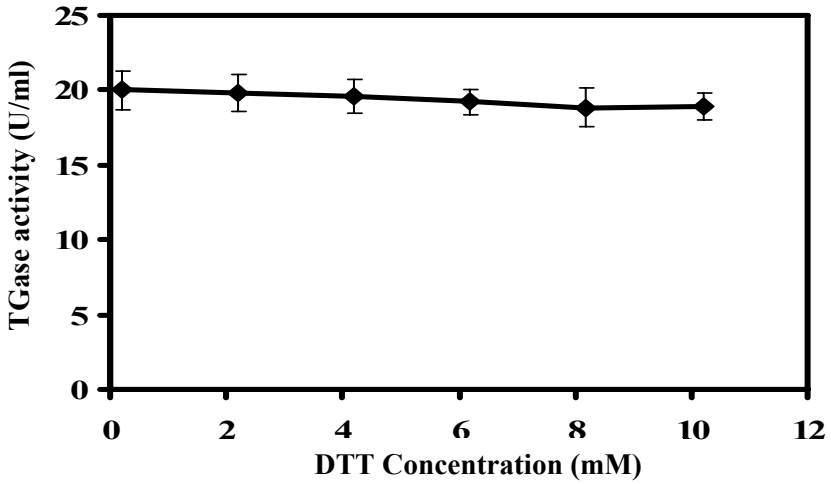

Figure 4.5 Effect of DTT on activity of TB liver TGase.

TGase activity of tropical tilapia, a freshwater fish, was markedly inhibited by NaCl (Worratao and Yongsawatdigul, 2005). The effect of NaCl on TGase activity seems to relate to environmental habitat of each species, which directly governs enzyme evolution. It should be noted that TGase activity of TB liver exhibits high activity at 0.6 M NaCl, which is the typical salt content (≈2% NaCl) used to solubilize muscle protein during preparation of fish protein gels (Lanier, 2000). Protein solubilization enables the exposure of available reactive amino residues for TGase.

These results indicated that TB TGase could be applied to food products containing NaCl up to 0.6 M without significant loss in activity.

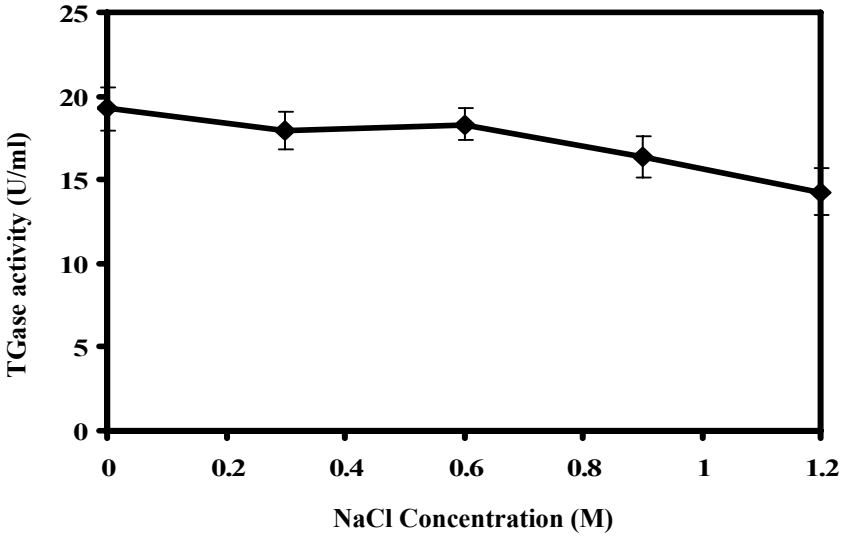

Figure 4.6 Effect of NaCl on activity of TB liver TGase.

4.3.2.5 Optimal pH and temperature

Optimal pH for the incorporation of MDC into DMC of TB liver TGase was 8.5 -9.0 (Figure 4.7). Relatively high pH optimum was also found in red sea bream TGase (Yasueda et al., 1994), walleye pollock liver TGase (Kumazawa et al., 1997). Optimum pH of fish liver TGase seems to be in the basic region. In contrast, pH optima of squid gill and tilapia muscle was 7.5-8.0 and 7.5, respectively. The optimal temperature of TB liver TGase was at 50 °C (Figure 4.8) similar to that reported in tropical tilapia (Worratao and Yongsawatdigul, 2005) and walleye pollock liver (Kumazawa et al., 1997). Slightly higher optimum temperature of 55 °C was reported in red sea bream liver TGase (Yasueda et al., 1994). It has been reported that optimum setting temperature of threadfin bream, a tropical fish, surimi is at 40 °C (Yongsawatdigul et al., 2002), while 4-25 °C is optimal for surimi made from Alaska

pollock and Pacific whiting whose habitat is cold and temperate environment, respectively (Lee and Park, 1998). Thus far, existing explanation of such a difference is the differences in thermal stability of muscle proteins. Muscle proteins of fish from tropical habitat require higher temperature to unfold and expose the reactive groups for both hydrophobic interactions and catalytic reaction of TGase. Our study suggested that higher temperature setting found in tropical fish could also be due to higher optimum temperature of endogenous TGase. Activity of the enzyme at 4-25 °C is lower than at 40 °C (Figure 4.8), resulting in a lesser extent of cross-linking reaction.

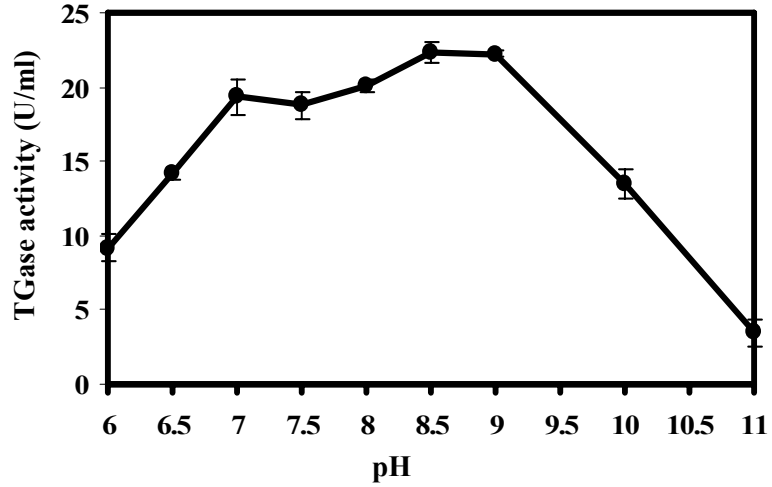

Figure 4.7 Effect of pH on activity of TB liver TGase

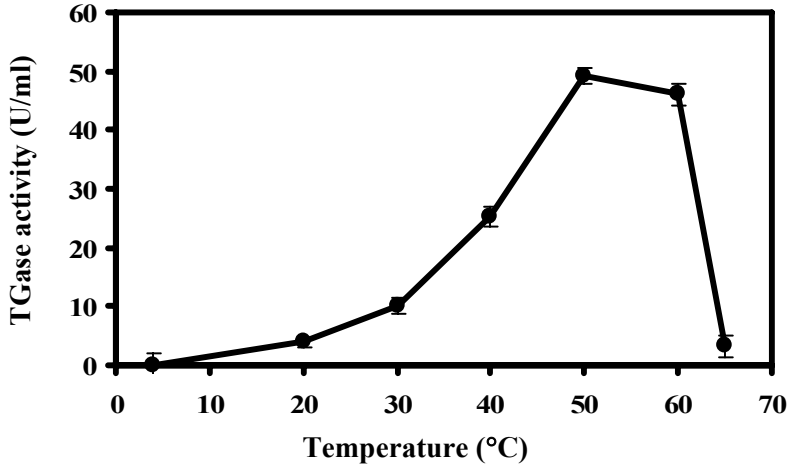

Figure 4.8 Effect of temperature on activity of TB liver TGase

4.4.3 Cross-linking of myosin catalyzed by partially purified TGase

Purified TB myosin was used as a substrate to investigate the cross-linking ability of the partially purified TB liver TGase. Cross-linked myosin appeared on the stacking gel in concomitant with a decrease of myosin heavy chains intensity (Figure 4.9). Myosin light chains were not affected even at relatively high $CaCl_2$ concentration of 50 mM. These results clearly demonstrated that only myosin heavy chain is a preferred substrate of TB liver TGase. These results agreed with those reported in red sea bream TGase (Yasueda et al., 1994), walleye pollack TGase (Kumazawa et al., 1996), tropical tilapia TGase (Worratao and Yongsawatdigul, 2005) and Japanese oyster TGase (Kumazawa et al., 1997). In addition, higher intensity of cross-linked products was observed in the presence of 30 and 50 mM $CaCl_2$ (Figure 4.9) in spite of only 1 mM $CaCl_2$ was needed for full activation of TGase (Figure 4.4). The previous study indicated that 10-100 mM Ca^{2+} induced conformational changes of TB myosin during incubation at 40 °C (Hemung and Yongsawatdigul, 2005). Such conformation changes could be critical for exposing reactive glutamine and lysine residues, which can be considered as a rate-determining step for TGase catalytic reaction. Thus, the optimal Ca^{2+} concentration for protein cross-linking mediated by TGase might not necessarily coincide with the optimal Ca^{2+} concentration for catalytic reaction of TGase. The effect of Ca^{2+} on conformation changes of myosin is also an important factor that should be taken into consideration in order to obtain the maximum cross-linked myosin.

According to our results, TGase from TB liver effectively catalyzed the cross-linking of myosin heavy chain, indicating its potential in the application of fish protein gel improvement. Nowadays, microbial TGase (MTG) has been commercially viable

and widely used for the same purpose. The optimum temperature of TB liver TGase was also similar to MTG at 50 °C (Ando et al., 1989). However, MTG activity decreased gradually after 50 °C, while that of TB liver TGase still remained at high level (≈ 90 %) until 60 °C. Moreover, TB liver TGase showed high activity (≥ 50 %) in a wide pH range (pH 6-10) rather than that MTG which was at pH 5-8 (Ando et al., 1989). Therefore, TB liver TGase could work comparably to MTG and might be better in terms of wider pH range and optimum temperature.

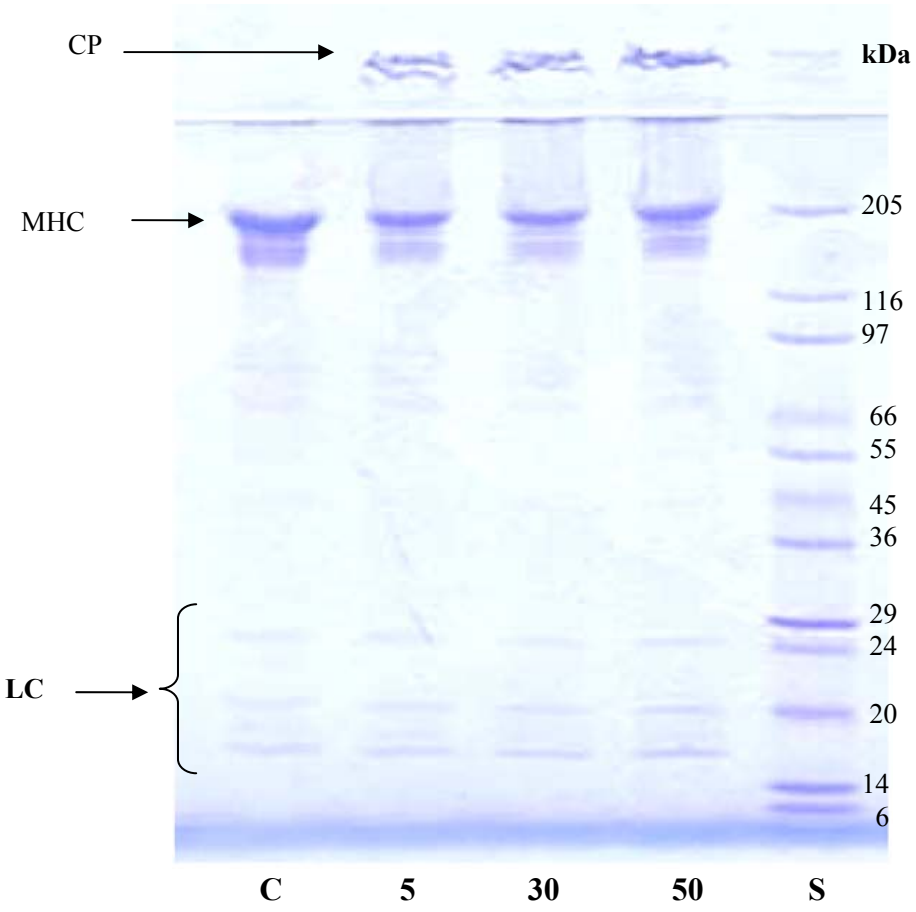

Figure 4.9 Cross-linking of TB myosin catalyzed by partially purified TB liver TGase. CP = Cross-linked products, MHC = Myosin heavy chain, LC = Myosin light chains, S = Standard markers. C = Control, The numbers indicate the $CaCl_2$ concentration (mM).

4.5 Conclusions

Partially purified TGase was obtained from TB liver and it showed Ca^{2+}-dependent characteristic. In addition, Sr^{2+} appeared to activate TGase activity by more than 50%. The enzyme activity was inhibited by metal ions (Mn^{2+}, Cu^{2+}, Co^{2+} and Fe^{2+}) and various TGase inhibitors. The optimum conditions for catalytic reaction were observed at 50°C and pH 8.5-9.0. The enzyme tended to retain activity at high salt concentration and it catalyzed cross-linking of TB myosin heavy chains.

4.6 References

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CHAPTER V

CROSS-LINKING REACTIVITY OF MICROBIAL AND FISH TRANSGLUTAMINASES TOWARDS NATURAL ACTOMYOSIN WITH DIFFERENT THERMAL STABILITY

5.1 Abstract

Changes in α-helical content determined by circular dichroism revealed that the transition temperatures of natural actomyosin (NAM) from Pacific whiting (PW) were at 31.8 and 43.1 °C, compared to at 35 and 49.3 °C for NAM from threadfin bream (TB). Endothermic transitions of PW-NAM measured by differential scanning calorimetry were at 31.8, 42.1, and 75.3 °C, while those of TB were at 36.1, 50.9, and 78.4 °C. PW-NAM unfolded greatly with a decrease in α-helical content and marked loss of Ca-ATPase activity after incubation at 25 °C, and the cross-linking of PW-myosin heavy chain (MHC) catalyzed by fish (FTG) and microbial (MTG) transglutaminases, TGases was observed. TB-NAM only slightly unfolded at 25 °C, resulting in less MHC cross-linking. A further unfolding of both NAM was observed at 40 °C. MTG catalyzed MHC cross-linking to the higher extent than did FTG resulting in more textural improvement of NAM gels. The unfolding of NAM played much more critical role for FTG in catalyzing protein cross-linking than MTG. The decreases in Ca-ATPase activity of TB- and PW-NAM after incubation at 25 and 40

°C was not changed by either TGase, suggesting that the cross-linking sites might not be at myosin head.

Key words: Threadfin bream, Pacific whiting, transglutaminase, natural actomyosin

5.2 Introduction

Natural actomyosin (NAM) is a major component responsible for gelation of muscle proteins. When fish NAM in the presence of salt is pre-incubated at the appropriate condition prior to heating, a stronger gel is formed. This process is typically known as "setting" (Lanier, 2000). One of the important mechanisms involved in the setting phenomenon is the formation of inter- and intra-molecular isopeptide bonds of NAM catalyzed by transglutaminases (TGases) (Kamath, Lanier, Foegeding, and Hamann 1992; Lee, Lanier, Hamann, and Knopp, 1997). The stability of NAM has been reported to be closely related to habitat temperatures (Ogawa, Ehare, Tamiya, and Tsuchiya, 1993). Muscle proteins extracted from fish living in tropical habitat, including tilapia, threadfin bream, and bigeye snapper unfolded at relatively high temperature and they can be set at about 40 °C (Klesk, Yongsawatdigul, Park, Viratchakul, and Virulhakul, 2000; Yongsawatdigul and Park, 2003; Howell, Matthews, and Donnelly,1991). In contrast, low setting temperatures at 4-25 °C are usually applied for muscle proteins from fish living at cold and temperate water temperatures, which contain low thermal stability, such as silver carp, Pacific whiting, walleye pollock, cod, and croaker (Imai, Hirayama, Kikuchi, Makinuma, and Watabe, 1997; Yoon, Kaninuma, Hirayama, Yamamoto, and Watabe, 2000; Togashi, Kanimuma, Nakaya, Ool, and Watabe, 2002; Kamath et al., 1992). Thermal unfolding

of NAM was reported to be a pre-requisite for setting (Ogawa, Kanamaru, Miyashita, Tamiya, and Tsuchiya, 1995). Kishi, Nozawa, and Seki (1991) observed that the reactivity of TGases toward NAM depended on the amount of reactive residues, which subsequently depend on conformational changes of NAM. The different thermal denaturation of NAM from fish living at different habitats would thus affect the reactivity of TGases. Catalytic reaction of TGase based on substrate with different thermal stability has never been systematically compared. Threadfin bream (TB) and Pacific whiting (PW) were selected as a representative of warm and temperate fish species, respectively, for NAM extraction. They are also important species for surimi production in Southeast Asia and North America, respectively. The effect of conformational changes of those NAM on the cross-linking ability of TGases was also investigated. These would lead to a better understanding of the effect of conformation of NAM on setting of fish proteins induced by TGases.

TGases generally catalyze an acyl-transfer reaction in which the γ-carboxamide groups of peptide-bound glutaminyl residues are the acyl donor. The lysine residues of proteins could act as acyl acceptors, resulting in the ε-(γ-glutamyl)lysine cross-links (Folk, 1980). TGase can be classified into Ca^{2+}-dependent and Ca^{2+}-independent types. Ca^{2+}-dependent TGases have been found in several tissues, including blood, plasma, and liver (Folk, 1977). TGases have been reported to play a role in food processing. Endogenous TGase from fish muscle proteins participated in polymerization of MHC during setting of surimi (Seki et al., 1990; Yongsawatdigul, Worratao, and Park, 2002). Besides muscle TGase, liver TGase from red sea bream (Yasueda, Kumazawa, and Motoki, 1994), walleye pollock (Kumazawa, Nakanishi, Yasueda, and Motoki, 1996), and TB (Hemung and Yongsawatdigul, 2007) could also catalyze cross-linking

of fish MHC. TB liver TGase is categorized as Ca^{2+}dependent TGase as muscle TGase. Its catalytic activity could explain cross-linking phenomenon during setting, which is normally catalyzed by muscle TGase. On the other hand, Ca^{2+}-independent TGases have been isolated from *Streptoverticillium mobaraense* (Ando et al., 1989) and *Streptoverticillium ladakanum* (Tsai, Lin, and Jiang, 1996), which are known as microbial TGase (MTG). MTG is commercially available and has been applied to increase the textural properties of surimi from some fish species (Ramírez, Santos, Morales, Morrissey, and Vázquez, 2000; Jiang, Hsieh, Ho, and Chung, 2000). However, MTG exhibits completely different characteristics from Ca^{2+}-dependent TGases, including molecular weight, crystal structure, active site arrangement, and catalytic mechanism (Kashiwagi et al., 2002). Substrate requirements between these types of TGase have been reported to be different (Ohtsuka, Ota, Nio, and Motoki, 2000). Cross-linking reactivity of MTG during setting of NAM from different habitats may also be different from Ca^{2+}-dependent TGase and should be elucidated. The results would lead to better understanding of the effect of different TGases on setting phenomena and provide critical information for setting of fish proteins from different habitat. Therefore, the objective was to compare the cross-linking of NAM from TB and PW catalyzed by TB liver TGase and MTG.

5.3 Materials and Methods

5.3.1 Materials

PW *(Merluccius productus)* were caught in July 15th and 25th, 2006 off the coast of Vancouver Island, British Columbia, Canada (around 48.5 °N and 124.7-125.5 °W). Fish were packed with ice in polystyrene foam boxes and transported to the

Food, Nutrition & Health Laboratory at the University of British Columbia, Vancouver. Tissue from the nape of the fish was removed to evaluate the level of infection by *Kudoa paniformis* and *Kudoa thyrsites* (Samaranayaka, Ho, and Li-Chan, 2006). The fish with undetectable infection were selected for this study and kept in vacuum-package at -20 °C until used of analysis. TB (*Nemipterus* sp.) was caught off the South China Sea and kept frozen at -20 °C until use.

Monodansylcadaverine (MDC), *N, N'*-dimethylated casein (DMC), phenylmethanesulfonyl fluoride (PMSF), and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemicals (St Louis, MO, USA). Dithiothreitol (DTT) was obtained from ICN Biomedicals (Aurora, Ohio, USA). BCA^{TM} Protein Assay Kit and bovine serum albumin (BSA) were purchased from Pierce (Rockford, IL, USA). The fluorescence probe, 6-propionyl-2-dimethylaminonaphthalene (PRODAN) was obtained from Molecular Probes (Eugene, OR., USA). Other chemicals and reagents used were of analytical grade**.**

5.3.2 NAM preparation

NAM from both TB and PW was prepared according to Ogawa et al. (1999) with slight modifications. The extraction was carried out at 0 - 4 °C to minimize protease activity. Fish muscle (10 g) was homogenized using an IKA homogenizer (Ultra Turrax T25 Basic Labortechnik, IKA Works, Inc., Wilmington, NC., USA) for 2 min with 10 volumes of low ionic strength buffer (20 mM Tris-Cl, pH 7.5 containing 50 mM NaCl and 0.05 mM PMSF). The homogenate was centrifuged at 5000 × g (Sorvall RC-5C Plus, Dupont, Wilmington, Del., USA) for 10 min before collecting the pellet. The process was repeated one more time. Subsequently, the pellet was homogenized with 100 ml of high ionic strength buffer (20 mM Tris-Cl, pH 7.5

containing 0.6 M NaCl) before centrifugation at 10,000 × g for 10 min. The supernatant was collected and diluted with 3 volumes of cold de-ionized (DI) water to precipitate NAM. The precipitates obtained by centrifugation at 10,000 × g for 10 min were dissolved in 20 mM Tris-Cl, pH 7.5 containing 0.6 M NaCl and the resulting preparation, referred to as NAM, was used within a week. Protein concentration of NAM was determined by the bicinchoninic acid (BCA) method with bovine serum albumin as a standard (Brown, Jarvis, and Hyland, 1989).

5.3.3 Thermal denaturation of NAM

5.3.3.1 Circular dichroism (CD)

NAM was dissolved in 0.6 M NaCl, 20 mM Tris-Cl, pH 7.5 and diluted to obtain final concentration of 0.05 mg/ml. CD spectra were measured using a J-810 spectropolarimeter (JASCO, Tokyo, Japan) equipped with a JASCO temperature control device (PFD-425S, JASCO,Tokyo, Japan). A quartz cell of 2 mm path length was used to measure the ellipticity at 222 nm$[\theta]_{222}$, with N_2 gas purging throughout the measurement. NAMs were heated from 10 to 80 °C at the heating rate of 1 °C/min. Molar mean ellipticities ([θ]) were calculated using a mean residue weight of 115 g/mol as described by Price (1996). ∝-Helical content (%) was estimated from $[\theta]_{222}$ according to the equation developed by Ogawa et al. (1993).

5.3.3.2 Differential scanning calorimetry (DSC)

DSC experiment was performed with a multiple cell differential scanning calorimeter model 4100 (Calorimetry Sciences Corp. Lindon, UT, USA). Approximately 0.5 g of NAM (15 mg/ml in 0.6 M NaCl, 20 mM sodium phosphate buffer at pH 7.5) was weighed into DSC ampoule, which was then sealed tightly with the lids to prevent evaporation during heating. The reference ampoule was filled with

buffer at the same weight. All ampoules were heated from 1-90 °C with a scan rate of 1 °C/min. The instrumental sensitivity was 10 μcal/ °C. The DSC data were analyzed using the software package, Origin, developed by MicroCal (MicroCal, Northampton, MA, USA) according to Nakaya, Kakinuma, and Watabe (1997).

5.3.4 Physicochemical changes of NAM after incubation

NAM from both species (1 mg/ml in 20 mM Tris-Cl, pH 7.5 containing 0.6 M NaCl) was incubated in the presence of 5 mM $CaCl_2$ at either 25 or 40 °C for 4 and 2 h, respectively, and then analyzed by SDS-PAGE. Those samples without $CaCl_2$ were analyzed as described below**:**

5.3.4.1 Protein patterns on SDS-PAGE

Protein patterns were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Sample was mixed with treatment buffer to contain final concentration of 2% SDS, 0.02%BME, and 0.01% bromophenol blue before boiling for 20 min. The boiled samples were centrifuged at 5,000 × g for 5 min. The soluble proteins in supernatant were separated on a PhastGel 10-15% gradient gel under reducing conditions using a PhastSystem^{TM} (GE Healthcare, Upsala, Sweden).

5.3.4.2 Changes in secondary structure analyzed by CD

The incubated NAM were diluted to 0.05 mg/ml with the same buffer and scanned at each incubated temperature from 195-300 nm at the speed of 100 nm/min using response of 2 s and the bandwidth of 1.5 nm. CD spectra of control (NAM without incubation) were scanned at 10 °C and the spectra were expressed as the averaged spectra from 3 measurements.

5.3.4.3 Changes in surface hydrophobicity

Surface hydrophobicity (So PRODAN) of NAM was determined according to Alizadeh-Pasdar and Li-Chan (2000) with slight modifications. PRODAN stock solution was prepared at 0.32 mg/ml in methanol and kept at -20 °C until use. The incubated samples and control (NAM without incubation) were diluted with buffer (20 mM Tris-Cl, pH 7.5 containing 0.6 M NaCl) to obtain the serial dilutions of 0, 0.0625, 0.125, 0.25, 0.5, and 1 mg/ml. To 2 mL of diluted protein solution, 10 μL of stock PRODAN was added, followed by incubation in the dark for 15 min. The fluorescence intensity (FI) of all samples was measured with a Shimadzu RF-540 spectrofluorometer (Shimadzu Corp., Kyoto, Japan) at excitation/emission wavelength of 365/445 nm with slits of 5 nm. FI of protein (without PRODAN) was also measured at each concentration, and subtracted from the FI of sample with PRODAN at each concentration to obtain the net FI. Surface hydrophobicity (S_0 PRODAN) was obtained from the regression slope of net FI versus protein concentration (%). S_0 PRODAN values of incubated NAM were compared to the control (without incubation) and expressed as the fold increase of S_0 PRODAN.

5.3.4.4 Changes in protein solubility

Soluble proteins of NAM were determined after incubating NAM (1 mg/ml in 20 mM Tris-Cl, pH 7.5 containing 0.6 M NaCl) at 25 and 40 °C for 4 and 2 h, respectively. All samples were centrifuged at 10,000 × g for 15 min before collecting the supernatant. The soluble protein content in the supernatants was determined by BCA method and expressed as percentage relative to the soluble protein content of the control NAM.

5.3.5 TGase preparation and activity assay

5.3.5.1 TGase preparation

Fish TGase (FTG) was obtained by partial purification from TB liver tissue as described in detail by Hemung and Yongsawatdigul (2007). In brief, the enzyme was purified using DEAE-Sephacel, hydroxyapatite, Sephacryl-200, and hi-trap heparin chromatographies. The partially purified TGase preparation was mixed with 20 % sucrose at a ratio of 1:1 before lyophilization. The lyophilized FTG was kept at -20 °C throughout the study. Lyophilized FTG was reconstituted with cold DI-water and was exchanged with a buffer (20 mM Tris-Cl, pH 7.5 containing 2 mM DTT) using a 10-kDa molecular weight cut-off membrane (Nanosep®, Pall Life Science, Ann Arbor, MI, USA). Microbial TGase (MTG), TG-K, from *Streptoverticilium mobaraense* was supplied by Ajinomoto Co., Inc (Tokyo, Japan). The enzyme was dissolved in DI-water to obtain final protein concentration of 5 mg/ml and was exchanged with the same buffer used for FTG.

5.3.5.2 TGase activity assay

TGase activity based on the incorporation of MDC into DMC was measured according to the method of Takagi, Saito, Kikuchi, and Inada, (1986) with slight modifications. For FTG assay, the reaction mixture contained 1 mg/ml DMC, 15 μM MDC, 70 mM Tris-Cl (pH 7.5), 5 mM $CaCl_2$, 3 mM DTT and 100 μL of enzyme. $CaCl_2$ was omitted in the reaction for MTG. After incubation at 37 °C for 10 min, ammonium sulfate was added to obtain final concentration of 42 mM in order to stop the reaction. The fluorescence intensity of sample (FI_S) was measured at excitation and emission wavelength of 350 and 480 nm, respectively, using a Shimadzu RF-540 spectrofluorometer (Shimadzu Corp., Kyoto, Japan). Blanks (FI_b) were prepared in a similar manner except that the enzyme was substituted with DI-water and FI was measured without incubation. TGase activity was defined as the amount of enzyme

that catalyzed the incorporation of 1 nmole of MDC into DMC within 1 min at 37 °C using enhancing factor of 3.2 and 1.5 for FTG and MTG, respectively.

5.3.6 Characterization of NAM after incubation with TGases

5.3.6.1 Ca-ATPase activity

Two hundred μL of NAM reaction mixture containing TGase was taken to determine residual Ca-ATPase activity according to Macdonald and Lanier (1994) with slight modifications. NAM solutions were pre-incubated at 25 °C for 5 min in the presence of 17 mM Tris-Cl, pH 7.5 and 3.3 mM $CaCl_2$. ATP was subsequently added to the final concentration of 0.67 mM before further incubation for 10 min. Chilled TCA was added at a final concentration of 5% to stop the reaction. After centrifugation at 3000 × g for 10 min, the supernatant was collected to determine inorganic phosphate (Pi) according to Black and Jones (1983) using KH_2PO_4 as a standard. Ca-ATPase activity was expressed as nmol of released Pi/mg protein/min at 25 °C.

5.3.6.2 NAM cross-linking

The cross-linking of NAMs was performed in the reaction mixture containing 3 mg/ml NAM in 0.6 M NaCl, 5 mM DTT, 5 mM $CaCl_2$, 20 mM Tris-Cl, pH 7.5, and 3 U TGase/ml. $CaCl_2$ was omitted in the MTG reaction. The enzyme was also replaced with DI-water in the control. All samples were incubated at either 25 or 40 °C for 4 and 2 h, respectively. Cross-linked proteins were determined by SDS-PAGE as described in actomyosin preparation (section 5.3.4.1).

5.3.7 Textural properties of NAM gels

NAM pastes containing final concentration of 123.2 ± 9.56 and 119.3 ± 2.40 mg/g for TB and PW, respectively, in 0.6 M NaCl, 20 mM Tris-Cl (pH 7.5) were prepared using mortar and pestle, before dividing into 3 portions. In the first portion,

$CaCl_2$ was added to obtain final concentration of 5 mM for the sample containing FTG. $CaCl_2$ solution was substituted with the equal volume of DI-water for the control and samples with added MTG. MTG and FTG were added to NAM pastes at 1 U/mg protein. The pastes were filled into a microplate with a diameter of 8 mm and 10 mm depth. The filled plates of PW-NAM were wrapped with a plastic bag and incubated at 25 °C for 4 h whereas those of TB-NAM were carried out at 40°C for 2 h. These temperatures are the optimal setting temperatures of each fish species. Samples were then heated at 90°C for 15 min before cooling at 4°C overnight. The breaking force and deformation were determined using a Texture Analyzer TA-XT2 (Stable Micro System, Surrey, England) equipped with a 3-mm diameter cylindrical probe at a test speed of 1mm/s. The average breaking force and deformation values were obtained from 7 measurements.

5.4 Results and discussion

5.4.1 Conformational changes of NAM

5.4.1.1 CD measurement

Changes of α-helical content of NAM upon heating are shown in Figure 5.1. α-Helical structure of PW-NAM started to decrease around 25 °C and showed the denaturation temperatures (Td) at 31.8 and 43.1 °C. TB-NAM exhibited higher thermal stability than PW-NAM as evident by higher Td at 35.0 and 49.3 °C. The changes in α-helical content were mainly attributed to the unfolding of myosin rod (Margossian and Lowey, 1982). Ogawa, Kanamaru, Miyashita, Tamiya, and Tsuchiya (1995) indicated that the most unstable portion of fish myosin rod was located at light meromyosin (LMM). Rogers, Karr, Biedermann, Ueno, and Harrington (1987)

reported that thermal stability of myosin rod shift along with the working temperature of their respective muscles. These studies led to speculate that myosin started to unfold at LMM region upon heating. The results from our study also confirmed that myosin rod of temperate water species was more thermally labile than that of tropical species.

5.4.1.2 DSC measurement

Transition temperature (Tm) and denaturation enthalpy values (ΔH_{cal}) of NAM measured by DSC are shown in Table 5.1. The multiple transition of NAM observed in this study corresponded to the changes in fish myofibrils as reported previously (Howell et al., 1991). Double transition of myosin from snapper and cod was also observed by Davies, Bardley, and Ledward (1988). Tm values of carp myosin acclimatized to 10 °C were found at 33.9 and 47.4°C and these values were also corresponded to denaturation of myosin rod as described by Nakaya et al. (1997). In addition, Td values of each NAM, representing the melting of helical structure (Figure 5.1), were similar to first two Tm values of respective NAM (Table 5.1). Thus, these two Td values would indicate denaturation temperature of myosin rod. Davies et al. (1988) also reported that the highest Tm value was arisen from denaturation of actin. The multiple transition of both NAM indicated the independent structural changes of their myosin subfragments and actin. Tm values of TB-NAM were higher than those of PW, confirming the higher thermal stability. Similar endothermic peaks of TB-NAM were also observed previously (Yongsawatdigul and Park, 2003). Nakaya and Watabe (1995) also reported that the multiple Tm values of myosin rod from carp changed with habitat temperature, while Togashi et al. (2002) also suggested that thermal stability of fish myosin is closely related to habitat temperature. Based on

these results, NAM from temperate water fish species showed less thermal stability and underwent unfolding at lower temperature when compared to that of tropical fish.

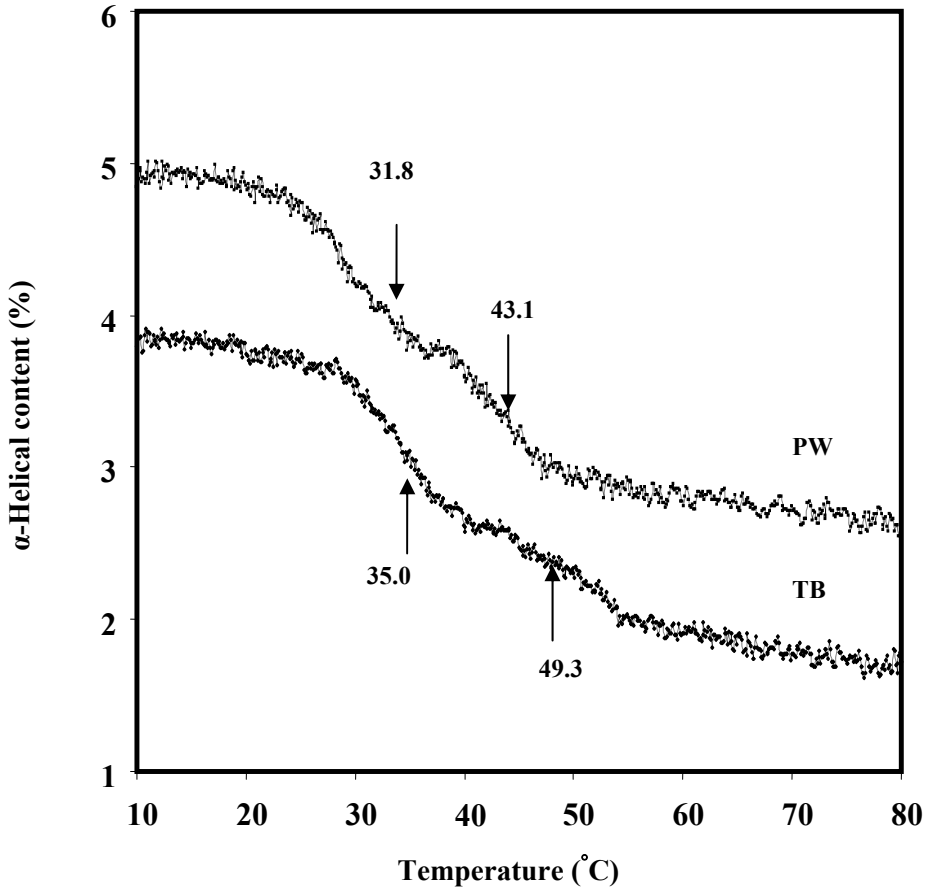

Figure 5.1 Changes in α-helical content of NAM in 0.6 M NaCl and 20 mM Tris-Cl, pH7.5 at heating rate of 1 °C/min. TB and PW = PW-NAM and TB-NAM. Denaturation temperatures (Td values) were indicated by the arrow.

Table 5.1 Transition temperature (T_m) and denaturation enthalpy (ΔH_{cal}) values of PW- and TB-NAM

NAM	Tm (°C)	ΔH_{cal} (J/mg)
TB-NAM	36.1 ± 0.5	0.168 ± 0.005
	50.9 ± 0.4	0.420 ± 0.002
	78.4 ± 1.2	0.640 ± 0.007
PW-NAM	31.8 ± 0.6	0.210 ± 0.003
	42.1 ± 0.2	0.736 ± 0.009
	75.3 ± 1.9	1.533 ± 0.004

Means and standard deviations were from 3 measurements

5.4.2 Physicochemical changes of NAM after incubation

5.4.2.1 Electrophoresis patterns

Protein components of extracted NAM from both PW and TB were determined by SDS-PAGE (Figure 5.2). Control NAM (without incubation) from both species showed similar molecular weight of the major proteins including, myosin heavy chain (MHC), actin, and tropomyosin of 200, 43.2, and 38.5 kDa, respectively. Similar protein patterns were also observed between the control and incubated NAM. This result suggested that the extracted NAM from both species contained minimal endogenous proteolytic and TGase activity. Thus, the extracted NAM from both species was considered to be the ideal substrate for our study.

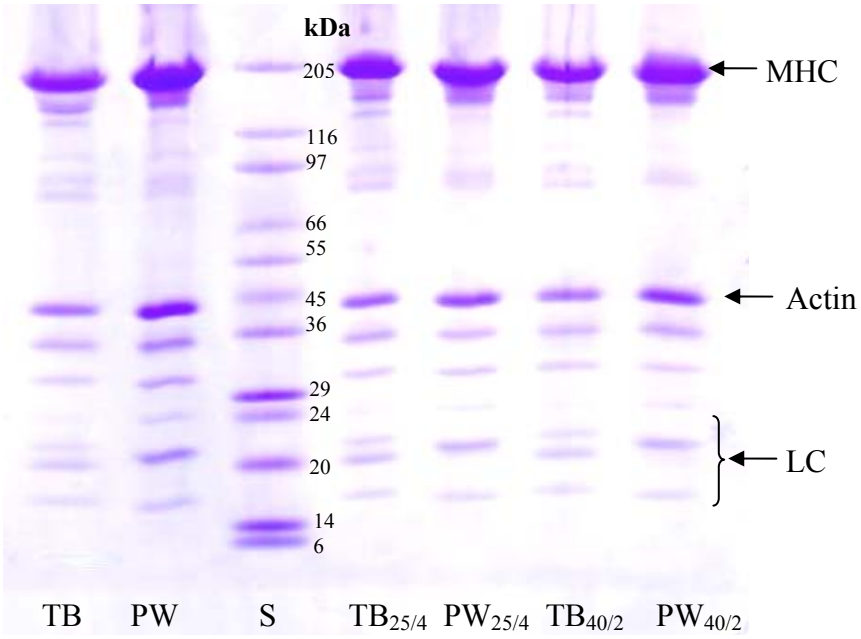

Figure 5.2 SDS-PAGE patterns of incubated NAM in the presence of 5mM $CaCl_2$ compared to control NAM. S = Broad molecular weight range standard markers. 25/4 and 40/2 = Incubation at 25 °C for 4h and at 40 °C for 2h, respectively; MHC = Myosin heavy chain; LC = Myosin light chains; TB and PW = TB-NAM and PW-NAM.

5.4.2.2 Changes in secondary structure

It has been reported that the α-helical structure is the dominant pattern of secondary structure at myosin rod (Lowey, Stayter, Weeds, and Baker, 1969). The changes in secondary structure of myosin rod from both NAM after incubation were observed as evidenced by the decreasing α-helical content, expressed as percentage of the α-helical reduction (Table 5.2). At 25 °C, α-helical content of NAM from TB and PW decreased by 33.1 and 69.7 %, respectively. Lower α-helical reduction of TB-NAM may reflect its higher stability when compared to PW-NAM. The α-helical reduction of TB-NAM

and PW-NAM after incubation at 40 °C was observed to be 78.0 and 89.7 %, respectively. Ogawa et al. (1995) indicated that setting was initiated by unfolding of α-helical structure. Optimum setting temperature of PW-NAM has been reported to be 25°C (Park, Yongsawatdigul, and Lin, 1994), which coincided with the high degree of unfolding of PW-NAM observed in this study. Yongsawatdigul et al. (2002) suggested the optimum setting temperature of TB-NAM at 40 °C. The present study also showed that the extent of TB-NAM unfolding at 40°C was comparable to that of PW-NAM at 25°C. In comparison, TB-NAM only unfolded slightly when incubated at 25 °C. This would explain why TB surimi hardly set at this temperature. These results suggested that the changes in secondary structure appeared to be the prime factor governing setting phenomenon of NAM from both species.

Table 5.2 α-Helical reduction of TB-NAM and PW-NAM after incubation at various conditions

NAM	**Incubation condition**	**α-Helical reduction** [a] **(%)**
TB-NAM	25°C/4h	33.1±1.35
	40°C/2h	78.0±0.43
PW-NAM	25°C/4h	69.7±1.48
	40°C/2h	89.7±1.09

[a]α-Helical reduction was relative to the α-helical content at 10 °C of each NAM.

Mean and standard deviation values of α-helical reduction were from 2 measurements.

5.4.2.3 Changes in surface hydrophobicity

PRODAN has been used as a fluorescence probe for the hydrophobic residues accounting for surface hydrophobicity (S_o PRODAN) and could also be used to monitor thermal denaturation of proteins by probing the hydrophobic patches that may be exposed from the hydrophobic core domain of proteins. After 25°C incubation, S_o PRODAN of PW-NAM markedly increased compared to the control NAM, while that of incubated TB increased slightly (Figure 5.3). An increase in S_o PRODAN of TB-NAM at 40 °C was higher than that at 25°C. In addition, S_o PRODAN of PW-NAM increased to the highest degree among studied conditions. An increase of So PRODAN correlated with the extent of secondary structural changes (Table 5.2). These results also implied that the exposure of hydrophobic residues resulted from unfolding of myosin rod, coiled coil domain. The amino acid sequences that adopt a coiled coil structure display a characteristic of seven-residue repeat pattern (haptad repeat), designated *a, b, c, d, e, f,* and *g* (McLachlan and Karn, 1982). The hydrophobic residues are concentrated at interior of the chain at the position of *a* and *d* to stabilize the coiled coil structure via hydrophobic interactions (Yoon, Kakinuma, Hirayama, Yamamoto, and Watabe, 1987). However, the presence of α-helix breakers (Pro and Gly) and α-helix unrelated residues (Asp and Thr) results in less stability of α-helical proteins (Kohn, Monera, Kay, and Hodges, 1995). The large gaps containing helix breakers were also observed in LMM of the least stable myosin, walleye pollock myosin (Togashi et al., 2002). Thus, thermal lability of NAM from PW, a temperate water fish, may possibly be a result of the presence of helix breakers at the myosin rod. This might be a reason why PW-NAM unfolded to the greater extent than did TB-NAM after incubation at the same condition.

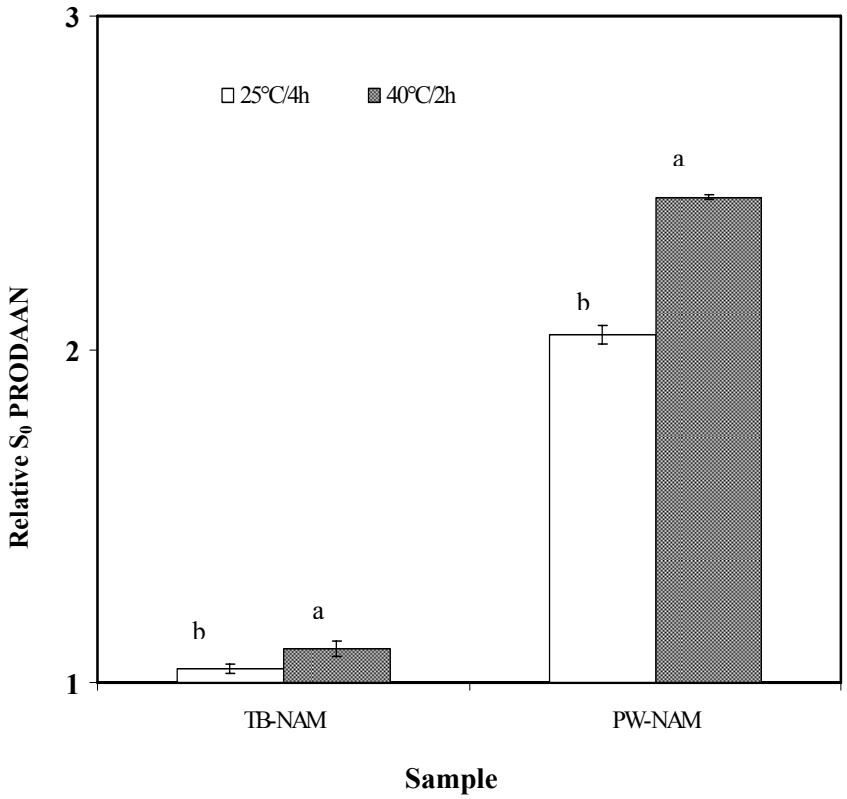

Figure 5.3 Relative S_0 PRODAN of incubated NAM expressed as the ratio of S_0 PRODAN of incubated NAM to that of control (without incubation). 25°C/4h and 40 °C/2h = Incubation at 25 °C for 4 h and 40 °C for 2h, respectively. Different letters within species indicate significant differences ($P < 0.05$).

5.4.2.4 Protein solubility

Solubility of PW-NAM after incubation at either 25 or 40 °C was lower than that of TB-NAM. These indicated that PW-NAM underwent intra- and inter-molecular interactions to form aggregates to a greater extent than did TB (Figure 5.4). Higher increase in surface hydrophobicity of (Figure 5.3) correlated to higher extent of aggregate formation of PW-NAM than TB-NAM. Hydrophobic interactions of myosin

rod were reported to initiate thermal aggregation of cod myosin (Chan, Gill, and Paulson 1993). The development of myosin gelation was mainly from the tail portion of myosin (Sano, Noguchi, Matsumoto, and Tsuchiya, 1990). Thus, hydrophobic interactions were responsible for aggregate formation of PW-NAM. These results may also infer that hydrophobic interactions were also important for setting of protein from cold and temperate fish species. High soluble protein content as well as a slight increase in S_0 PRODAN of incubated TB-NAM (Figure 5.4) implied that hydrophobic interactions were formed slightly and these interactions may also play less important role in setting phenomena of TB.

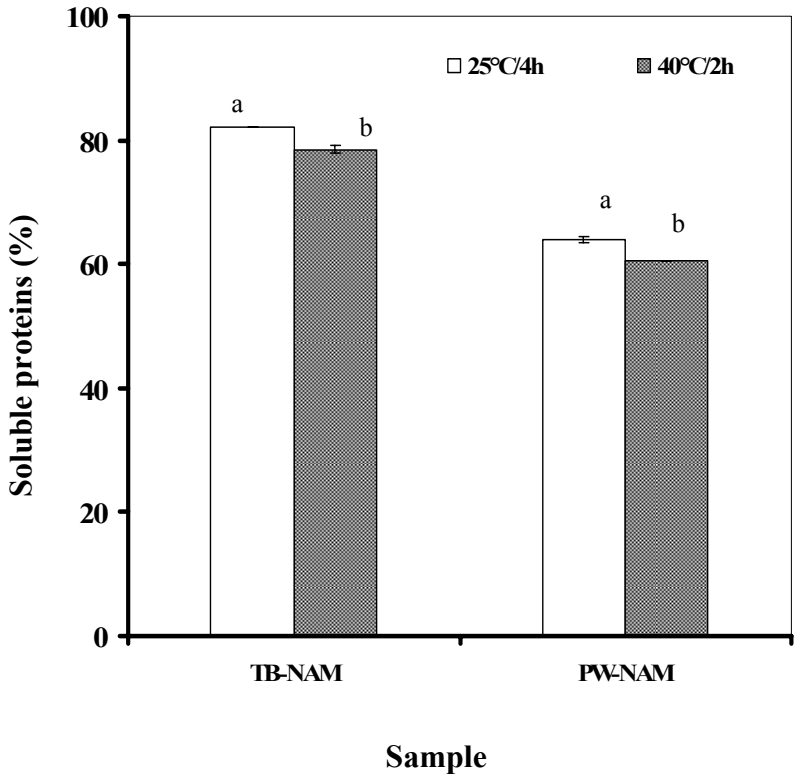

Figure 5.4 Soluble proteins of incubated NAM expressed as percentage compared to control (without incubation). Abbreviations are the same as Figure 5.3.

5.4.3 Changes of NAM after incubation with TGases

5.4.3.1 Changes in Ca-ATPase activity

Ca-ATPase activities of NAM from TB and PW dramatically decreased after incubation at 25 °C and remained approximately at 60 and 40 % compared to control counterparts, respectively (Figure 5.5a and b). The remaining Ca-ATPase activity of PW-NAM after incubation at 25°C was slightly higher than that at 40°C, implying that PW-NAM at 25 °C underwent denaturation. Ca-ATPase activity of incubated TB-NAM remained at higher level than that of PW at studied conditions ($P<0.05$), confirming higher thermal stability of NAM from tropical habitat. Johnston, Frearson, and Goldspink (1973) also found that the inactivation half-life of myofibrillar ATPase was correlated well with habitat temperature of fish.

In addition, ATPase activity of rabbit myosin head were not significantly affected by the presence of cross-linking catalyzed by MTG as reported by Huang et al. (1992). They also demonstrated that the isolated globular head of myosin (S1) was not cross-linked, while the myosin rod portion was quickly cross-linked. Seki, Nakahara, Takeda, Maruyama, and Nozawa (1998) also suggested that glutamine residues at S2 region of carp myosin were the preferential sites for endogenous TGase. Sano, Ohno, Otsuka-Fuchino, Matsumoto, and Tsuchiya (1994) reported that thermal unfolding of myosin head induced the formation of disulfide bonds between myosin head, resulting in a decreased in Ca-ATPase of carp-NAM. The disulfide bond formation was also proposed to be the initiation step of heat-induced gelation of myosin (Samijima, Ishioroshi, and Yasui, 1981). Thus, a decrease in Ca-ATPase activity during setting might be partly contributed from disulfide bond formation between myosin heads.

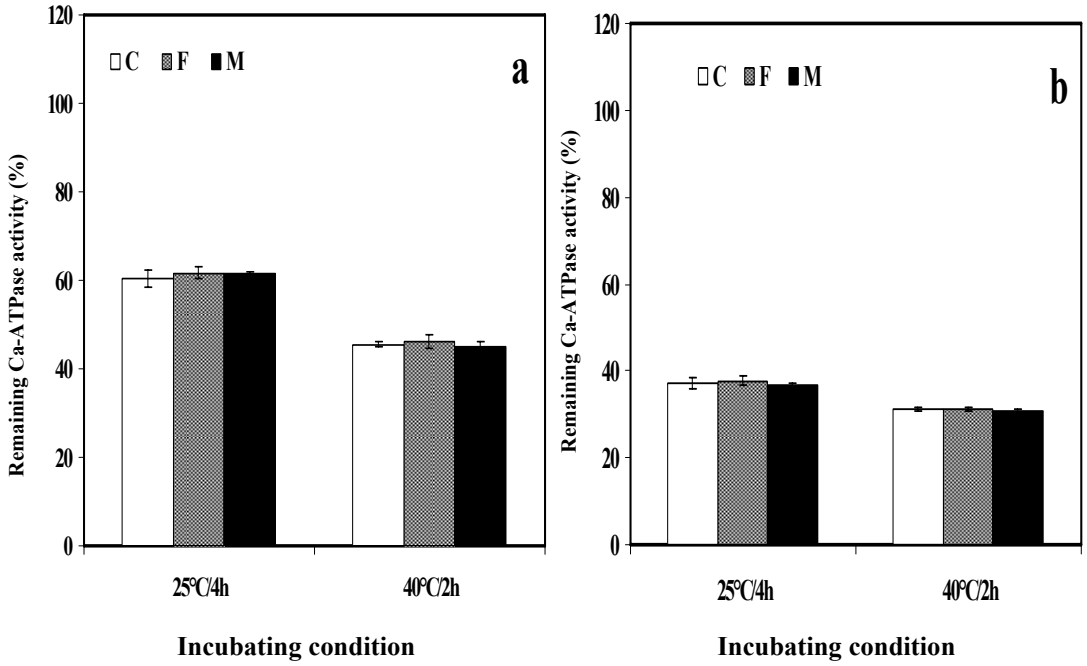

Figure 5.5 Remaining Ca-ATPase activity of TB-NAM **(a)** and PW-NAM **(b)** incubated at various conditions in the presence of TGases. C = Control (without enzyme); M = MTG; F = FTG; 25°C/4h and 40 °C/2h = Incubation at 25 °C for 4h and 40 °C for 2h, respectively; Ca-ATPase activity of NAM without incubation of respective species was considered to be 100%.

5.4.3.2 Protein patterns

The electrophoretograms of cross-linked TB-NAM incubated at 25 °C for 2h are shown in Figure 5.6a. Cross-linking of TB-NAM by FTG was noticed and MHC appeared to be the favored substrate as evidenced by a decrease in MHC concomitant with the formation of cross-linked polymers (CP). These may infer that setting of TB at this condition could be possible when endogenous TGase is activated. Yongsawatdigul et al. (2002) also found cross-linking of TB-MHC after incubation TB

surimi at this condition in the presence of 0.1% $CaCl_2$. However, it can be clearly seen that MTG catalyzed cross-linking of TB-MHC to the higher extent than did FTG at 25 °C. These indicated that MTG catalyzed MHC cross-linking to the higher ability than did FTG at this condition, 25 °C/2h, in which TB-NAM was unfolded slightly (33.1 % in Table 5.2). This also suggested that the effect of NAM conformation played less important role on MTG in catalyzing protein cross-linking than FTG. A small molecular size of MTG may facilitate more accessible to reactive groups within NAM than such a high molecular weight of FTG. In addition, the reactive groups for MTG may be located at the surface of NAM molecule, while those for FTG might be at the buried position. This would lead to observe less effect of NAM conformation on MHC cross-linking catalyzed by MTG. Investigation of TGase substrate specificity toward muscle proteins would lead to a better understanding of such differences between these TGases.

Cross-linking of TB-MHC catalyzed by both enzymes at 40 °C was observed to the higher extent than at 25 °C (Figure 5.6b). TB-MHC completely disappeared by the action of MTG, while the monomeric TB-MHC was still observed in the presence of FTG. It can be clearly seen that TB-MHC was disappeared completely by the action of MTG (Figure 5.6b) when high degree of α-helical reduction of TB-NAM was found (Table 5.2). In contrast, monomeric TB-MHC was still observed in the presence of MTG (Figure 5.6a) at the condition, in which α-helical reduction of TB-NAM was observed slightly (Table 5.2**)**. The results clearly showed that degree unfolding of NAM directly governed MHC cross-linking. These might be because the exposure of reactive groups within protein substrates, namely glutamine (Q) and lysine (K), are critical for TGases reactivity.

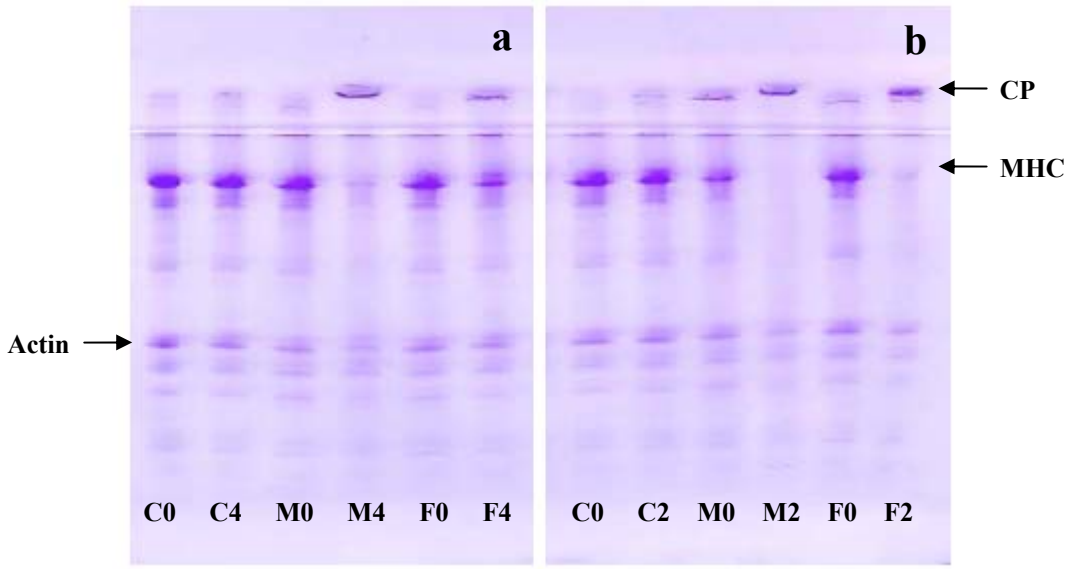

Figure 5.6 SDS-PAGE patterns of TB-NAM incubated at 25 °C **(a)** and 40 °C **(b)** in the presence of TGases. Numbers designate incubation time (h); 0 h = Incubation for 5 min; CP = Cross-linked polymers; MHC = Myosin heavy chain; C = Control (without enzyme); M = MTG; F = FTG.

The marked unfolding of PW-NAM was observed at either 25 or 40 °C (Table 5.2), suggesting that conformational changes of PW-NAM at these conditions induced favorable substrates for TGases. This was confirmed by a complete disappearance of PW-MHC catalyzed by MTG was also found after incubation PW-NAM at 25 and 40 °C (Figure 5.7a and b). These results clearly indicated that conformational changes of NAM rendered the more favorable substrates for TGase. Kishi et al. (1991) also reported that conformational changes of carp-NAM allowed the certain Q residues to become reactive. Thus, the reactivity of NAM as substrates for TGase mainly depended on their conformation induced by thermal unfolding. However, PW-MHC was not disappeared completely by the action of FTG at either incubating conditions,

25 or 40 °C (Figure 5.7a and b). This clearly showed that FTG catalyzed MHC cross-linking at the less ability than did MTG at the same unit of TGase. However, the same unit of TGase was based on the ability of both enzymes in incorporating MDC into DMC, which were different systems from the proteins substrates in the cross-linking reaction. Thus, such different cross-linking ability might be due to the different substrate specificity between MTG and FTG.

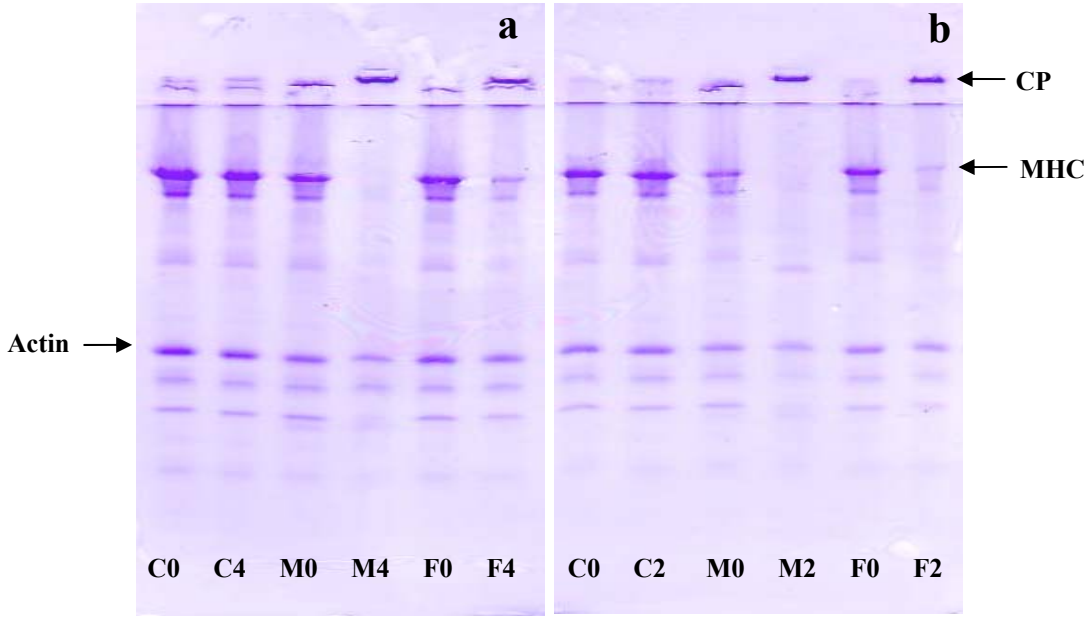

Figure 5.7 SDS-PAGE patterns of PW-NAM incubated at 25 °C **(a)** and 40 °C **(b)** in the presence of TGases. Abbreviations are the same as Figure 5.6.

5.4.4 Textural properties of NAM gels

Textural properties of TB-NAM and PW-NAM gels were measured after setting at 40 and 25 °C, respectively. These conditions are typically applied for setting of these species. FTG improved breaking forces and deformation values of TB-NAM gels by 1.3 and 1.5 fold, respectively (Figure 5.8a and b). A1.7 fold increase in those

values was also observed for PW-NAM gels (Figure 5.8c and d). Joseph, Lanier, and Hamann (1994) also reported that adding guinea pig liver TGase enhanced gelation of NAM extracted from Alaska Pollock and Atlantic croaker during setting. Since FTG is Ca^{2+}-dependent TGase and showed comparable characteristic as fish muscle TGase (Hemung and Yongsawatdigul, 2007), its catalytic reaction would represent the action of endogenous TGase in setting.

Textural properties of NAM were much more greatly improved by adding of MTG with increases in breaking force and deformation values of up to 3.1 and 1.7 folds, respectively, for TB-NAM gels (Figure5.8a and b). Jiang et al. (2000) also reported that MTG enhanced breaking force of golden threadfin bream by almost 3 folds after setting at 40 °C. Breaking force and deformation values of PW-NAM gels wet with MTG were also improved by 6.1 and 3.6 folds, respectively (Figure5.8a and b). It can be noticed that textural improvement of NAM gels corresponded well with the degree of MHC cross-linking observed by SDS-PAGE (Figure 5.6 and 5.7). Seguro, Kumazawa, Ohtsuka, Toiguchi, and Motoki (1995) also reported that gel strength of kamaboko made from Alaska pollock steadily increased as MHC cross-linking increased. The higher degree of MHC cross-linking of both species catalyzed by MTG resulted in greater textural improvement of NAM gels when compared to that did by FTG. Textural improvement of NAM gels depends on the extent of MHC cross-linking, which ultimately depended on the types of TGase.

It can be noticed that setting of PW-NAM was pronounced even at low temperature (25 °C), which is far from the optimum temperature of FTG (50 °C) and MTG (55 °C) (Ando et al., 1989; Hemung and Yongsawatdigul, 2007). These results

indicated the importance of NAM conformation on catalytic reaction of TGase since textural improvement of set gels depended on MHC cross-linking.

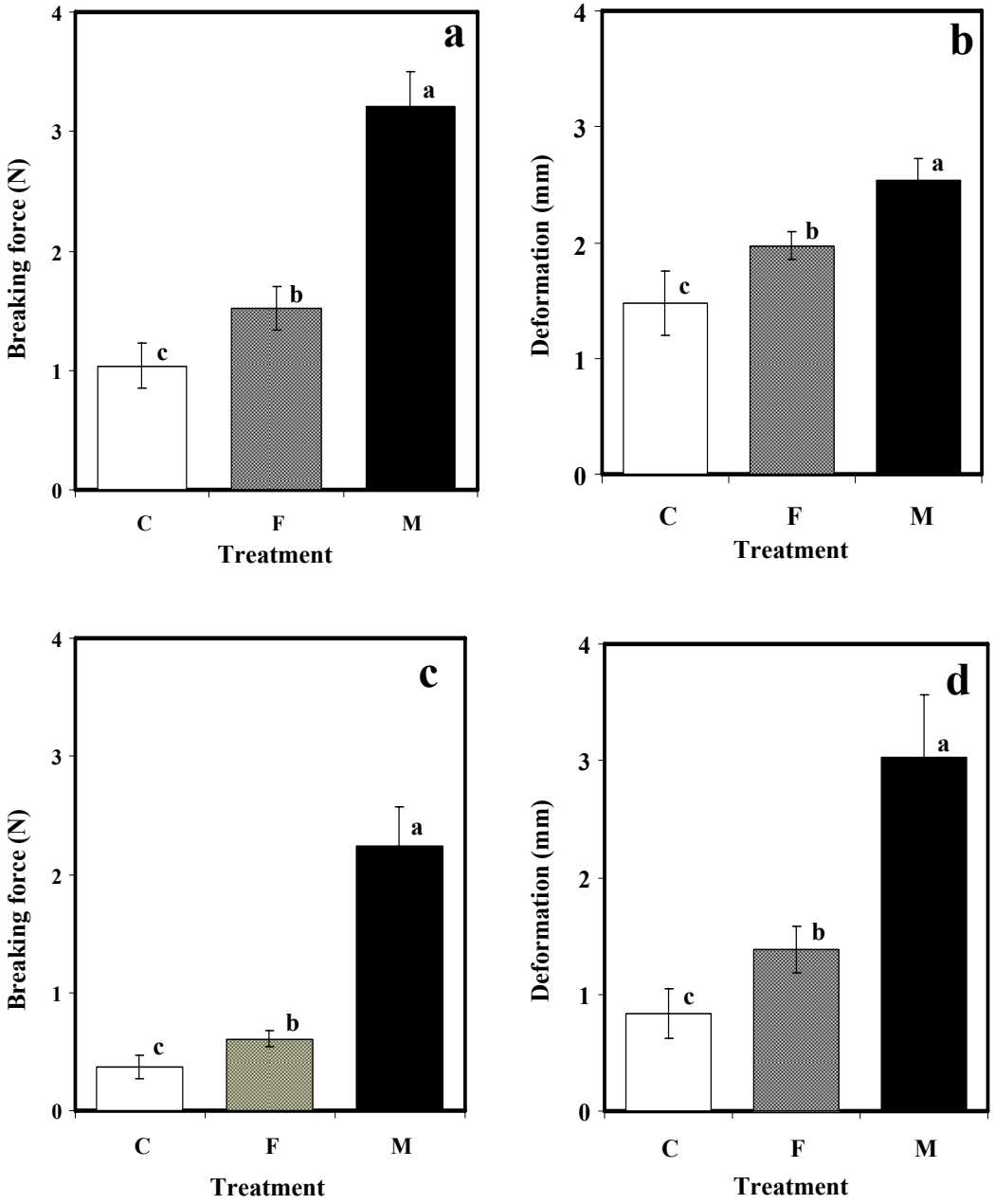

Figure 5.8 Breaking force and deformation values of TB-NAM gels (**a** and **b**) and PW-NAM gels (**c** and **d**) prepared by setting at 40°C for 2h and at 25°C for 4h, respectively. C = Control; F = FTG; M = MTG; Different letters within each figure indicate significant difference ($P < 0.05$).

However, textural properties of PW-NAM gels set at 25 °C appeared to be lower than those of TB-NAM gels set at 40°C. This might be due to the lower activity of TGases at 25 °C than at 40 °C. It was supported by observation that MTG and FTG catalyze the reaction at 25 °C to the lower extent than at 40 °C (Ando et al., 1989; Hemung and Yongsawatdigul, 2007).

5.5 Conclusions

NAM from fish living in temperate habitat underwent greater conformational changes than tropical fish under setting conditions at either 25°C for 4h or 40°C for 2h and such changes directly governed NAM cross-linking of catalyzed by TGases. NAM conformation and types of TGase were important for MHC cross-linking reaction. Unfolding of NAM played a much more critical role for FTG in catalyzing protein cross-linking than for MTG. TB-MHC could be cross-linked even at 25°C and cross-linking sites by both MTG and FTG might not be located at myosin head. MHC cross-linking correlated to the textural improvement and more extensive MHC cross-links catalyzed by MTG resulted in greater textural improvement than obtained by FTG.

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CHAPTER VI

REACTIVITY OF TRANSGLUTAMINASES ON GLUTAMYL SITES OF PEPTIDES DERIVED FROM THREADFIN BREAM MYOSIN

6.1 Abstract

MTG exhibited higher thermal stability, leading it to catalyze cross-linking of TB-myosin heavy chain (MHC) and the 5-(biotinamido) pentylamine ($BPNH_2$) incorporation into peptides to the greater extent than FTG. $BPNH_2$-tagged peptides were identified for their amino acid sequences and $BPNH_2$ modification sites by tandem mass spectrometry based on amberjack-MHC sequence. Peptides derived from TB-MHC were matched to those of amberjack-MHC using Mascot database search. The identified sites of $BPNH_2$ modification catalyzed by both TGases were at the myosin rod and most $BPNH_2$-tagged peptides contain hydrophilic amino acids e.g. lysine, arginine, and glutamic acid at glutaminylamide site of reactive glutamine (Q*). FTG tended to catalyze $BPNH_2$ incorporation of peptides that contain hydrophobic amino acids glycine, valine, leucine, and isoleucine at the α-acrylamide site of Q*. Those hydrophobic amino acids locate at the hydrophobic core of the intact myosin rod. Amino acids at α-acrylamide site of Q* for MTG were either hydrophobic or hydrophilic amino acids, suggesting broader glutamyl substrate specificity.

Key word: Fish liver TGase, microbial TGase, glutamyl substrate, threadfin bream myosin, tandem mass spectrometry

6.2 Introduction

Transglutaminases (TGases) are enzymes that catalyze the formation of isopeptide bond between protein–bound glutamine and lysine residues, resulting in cross-linked proteins or peptides (Folk, 1980). Primary amines, including the ε-amino groups of lysine (K) residues, typically serve as the acyl acceptor, while the amide groups of glutamine (Q) serve as the acyl donor.

TGases can be distinguished into two types depending on Ca^{2+} requirement for full activation. Ca^{2+}-dependent TGases have been found in several tissues, including blood factor XIIIa, guinea pig liver, red sea bream liver, and threadfin bream liver (Bohn, 1972; Folk and Cole, 1965; Yasueda, Kumazawa, and Motoki, 1994; Hemung and Yongsawatdigul et al., 2007). Molecular weight (Mw) of these enzymes ranges from 80 to 90 kilodalton (kDa) and Ca^{2+} is required for catalytic reaction. Crystal structure of red sea bream liver TGase showed four sequential domains and the overall active site structures were similar to those of factor XIIIa (Noguchi et al., 2001). Another type of TGase is Ca^{2+}-independent TGase, which has been isolated from microorganisms, *Streptoverticillium* sp., and is usually referred to microbial TGase (MTG). Mw of MTG is 38 kDa and its crystal structure showed a single compact domain, which is completely different from that of fish liver TGase (Ando et al., 1989; Kashiwagi et al., 2002). Moreover, the active site arrangement of MTG was superimposed well to that of fish liver TGase. These characteristics could lead to the different catalytic mechanism between these TGases, particularly their substrate specificities. Ohtsuka, Ota, Nio, and Motoki (2000) demonstrated that fish liver TGase and MTG specifically catalyze L-glutamine as glutamyl substrate. However, the amino acid sequences around the reactive Q also influenced the recognition of these TGases.

The preferential feature of glutamyl substrates in gliadin peptides for guinea pig liver TGase (GTG) was reported to be the consensus sequence Q-X-P (Mamone et al., 2004), while other peptides or proteins showed different preferred sequences (Gorman and Folk, 1981; Ruoppolo et al., 2003). This consensus sequence was also catalyzed slowly by MTG (Piersma, Pijpekamp, Wijngaards, Gruppen, and Boumans, 2002). It is seen that TGase specificities are varied, depending on the type of TGase and substrates. The glutamyl substrates of TGases in specific protein substrates should be investigated in order to understand the differences in catalytic reaction and specificities between Ca^{2+}– dependent and Ca^{2+}– independent TGases.

Myosin is a major component responsible for heat induced gelation of muscle proteins (Chan and Gill, 1994). Each myosin molecule consists of two 200 kDa heavy chains (MHC) and four light chains (LC) with the size of 15-20 kDa (Gershman, Stracher, and Dreizen, 1969). The N-terminal of each heavy chain folds together with two types of LC into subfragment-1 (S1). The remaining parts of the two MHC participate in a coiled-coil rod structure, so called myosin rod, exhibiting two distinct regions, subfragement-2 (S2) and light meromyosin (LMM) (Margossian and Lowey, 1982).

MHC has been reported to be a preferential substrate for TGase during pre-incubation of surimi-based products, which is known as “setting” (Kumasawa, Numazawa, Seguro, and Motoki, 1995; Jiang, Hsieh, Ho, and Chung, 2000). Cross-linking of MHC by TGase improves textural properties of food protein gels. Lee, Lanier, Hamann, and Knopp (1997) reported that fish protein gels treated by MTG showed different textural properties from that by endogenous TGase, suggesting the different cross-linking ability of these TGases. Identification of glutamyl substrates for

TGases on myosin molecules would lead to in depth information on the cross-linking sites and substrate specificities of fish and microbial TGases. More understandings on myosin cross-linking catalyzed by those TGases would be critical knowledge for textural modification of muscle proteins.

Identification of glutamyl substrates for TGases on myosin molecule is difficult due to the extremely large size and poor solubility of cross-linked proteins (Nemes, Petrovski, and Fesus, 2005). Peptide mapping analysis could differentiate the cross-linking of Alaska pollock-MHC catalyzed by MTG and endogenous TGase but it could not identify the reaction sites due to the complexity of digested peptides (Sato et al., 2001). Introduction of mass spectrometry (MS)-based analysis and the development of tandem mass spectrometry (MS/MS) procedures leads to the introduction of new peptidomic strategy that enables rapid identification of digested peptides from complex mixtures (Piersma et al., 2002). Moreover, labeling glutamyl substrates with affinity probe reduced the complexity after selective purification of the glutamyl substrates by affinity chromatography (Rouppolo et al., 2003). Thus, modification of myosin peptides with affinity probe and followed by peptidomic approach could facilitate identification of TGase-mediate modification of myosin peptides. Therefore, our objective was to determine the glutamyl substrates in myosin peptides for fish liver TGase and MTG, using a peptidomic strategy.

6.3 Materials and methods

6.3.1 Materials

Monodansylcadaverine (MDC), *N, N'*-dimethylated casein (DMC), hexadecyl

trimethyl-ammonium bromide (HABA), sodium dodecylsulfate (SDS), and trypsin were purchased from Sigma Chemicals (St Louis, MO, USA). Dithiothreitol (DTT) was obtained from ICN Biomedicals (Aurora, Ohio, USA). 5-(biotinamido) pentylamine ($BPNH_2$) and the pre-packed monomeric avidin column kit were from Pierce (Rockford, IL, USA). BCA™ Protein Assay Kit and bovine serum albumin (BSA) were purchased from Pierce (Rockford, IL, USA). Threadfin bream (TB) myosin was prepared as described by Hemung and Yongsawatdigul (2005). Other chemicals and reagents used were of analytical grade.

6.3.2 Transglutaminases preparation and activity assay

Fish TGase (FTG) was partially purified from TB liver tissue using DEAE-Sephacel, hydroxyapatite, Sephacryl-200, and hi-trap heparin chromatography as described by Hemung and Yongsawatdigul (2007). Partially purified TGase preparation was mixed with 20% sucrose at a ratio of 1:1, lyophilized, and kept at -20 °C until use. Lyophilized FTG was dissolved with cold de-ionized water (DI-water) and was diafiltered with buffer (20 mM Tris-Cl, pH 7.5 containing 2 mM DTT) to eliminate residual sucrose using Nanosep® centrifugal devices (Pall Life Science, Ann Arbor, MI, USA) with a 10-kDa-molecular weight cut-off (MWCO) membrane. The retentate was used as FTG.

Microbial TGase (TG-K) from *Streptoverticillium mobaraense* was supplied by Ajinomoto Co., Inc, Tokyo, Japan. The enzyme was dissolved with DI-water at the concentration of 5 mg/ml and was diafiltered with the same buffer and protocol as described in fish TGase preparation.

TGase activity was measured based on the incorporation of MDC into DMC according to the method of Takagi, Saito, Kikuchi, and Inada (1986) with slight

modifications. The reaction mixtures contained 1 mg/ml DMC, 15 μM MDC, 70 mM Tris-Cl (pH 7.5), 5 mM $CaCl_2$, 3 mM DTT, and 100 μL of TGase solution. $CaCl_2$ was omitted in the reaction for MTG. After incubation at 37 °C for 10 min, ammonium sulfate was added to obtain final concentration of 42 mM in order to stop the reaction. The fluorescence intensity of sample (FI_S) was measured at excitation and emission wavelength of 350 and 480 nm, respectively, using a Shimadzu RF-540 spectrofluorometer (Shimadzu Corp., Kyoto, Japan). Blanks (FI_b) were prepared in a similar manner except that enzyme was substituted with DI-water and FI was measured without incubation. TGase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmole of MDC into DMC within 1 min at 37 °C using enhancing factor of 3.2 and 1.5 for FTG and MTG, respectively.

6.3.3 Thermal stability of TGases

MTG and FTG solutions (10 U/ml) were incubated at 40 °C for 0, 0.5, 1, 1.5, and 2 h. After the incubation time was attained, enzymes were immediately cooled on ice and the residual TGase activity was then determined. Thermal stability of TGases was expressed as the percentage of remaining activity after incubation at 40 °C and the activity without incubation was considered to be 100 %.

6.3.4 TB-myosin cross-linking

Cross-linking of TB-myosin was performed in the reaction mixture containing 5 mg/ml of TB myosin, 20 mM Tris-Cl (pH 7.5), 5 mM $CaCl_2$ 5 mM DTT, and 5 U/ml TGase. The enzyme was replaced with DI-water in the control and $CaCl_2$ was omitted in the reaction catalyzed by MTG. All samples were incubated at 40 °C for 2 h. The reaction was stopped by adding SDS solution to obtain final concentration of 10 %. In

addition, the reaction catalyzed by MTG was carried out in the presence of 0-12 mM $BPNH_2$ to investigate the inhibitory effect of $BPNH_2$ on TB myosin cross-linking.

Protein profiles of TB myosin after incubation with and without $BPNH_2$ in the presence of either FTG or MTG were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Cross-linked sample (4 mg/mL) was mixed with SDS, BME, and bromophenol blue to obtain final concentration of 2, 0.02, and 0.01 %, respectively, before boiling for 20 min. The boiled samples were centrifuged at 5,000 × g for 5 min. Then, 1μL of soluble proteins in supernatant were separated on a PhastGel 10-15% gradient gel (GE Healthcare, Uppsala, Sweden) under reducing conditions using a PhastSystem^{TM} (GE Healthcare, Upsala, Sweden).

6.3.5 Amino acid composition of TB-myosin

TB myosin solution (10 mg/ml in 0.6 M NaCl, 20 mM Tris-Cl, pH 7.5) was dialyzed against DI water to remove salts before lyophilization. Amino acid analysis was performed at the Hospital for Sick Children-Advanced Protein Technology Centre (University of Toronto, Toronto, ON, Canada). Lyophilized myosin was subjected to vapor phase hydrolysis by 6N HCl with 1% phenol at 110°C for 24 h under nitrogen gas. The hydrolysate was derivatized with phenylisothiocyanate (PITC), producing phenylthiocarbamyl (PTC) amino acids according to the protocol described by Hirs (1967). Derivatized amino acids were re-dissolved in phosphate buffer and were separated by a reverse phase HPLC using a Pico-Tag column (3.9 mm × 15 cm) (Waters, Milford, MS, USA). Amino acid derivatives were detected at 254 nm by Waters 24B7 Dual wavelength absorbance detector (Waters, Milford, MS, USA). Tryptophan content of TB-myosin was determined using the same method except that TB-myosin was hydrolyzed in the presence

of 4 M methanesulfonic acid containing 0.2 % (w/v) tryptamine hydrochloride to protect tryptophan destruction during hydrolysis. The hydrolysate was neutralized with KOH, derivatized with triethylamine-PITC, and quantified using the Waters Pico-Tag System. For cysteine content, TB-myosin was mixed with 10 volumes of performic acid (5:95 of 30% hydrogen peroxide: 88% formic acid) before drying in a centrifugal evaporator. Dried sample was hydrolyzed with 6 N HCl at 110 °C for 24 h as described in total amino acid analysis.

The amount of amino acids was expressed as the mole percentage of each amino acid and compared to that of MHC from various fish species, including fast skeletal salmon (Iwami, Ojima, Inoue, and Nishita, 2002), carp (Guo, Nakaya, and Watabe,1999), pollock (Togashi et al., 2000), croaker (Yoon, Kakinuma, Hirayama, Yamamoto, and Watabe, 2000), and amberjack (Kawabata, Kanzawa, Ogawa, and Tsuchiya, 2000) with the accession numbers from GenBank of BAC00871, BAA22069, BAA33452, BAB12571, and Q9IBD7_SERDU, respectively.

6.3.6 Primary sequence of protein related to TB-MHC

Since the sequence of TB-myosin has not been reported in the literature, TB myosin was subjected to tryptic digestion followed by mass spectrometry for comparison to the reported sequences from the Mascot database.

TB myosin was separated on SDS-PAGE using 10-15% acrylamide gradient gel (GE Healthcare, Uppsala, Sweden) under reducing condition using the Phast System (GE Healthcare, Uppsala, Sweden). Gels were stained by Coomasie brilliant blue. The protein band corresponding to MHC with Mw of about 200 kDa was excised and placed into the microcentrifuge tube before chopping into small pieces (approximately 1 mm) using a scalpel. Protein identification of TB-MHC was carried

out at the Proteomic Core Facilities (University of British Columbia, Vancouver, B.C., Canada). TB-MHC was digested with trypsin as described by Shevchenko, Wilm, Vorm, and Mann (1996). Digested peptides were extracted with extraction solution (1% trifluoroacetic acid, 0.5% acetic acid, and 3% acetonitrile). The extracted peptides were separated on a Jupiter C_{18} column (1.0×150 mm; Phenomenex Inc., Torrance, ON, Canada) equilibrated with solvent A (0.05 % trifluoroacetic acid and 2 % CH_3CN in water) and eluted with a linear gradient of 0-60 % solvent B (0.045 % trifluoroacetic acid and 80 % CH_3CN in water). Mass profile of peptides was determined using a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, ON, Canada). The full-scan spectrum was followed by tandem mass spectrum analysis using a data-dependent MS/MS mode of mass spectrometer. Amino acid sequences of peptides were obtained by submitting the MS/MS data for protein identification using the Mascot search engine available on the web at http://www.matrixscience.com, which is based on the probability-based scoring algorithm of Perkins, Pappin, Creasy, and Cottrell (1999). The protein sequence was identified based on the tryptic peptide sequences by searching for non-redundant proteins in the database within a class of Actinopterygii, using molecular weight of 200 kDa and specifically digested by trypsin with up to two missed cleavages.

6.3.7 $BPNH_2$ modification of TB-myosin peptides

TB myosin was digested in 50 mM ammonium bicarbonate, pH 8.0 at 37 °C overnight using a trypsin: myosin ratio of 1: 10. The digestion was stopped by boiling the sample for 10 min. The digested solution was evaporated using centrifugal evaporator. $BPNH_2$ was incorporated into TB myosin peptides in the reaction mixture containing 5 mg peptides/ml, 20 mM Tris-Cl, pH 7.5, 5 mM DTT, 5 mM $CaCl_2$, 2 mM

$BPNH_2$, and 5 U/ml TGase at 40 °C for 4 h. $CaCl_2$ was omitted in the reaction catalyzed by MTG. Subsequently, the sample was boiled for 10 min to stop the cross-linking reaction and then dialyzed against DI-water using Spectra/Por® Biotech Cellulose Dialysis membrane with MWCO of 500 Da (Spectrum® Laboratories, Inc., Roncho Dominguez, CA, USA) to remove free $BPNH_2$. The dialysate was kept for biotin quantification and $BPNH_2$-tagged peptides purification.

6.3.8 Biotin quantification

The degree of biotinylation of TB myosin peptides catalyzed by TGases was determined as biotin content in the peptide mixture according to Green (1970) with slight modifications. HABA solution (25 mM HABA in 0.01 M NaOH) was prepared and filtered through Whatman filter paper No. 1 (Whatman International Ltd, Maidstone, England). The filtrate was used as a stock solution and was kept at -20°C throughout the study. HABA stock solution was mixed with avidin to obtain HABA-avidin complex solution at final concentration of 0.75 mM HABA and 2 mg/ml avidin in 50 mM ammonium bicarbonate, pH 8.0. Absorbance at 500 nm (A_{500}) of HABA-avidin complex (0.9 mL, V_1) was recorded as A_1. Then, 0.1 mL (V_2) of sample solution was added before reading A_{500} (A_2). Biotin content was calculated according to the equation developed by Green (1970) using extinction coefficient of 34 as described in the following equation**.**

$$[Biotin]\ mM = \frac{A_1 - \frac{A_2 \times (V_2 + V_1)}{V_1}}{34}$$

6.3.9 Purification of $BPNH_2$-tagged peptides

$BPNH_2$-tagged peptides were loaded onto a monomeric avidin column equilibrated with phosphate buffer saline, PBS, (0.1 M NaCl, 0.1 M phosphate buffer pH 7.2). The column was incubated at room temperature for 1 h to enhance binding ability. Subsequently, unbound peptides were washed with PBS buffer until the A_{280} reached the baseline of approximately zero. Bound peptides were selectively eluted by elution buffer (2 mM D-biotin in PBS) and the fractions containing high A_{280} were collected and dialyzed against DI-water using a Spectra/Por® Biotech Cellulose Dialysis membrane with MCOW of 500 Da (Spectrum® Laboratories, Inc., Roncho Dominguez, CA, USA). Dialyzed samples were lyophilized and submitted to LC-MS and LC-MS/MS analyses.

6.3. Identification of $BPNH_2$-tagged peptides by LC-MS/MS

$BPNH_2$-tagged peptides were separated on a Jupiter C_{18} column (1.0×150 mm; Phenomenex Inc., Torrance, ON, Canada) equilibrated with solvent A (0.05% trifluoroacetic acid and 2 % CH_3CN in water) and eluted with a linear gradient of 0-60% solvent B (0.045 % trifluoroacetic acid and 80 % CH_3CN in water) over 60 min at a flow rate of 50 µl/min. ESI-MS spectra of $BPNH_2$-tagged peptides were analyzed on a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, ON, Canada) equipped with an electrospray source. The ion source voltage was used at 5 kV with the orifice voltage of 50 V. For LC-MS mode, the quadrupole mass analyzer was scanned over an m/z range of 500-3000 atomic mass units with a step size of 0.5 Da and a dwell time of 1.0 ms/step. For LC-MS/MS mode, the spectra were obtained by selectively introducing the precursor ions with the m/z value of interest from the first quadrupole into the collision cell before fragmenting in the second quadrupole,

and the fragment ions were subsequently analyzed by the third quadrupole. The scan range was 50-1900 atomic mass units with the same step size of 0.5 Da.

6.4 Results and discussion

6.4.1 Cross-linking of TB myosin

Cross-linking of TB-myosin catalyzed by either FTG or MTG was performed at 40°C in order to compare the reactivity of TGases toward intact myosin. The studied condition is a typical setting condition applied for TB muscle protein (Yongsawatdigul, Worratao, and Park, 2002). After incubation with FTG for 2 h, a decreased intensity of MHC was evident, concomitant with the formation of higher molecular weight components (Figure 6.1). These aggregates were not noticed in the control incubated without added TGase. Moreover, MHC completely disappeared in the presence of MTG. These results indicated that TB-MHC was a substrate for both FTG and MTG, but MTG catalyzed cross-linking of TB-MHC to a greater extent than did FTG.

The different thermal stability between MTG and FTG could contribute to the different TB-MHC cross-linking reaction. The remaining activity of MTG and FTG after incubation at 40 °C for 2 h was about 90 and 40 %, respectively (Figure 6.2). Lower thermal stability of FTG may have resulted in the lesser extent of MHC cross-linking. Ando et al. (1989) also suggested that MTG was stable at temperatures up to 40 °C. However, Sato et al. (2001) also observed that MTG produced more ε-(γ-glutamyl)lysine cross-links in Alaska pollock surimi than did endogenous TGase. They suggested that MTG and endogenous TGase reacted at different sites of MHC, resulting in the different amounts of cross-links formed. The different catalytic sites of

both FTG and MTG may also lead to different extent of cross-linking. Therefore, the glutamyl substrates for both TGases were investigated in order to understand the different substrate specificities between FTG and MTG.

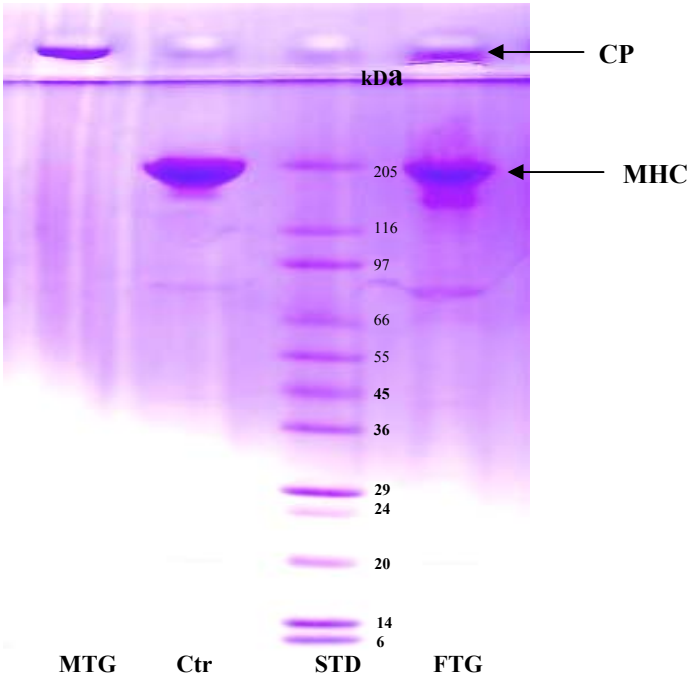

Figure 6.1 SDS-PAGE patterns of cross-linked myosin catalyzed by MTG and FTG at 40 °C for 2 h. CP = Cross-linked products, MHC = Myosin heavy chain, STD = Standard markers. Ctr = Control.

6.4.2 $BPNH_2$ labeling of TB-myosin peptides

$BPNH_2$ was used to label Q residues within the myosin molecule. The amino terminus of $BPNH_2$ serves as an acyl acceptor, while the functional group of biotin can bind specifically to avidin. The ability to incorporate $BPNH_2$ into intact myosin by MTG was investigated. However, low amount of $BPNH_2$ was incorporated into TB-myosin (0.29 ± 0.085 μmol biotin/g protein). TB-MHC cross-linking was still observed in the presence of $BPNH_2$**.** Moreover, the comparable CP content was

obtained with the addition of 0-12 mM $BPNH_2$ (Figure 6.3). It is possible that the conformation of TB-myosin provides highly reactive lysine residues, which favor their reactivity as acyl acceptor compared to $BPNH_2$.

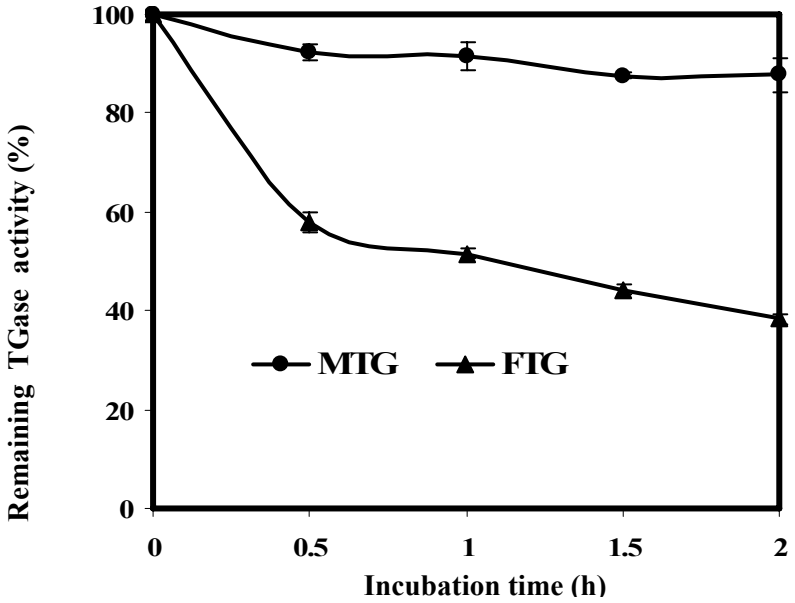

Figure 6.2 Thermal stability of FTG and MTG at 40 °C. Means were from duplicate measurement and bars indicate standard deviation from 2 measurements.

Seki, Nakahara, Takeda, Maruyama, and Nozawa (1998) suggested that MDC did not inhibit cross-linking of carp-MHC catalyzed by endogenous TGase. Since FTG is also categorized as Ca^{2+}-dependent TGase like carp TGase, its ability to incorporate external amine into muscle protein would also be similar to carp TGase. It could be hypothesized that $BPNH_2$ could be incorporated into TB-myosin by FTG for a lesser extent than MTG. To eliminate the effect of conformation on TB-MHC cross-linking and increase the $BPNH_2$ incorporation, $BPNH_2$ was incorporated into TB-myosin peptides instead of the intact myosin molecule. $BPNH_2$ and internal lysine residues

from myosin would be able to freely compete for Q residues in the peptide system without conformational effect.

FTG and MTG incorporated $BPNH_2$ into TB-myosin peptides at 19.90 ± 0.69 and 122.36 ± 0.41 μmol biotin/ g peptide, respectively. $BPNH_2$- tagged peptides were selectively purified on the avidin column before lyophilizing to obtain the dried peptides. Dried weight of $BPNH_2$-tagged peptides obtained from the action of MTG and FTG were 9.1 and 1.3 mg, respectively, which recovered from starting peptides of 20 mg. The higher amount of $BPNH_2$ -tagged peptides catalyzed by MTG confirmed the greater ability of MTG catalyzed either TB-MHC cross-linking reaction or $BPNH_2$ incorporation. These results could be partly explained by the differences in thermal stability between these two TGases. However, possible differences in specificity toward glutamyl sites may also be involved. Therefore, identification of the reactive glutamyl sites was carried out.

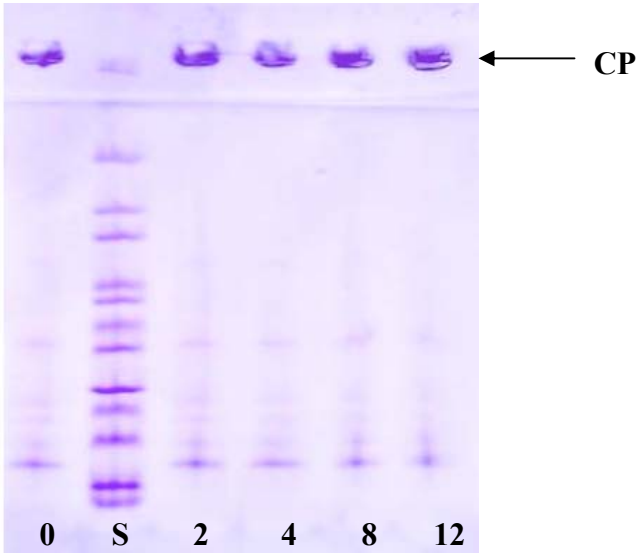

Figure 6.3 Effect of $BPNH_2$ on cross-linked TB-myosin catalyzed by MTG. S = Standard markers. CP = Cross-linked products, Numbers indicate $BPNH_2$ concentration (mM).

6.4.3 Amino acid composition of TB-myosin and TB-MHC identification

The growing body of nucleotide and protein sequences reported in public domain databases has made rapid protein identification feasible by combining mass spectrometry data with database search (Perkin et al., 1999). This approach is applicable for proteins in which the sequences are already known. The partial or complete amino acid sequences of the target protein can be used to search from databases to reveal similar or homologous sequences. Since neither DNA nor amino acid sequences of TB-myosin are presently available, the identification of $BPNH_2$-tagged peptides was investigated based on similar amino acid sequences in the database using the Mascot search engine. The $BPNH_2$ modification sites on TB-myosin peptides could infer the reactive sites on the intact myosin since the sequence of $BPNH_2$-tagged peptide can be used to search for the location within the intact molecule based on the reported sequence of homologous proteins. This approach was also applied to localize the acyl donor for TGase on β-casein (Ruoppolo et al., 2000). Therefore, amino acid compositions of TB-myosin and probability-based protein identification of TB-MHC were determined in order to obtain the putative amino acid sequence.

Amino acid composition of TB-myosin was determined and mole percentage of amino acids from TB-myosin was similar to that of MHC from other fish species as shown in Table 6.1**.** These results indicated that TB-myosin was probably similar to other species, including amberjack, salmon, pollock, croaker, and carp.

Since protein similarity also depends on the sequence of amino acids, a probability-based protein identification approach was applied to compare TB-MHC sequence to other MHC sequences from the Mascot database. Peptides derived from

TB-MHC were matched with those of MHC from several fish species as shown in Table 6.2. The highest score was obtained for MHC from amberjack and amino acid sequence deduced from the nucleotide encoding is shown in Figure 6.4.

Table 6.1 Amino acid composition (mole percentage) of TB-myosin compared to that of MHC from various fish species.

Amino acids	TB	Amberjack	Salmon	Pollock	Croaker	Carp
G	6.22	4.67	4.68	4.65	4.18	4.62
H	1.66	1.87	1.56	1.86	1.39	1.85
K	7.05	10.75	10.91	10.70	11.15	10.63
I	4.56	4.21	4.16	4.19	4.18	4.16
A	9.53	8.41	8.57	8.79	8.51	8.47
C	0.83	0.93	1.04	0.93	0.93	0.92
E+Q	19.09	19.16	19.22	18.61	19.04	19.41
F	2.90	2.80	2.60	2.33	2.79	2.77
L	10.37	10.28	9.87	9.77	9.75	9.71
M	2.07	2.80	3.12	2.79	3.25	3.24
D+N	9.54	9.81	9.87	10.24	9.75	9.24
P	2.90	1.40	1.56	1.86	1.86	1.85
R	5.81	5.14	5.19	5.12	5.11	5.08
S	4.56	5.14	4.68	5.12	5.11	5.08
W	0.41	0.47	0.52	0.47	0.46	0.46
T	4.15	4.67	4.68	4.65	4.64	4.62
V	5.81	5.14	5.19	5.58	5.57	5.55
Y	2.49	2.34	2.60	2.33	2.32	2.31

Amino acid composition of myosin heavy chain from amberjack, salmon, Pollock, croaker, and carp were from the GenBank accession numbers Q9IBD7_SERDU (Kawabata et al., 2000), BAC00871 (Iwami et al., 2002), BAA33452 (Togashi et al., 2000), BAB12571 (Yoon et al., 2000), BAA22069 (Gou et al., 1994), respectively.

S1 ⟶

1	MSTDAEMEQY	GPAAIYLRKT	ERERIEAQTT	PFDAKTAYFV	ADADEMYLKG
51	KLVKKEGGKA	TVETDTGKTL	TVKEDDIHQR	NPPKFDKIED	MAMMTHLNEP
101	CVLYNLKDRY	ASWMIYTYSG	LFCVVVNPYK	WLPVYDAVVV	GAYRGKKRIE
151	APPHIFSISD	NAYQAMHTDR	ENQSVLITGE	SGAGKTVNTK	RVIQYFATIA
201	ALGAKKAEAT	PGKMQGSLED	QIVAANPLLE	AYGNAKTVRN	DNSSRFGKFI
251	RIHFGSAGKL	SSADIETYLL	EKSRVTFQLS	AERSYHIFYQ	LMTGHKPELL
301	EALLITTNPY	DYHMISQGEI	TVKSIDDVEE	FIATDTAIDI	LGFTAEEKLG
351	IYKLTGAVMH	HGNMKFKQKQ	REEQAEPDGN	EEADKIAYLL	GLNSADMLKA
401	LCYPRVKVGN	EMVTKGQTVP	QVNNSVSALC	KSIYEKMFLW	MVIRINEMLD
451	TKQPRQYFIG	VLDIAGFEIF	DFNSLEQLCI	NFTNEKLQQF	FNHHMFVLEQ
501	EEYKKEGIEW	EFIDFGMDLA	ACIELIEKPM	GIFSILEEEC	MFPKASDTTF
551	KNKLHDQHLG	KTKAFEKPKP	AKGKAEAHFS	LVHYAGTVDY	NISGWLDKNK
601	DPLNDSVVQL	YQKSSNKLLA	FLYAAHGGAD	DAAGGGGKKG	GKKKGGSFQT
651	VSALFRENLG	KLMTNLRSTH	PHFVRCLIPN	ETKTPGLMEN	FLVIHQLRCN
701	GVLEGIRICR	KGFPSRILYG	DFKQRYKVLN	ASVIPEGQFI	DNKKASEKLL
751	GSIDVDHTQY	KFGHTKVFFK	AGLLGALEEM	RDDKLATLVT	MTQALCRGYL

S2 ⟶

801	MRKEFVKMME	RRESIFSIQY	NIRSFMNVKN	WPWMNLY FKI	KP LLKSAETE
851	KELQQMKENY	EKMQSDLATA	LAKKKELEEK	MVSLLQEKND	LQLQVASEVE
901	NLSDAEERCE	GLIKSKIQLE	AKLKETTERL	EDEEEINAEL	TAKKRKLEDE
951	CSELKKDIDD	LELTLAKVEK	EKHATENKVK	NLTEEMASQD	ESIAKLTKEK
1001	KALQEAHQQT	LDDLQAEEDK	VNTLTKAKTK	LEQQVDDLEG	SLEQ*EKKLRM
1051	DLERAKRKLE	GDLKLAQESI	MDLENDKQQS	DEKIKKKEFE	TSQLLSK**IED**
1101	**EQSLGAQ*LQK**	KIK**ELQ*AR**IE	ELEEEIEAER	AARAKVEKQR	ADLSRELEEI
1151	SERLEEAGGA	TAAQIEMNKK	R**EAEFQ*K**LRR	DLEESTLQHE	STASALRKKQ

Figure 6.4 Amino acid sequence of amberjack-MHC. Letters with under line indicates $BPNH_2$ tagged peptides by MTG, bold letters indicate $BPNH_2$ tagged peptides by FTG, and bold and underline letters indicate $BPNH_2$ tagged peptides by both MTG and FTG. $BPNH_2$ tagged Q residues were indicated by asterisk. Starting point of S1 and S2 are indicated by the arrow.

1201 ADSVAELGEQ IDNLQRVKQK LEKEKSEYKM EIDDLSSNME AVAKSKGNLE

1251 KMCRTLEDQL SELKAKNDEN VRQLNDINAH KARLQTENGE FSRQLEEKEA

1301 LVSQLTRGKQ AFTQQIEELK RHIEEEVKAK NALAHAVQSA RHDCDLLREQ

LMM ⟶

1351 FEEEQEAKAE LQRGMSKANS EVAQW RTK**YE** **TDAIQ*R**TEEL EEAKKKLAQR

1401 LQDAEESIEA VNSKCASLEK TKQRLQGEVE DLMIDVERAN SLAANLDKKQ

1451 RNFDKVLAEW KQKYEEGQAE LEGAQKEARS LSTELFKMKN SYEEALDHLE

1501 TMKRENK**NLQ** **Q*EISDLTEQI** **GETGK**SIHEL EKAKKTVETE KTEIQSALEE

1551 AEGTLEHEEA KILRVQLELN QVKGEVDRKL AEKDEEMEQI KRNSQRVMDS

1601 MQSTLDAEVR SRNDALRVKK KMEGDLNEME IQLSHANRQA AEAQKQLRNV

1651 Q*GQLKDALLH LDDAVRGQED MKEQVVMVER RNGLMVAEIE ELRVALEQTE

1701 RGRK**VAEQ*EL** **VDASER**VGLL HSQNTSLLNT KKKLESDLVQ VQSEVDDSIQ

1751 EARNAEEKAK KAITDAAMMA EELKKEQDTS AHLERMKKNL EVTVKDLQHR

1801 LDEAENLAMK GGK**KQLQ*K**LE SRVR**ELEAEV** **DAEQ*R**RGSDA VKGVRKYERR

1851 VKELTYQTEE DKKNVHRLQD LVDKLQLKVK AYKRQAEESE EQANTHLSRL

1901 RKVQHEMEEA QERADIAESQ VNKLRAKSRD TGKSESAE

Figure 6.4 Amino acid sequence of amberjack-MHC. (Continued)

Letters with underline indicates $BPNH_2$-tagged peptides by MTG, bold letters indicate $BPNH_2$-tagged peptides by FTG, and bold letters with underline indicate $BPNH_2$-tagged peptides by both MTG and FTG. $BPNH_2$-tagged Q residues were indicated by asterisk. Starting point of LMM is indicated by the arrow.

Amberjack (*Seriola dumerili*) is a fish living in warm habitat at the temperature 25-30 °C, which is closed to that of TB (Kawabata et al., 2000). The changes in amino acid composition of MHC induced by water temperature were found in carp (Watabe et al., 1995; Imai, Hirayama, Kikuchi, Kakinuma, and Watabe, 1997). In addition, habitat temperature also induced expression of either S1 or LMM isoform (Guo,

Nakaya, and Watabe, 1994; Watabe, Guo, and Hwang, 1994). The different amino acid composition might be a molecular mechanism for thermal adaptation of myosin.

Table 6.2 Significant hits of TB-MHC peptides from Mascot search

Accession numbers	Score	Protein	Species
Q9IBD7_SERDU	4815	Myosin heavy chain	*Seriola dumerili*
	116/556		(Amberjack)
Q6SNT2_9PERO	4602	Fast skeletal muscle	*Siniperca chuatsi*
	116/556	myosin heavy chain	(Chinese perch)
Q6IQX1_BRARE	4189	Muscle myosin, heavy	*Brachydanio rerio*
	104/556	polypeptide 2	(Zebra fish)
Q2HX57_CYPCA	4160	Myosin heavy chain	*Cyprinus carpio*
	97/556	embryonic type 2	(common carp)
Q90YF6_PARFO	4277	Myosin heavy chain	*Paracirrhites forsteri*
	102/556		(Blackside hawkfish)
Q90337_CYPCA	3358	Myosin heavy chain	*Cyprinus carpio*
	82/556		(common carp)
P87344_THECH	3038	Myosin heavy chain	*Theragra chalcogramma*
	77/556	(fragment)	(Alaska pollock)
Q2PMW9_DICLA	2912	Myosin heavy chain	*Dicentrarchus labrax*
	67/556		(European sea bass)
Q91520_THUTH	2816	Myosin heavy chain	*Thunnus thynnus*
	66/556	(fragment)	(Bluefin tuna)

6.4.4 Glutamyl substrate identification by LC-MS/MS

$BPNH_2$-tagged peptides catalyzed by FTG and MTG were analyzed by LC-MS as shown in Figure 6.5 and 6.6, respectively. The number of $BPNH_2$-tagged peptides for FTG and MTG were comparable in the mass ranges at the m/z of 500-3000. The

same mass values were also observed for $BPNH_2$-tagged peptides catalyzed by MTG and FTG.

The amino acid sequences of those $BPNH_2$-tagged peptides were identified by tandem mass spectrometry (MS/MS) using amberjack-MHC sequence as a model in order to localize the $BPNH_2$ modification sites. Amino acid sequence of amberjack-MHC was submitted to *in-silico* digestion by trypsin without modification, up to 2 missed cleavages, using the ProteinProspector MS-digest program at http://prospector.ucsf.edu. The Q-containing peptides were then selected to be the candidates for $BPNH_2$ modification. The predicted mass after $BPNH_2$ modification was also calculated for each peptide. The calculated mass of $BPNH_2$-tagged peptides was compared with experimental mass from LC-MS (Figure 6.5 and 6.6) to obtain the possible putative amino acid sequences of $BPNH_2$-tagged peptides. These putative amino acid sequences were then confirmed by LC-MS/MS. For example, $BPNH_2$-tagged peptide at m/z of 955 was found in both experimental and calculated mass. This mass was subtracted by 311.14 Da (mass of $BPNH_2$ after releasing ammonia, 328.14 - 17.00) to obtain the mass of unmodified peptide at 642.8 Da. It was likely to be the sequence of KQLQK, locating at the position of 1814-1818 in the LMM subfragment of amberjack-MHC.

The doubly charged ion of $BPNH_2$-tagged peptide (KQLQK + 1 $BPNH_2$) at m/z of 478.1 was selected for analysis by tandem mode mass spectrometry. Generally, the fragmentation process generates a complex set of fragmented ions mainly belonging to the b- and y- ion series. The b- and y- ion series can lose ammonia, generating the b - (NH_3) and y - (NH_3) series. The related patterns of b- and y- ions for

KQLQK without $BPNH_2$ modification could be depicted as in Figure 6.7. The expected masses of those b- and y- ions are shown in Table 6.3.

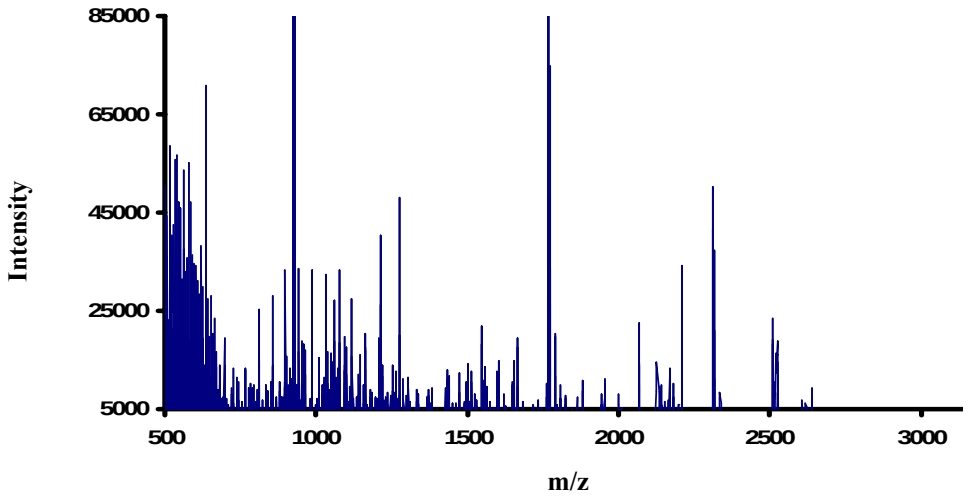

Figure 6.5 Mass profile of $BPNH_2$-tagged peptides catalyzed by FTG

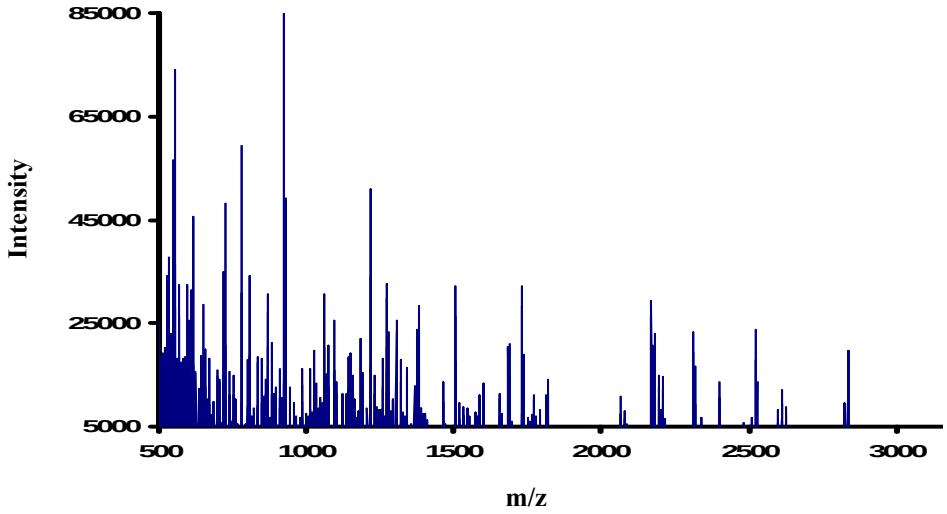

Figure 6.6 Mass profile of $BPNH_2$ tagged peptides catalyzed by MTG

The tandem mass spectrum of the selected ion (m/z of 478.2) generated several b- and y-ions and some of b- or y- ions without ammonia were also observed directly (Figure 6.7). Masses of b_1, b_2, b_3, (y_3)-NH_3, and y_4 ions were observed at the signal of

m/z 129.1, 257.1, 370.2, 257.1, and 147.1, respectively. The signals corresponding to y_1- NH_3, y_2, and y_3 were not observed whereas other signals were shown at m/z of 810.5, 699.4, and 586.4, corresponding to (y_1-NH_3) + $BPNH_2$, (y_2) + $BPNH_2$, and (y_3) + $BPNH_2$, respectively.

Table 6.3 Principle ion series of peptide KQLQK upon fragmentation by MS/MS

No.	**AA**	**Mass**	**b-series**			**y-series**		
			series	**b**	**b - (NH_3)**	**series**	**y**	**y - (NH_3)**
1	K	128.095	1	**129.102**	112.076	-	-	-
2	Q	128.059	2	**257.161**	240.134	1	516.314	499.288
3	L	113.084	3	**370.245**	353.218	2	388.255	371.230
4	Q	128.059	4	498.304	481.277	3	275.171	**258.145**
5	K	128.095	-	-	-	4	**147.113**	**130.086**

Bold entries indicate b- and y- ions that were observed directly by LC-MS/MS

These results strongly indicated that $BPNH_2$ was incorporated into the 4^{th} (Q) residue as NH_2-K-Q-L-Q-($BPNH_2$)-K-COOH. The $BPNH_2$ modification sites on other $BPNH_2$-tagged peptides catalyzed by FTG and MTG were determined in the similar fashion, as shown in the results in Table 6.4 and Table 6.5, respectively. However, not all $BPNH_2$-tagged peptides could be identified by this approach due to the lack of exact amino acid sequence of TB-myosin.

Amino acid sequences of $BPNH_2$-tagged peptides can be used to localize the probable modification sites on the myosin molecule since amino acid sequences of MHC have been reported to be conserved (Iwami, Ojima, Inoue, and Nishita, 2002). It

can be noticed that all identified peptides were located at the myosin rod (Table 6.4 and 6.5). Those sequences were at the extremely conserved region of myosin rod. Seki et al. (1998) suggested that myosin rod from carp was the preferential site for endogenous TGase. In addition, MDC was labeled by the action of guinea pig liver TGase on S2 near the S2/LMM junction (Kunioka and Ando, 1996). Our results also showed that FTG incorporated $BPNH_2$ into YETDAIQ*R, locating at LMM near the S2/LMM junction (Figure 6.4). Huang, Seguro, Motoki, and Tawada (1992) reported that MTG also quickly cross-linked myosin rod from rabbit filamentous myosin. It might be inferred that Q residues within myosin rod were glutamyl substrates for TGases. These are important reactive sites for catalytic reaction of TGase, contributing to textural improvement of fish protein gels. The identified reactive Q (Q*) residues for FTG were consistent with the rule proposed by Folk and Cole (1965). They reported that Q can serve as a substrate for TGases when they are located at least the third amino acid position from the NH_2 terminus and at least the second amino acid position from the COOH terminus.

When the "window" of five amino acids around Q* of $BPNH_2$-tagged peptides was designed to be (-2) - (-1) - Q* - (1) - (2), hydrophobic amino acids often occupy at the (-2) or (-1) positions (Table 6.4). Similar patterns were also observed in the mixtures of VIP, SV-IV, and casein substrate for GTG (Rouppolo et al., 2003). In addition, substitution with hydrophobic amino acids, V and F, at (-1) position enhanced reactivity of red sea bream liver TGase (Ohtsuka et al., 2000). Folk (1983) also suggested that amino acids at the α-acrylamide site of Q* bind with enzyme molecule, suggesting that hydrophobic amino acids play a role on binding to the enzyme. Amino acids with hydrophilic side chain were important to arrange the

Carboxyamide in the direction of the active center of TGase (Folk, 1983).

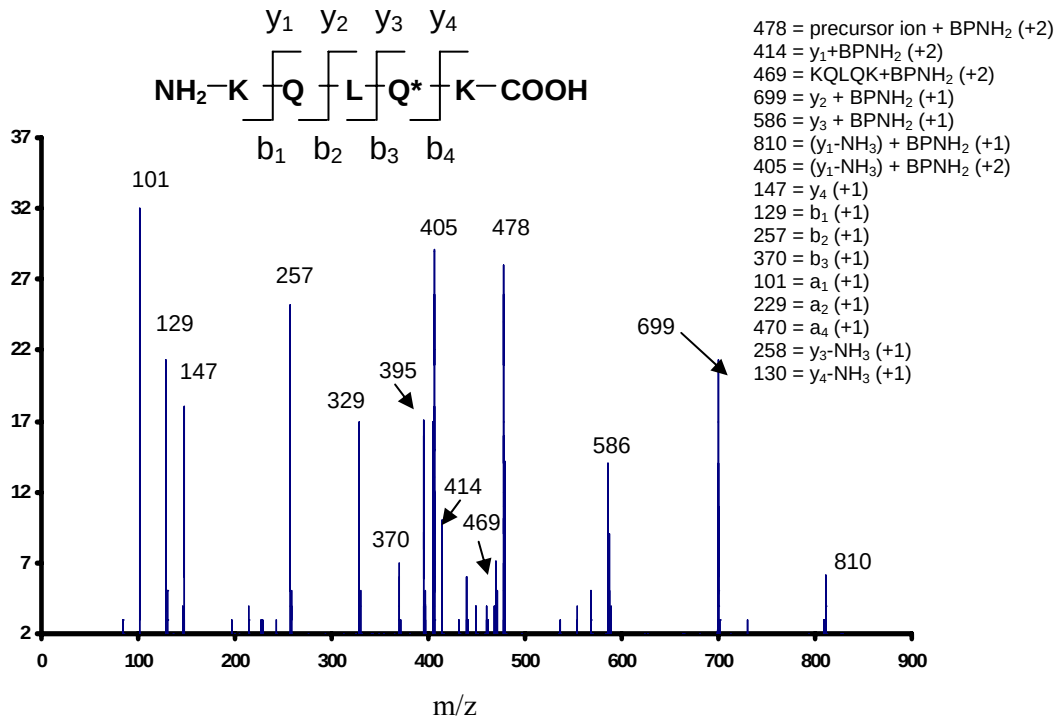

Figure 6.7 Tandem mass spectrum obtained from fragmentation of the precursor ion at m/z of 478.1. The peptide sequence and interpretation of b- and y-ions are shown. Q* indicates the $BPNH_2$ tagged Q.

Hydrophilic amino acids (K, Q, E, or R) were also found in position (1) or (2) (Table 6.4). Ohtsuka et al. (2000) reported that activity of red sea bream liver TGase increased when E, N, or R was at the position of (1) or (2). Thus, amino acids at glutaminylamide site of Q* also play a role on the formation of substrate-enzyme intermediate.

McLachlan and Karn (1982) reported that amino acids at myosin rod display a characteristic seven-residue repeat pattern (haptad repeat), designated *a, b, c, d, e, f,* and *g*. This haptad repeat also arranged into 28 residues repeat zones. The hydrophobic

residues often locate at the positions of *a* and *d*, hydrophobic core, and stabilize the coiled coil structure via hydrophobic interactions (Moitra, Szilak, Krylov, and Vinson, 1997). In deed, most of amino acids at (-1) position of $BPNH_2$ tagged peptide catalyzed by FTG (Table 6.4) were arranged at positions of *a* or *d* of the 28 residue repeat zones (Togashi et al., 2000). The results inferred that Q* located near the hydrophobic core within the intact myosin rod. Coussons and Price (1992) proposed that the accessibility to enzyme was the first requirement of Q to be reactive residue for TGase. Thus, Q* residues on the intact myosin molecule could serve as glutamyl substrates for TGase when those Q* are exposed from the hydrophobic core to solvent. Amino acids at hydrophobic core of myosin rod could be exposed to the solvent upon thermal denaturation (Chan, Gill, and Paulson, 1993). Ogawa, Kanamaru, Miyashita, Tamiya, and Tsuchiya (1995) also reported that thermal unfolding of myosin rod is the pre-requisite for setting phenomenon, which is induced by TGase (Lee et al., 1997).

The general patterns of neighboring residues for Q* catalyzed by MTG have not been reported. Some of the $BPNH_2$-tagged peptides and $BPNH_2$ modification sites catalyzed by MTG were the same as those catalyzed by FTG. These results were in agreement to those reported by Sato et al. (2001). They revealed that MTG competed with fish TGase for same reactive groups on surimi proteins. It can be noticed that the equal amount of hydrophobic (50%) and hydrophilic (50%) amino acids were found at either (-2) or (-1) positions (Table 6.5), suggesting the broader glutamyl substrate specificity of MTG than FTG. Shimba, Yokoyama, and Suzuki (2002) suggested that MTG showed broader substrate specificity than red sea bream liver and guinea pig liver TGases when their glutamyl substrates within ovalbumin were analyzed by nuclear magnetic resonance.

Table 6.4 Identification of $BPNH_2$-tagged Q* residues of TB-myosin peptides catalyzed by FTG

No.	M	M*	Subfragment	Site	Sequence
1	**615**	**927**	**S2**	**1114-1118**	**ELQ*AR**
2	**750**	**1062**	**S2**	**1172-1177**	**EAEFQ*K**
3	1459.6	1770.9	S2	1098-1110	IEDEQSLGAQ*LQK
4	643.8	955.3	LMM	1814-1818	KQLQ*K
5	2003.2	2314.7	LMM	1508-1525	NLQQ*EISDLTEQIGETGK
6	996	1306.5	LMM	1379-1386	YETDAIQ*R
7	**1344**	**1656.1**	**LMM**	**1705-1716**	**VAEQ*ELVDASER**
8	**1289.4**	**1599.8**	**LMM**	**1825-1835**	**ELEAEVDAEQ*R**

Q* = $BPNH_2$ modified Q

M = Mass of singly charged peptide

M* = Mass of singly charged $BPNH_2$-tagged peptide

Bold entries indicate $BPNH_2$-tagged peptides that were also obtained by the action of MTG (see Table 6.5).

Kashiwaki et al. (2002) also reported that the catalytic group of MTG (Cys-64) is sufficiently exposed to the solvent and promptly react with substrate. In addition, the flexibility of the side wall of active site cleft of MTG also decreases the steric hindrance between enzyme and substrates. In contrast, the catalytic residue of red sea bream liver TGase (Cys-272) is inaccessible to the solvent and also hydrogen bond with the Oγ of Tyr-515 on the loop of the barrel domain, covering the active site (Yee et al., 1994). The complicated activation of Ca^{2+} dependent TGases was also proposed that binding of Ca^{2+} and acyl donor substrate causes conformational changes, leading

to removal of the covering Tyr from the active site and the acyl-enzyme intermediate can be formed. Hydrophobic amino acids at the possible acyl donor binding site of red sea bream liver TGase and Factor XIIIa is scarcely charged (Noguchi et al., 2001). These differences may be the reason for the different specificity between MTG and other Ca^{2+}– dependent TGases.

Table 6.5 Identification of $BPNH_2$-tagged Q* residues of TB myosin peptides catalyzed by MTG

No.	M	M*	Subfragment	Site	Sequence
1	**615**	**927**	**S2**	**1114-1118**	**ELQ*AR**
2	**750**	**1062**	**S2**	**1172-1177**	**EAEFQ*K**
3	2089.3	2400.7	S2	1029-1046	TKLEQQVDDLEGSLEQ*EK
4	**1344**	**1656.1**	**LMM**	**1705-1716**	**VAEQ*ELVDASER**
5	786.9	1097.6	LMM	1649-1655	NVQ*GQLK
6	**1289.4**	**1599.8**	**LMM**	**1825-1835**	**ELEAEVDAEQ*R**

Q* = $BPNH_2$ modified glutamine

M = Mass of singly charged peptide

M* = Mass of singly charged BPNH-tagged peptide

Bold entries indicate $BPNH_2$-tagged peptides that were also obtained by the action of FTG (see Table 6.4).

The different amounts of $BPNH_2$-tagged peptides and biotin content catalyzed by MTG and FTG were observed at the same unit of enzyme, which based on the ability of enzyme to incorporate MDC into DMC. However, the reactivity of both enzymes in incorporating $BPNH_2$ into TB-myosin peptides might exhibit to be

different from that in the assay. This suggested that the different specificities between MTG and FTG resulted in different abilities to incorporate $BPNH_2$ into TB-myosin peptides. Thus, the different amount of biotin content and $BPNH_2$-tagged peptides catalyzed by FTG and MTG were possibly due to the different thermal stabilities as well as substrate specificities of these two enzymes.

6.5 Conclusions

Higher thermal stability allowed MTG to catalyze TB-MHC cross-linking and also to incorporate $BPNH_2$ into TB-myosin peptides to the higher extent than by the more thermally labile enzyme, FTG. Amino acid sequence of peptides derived from TB-MHC exhibited highest similarity to that of amberjack-MHC. Tandem mass spectrometry successfully identified the amino acid sequence of $BPNH_2$-tagged peptides catalyzed by MTG and FTG, locating at the myosin rod. Most of amino acids at the glutaminylamide site of the reactive Q within peptides catalyzed by both enzymes were hydrophilic amino acids. FTG tended to catalyze peptides that contain hydrophobic amino acids at the α-acrylamide site of reactive Q. Amino acids at α-acrylamide site of reactive Q for MTG were either hydrophobic or hydrophilic amino acids, suggesting broader glutamyl substrate specificity.

6.6 References

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CHAPTER VII

SUMMARY

Ca^{2+} induced conformational changes of threadfin bream (TB) myosin and actin, leading to unfolding and exposure of hydrophobic amino acids. Hydrophobic interactions and disulfide linkages were important for aggregate formation of TB-myosin and actin incubated at either 25 or 40 °C. These interactions may be important in gel-forming of muscle proteins from TB during setting.

FTG showed Ca^{2+}- dependent characteristic similar to other tissue-TGases. The enzyme activity was inhibited by metal ions (Mn^{2+}, Cu^{2+}, Co^{2+} and Fe^{2+}) and various TGase inhibitors. The optimum conditions for catalytic reaction were observed at 50 °C and pH 8.5-9.0. FTG tended to retain activity at 0.6 M NaCl.

Myosin heavy chain (MHC) from TB-myosin, Pacific whiting natural actomyosin (PW-NAM), and TB-NAM was cross-linked by FTG to the lesser ability than did by microbial TGase (MTG) due to less thermal stability. NAM from fish living in temperate habitat underwent greater conformational changes than that from tropical fish. Conformational changes of NAM played much more critical for FTG in catalyzing protein cross-linking than MTG. Cross-linking sites for both MTG and FTG unlikely be at myosin head. MHC was extensively cross-linked by MTG than did FTG, resulting in higher textural improvement.

MTG incorporated 5-(biotinamido) pentylamine ($BPNH_2$) into TB-myosin peptides to the higher amount than did FTG. Identification of $BPNH_2$ modification

sites and the sequence of $BPNH_2$-tagged peptides were performed successfully by tandem mass spectrometry based onamberjack-MHC sequence. The identified sites of $BPNH_2$ modification catalyzed by both TGases were at the myosin rod and most amino acids at the glutaminylamide site of reactive glutamine (Q*) were hydrophilic amino acids. FTG preferentially catalyzed $BPNH_2$ modification of peptides that contain hydrophobic amino acids at α-acrylamide site of Q*. Amino acids at α-acrylamide site of Q* for MTG were either hydrophobic or hydrophilic amino acids, suggesting broader glutamyl substrate specificity.

Therefore, thermal stability and substrate specificity of TGases might be the critical factors in application of TGase for protein cross-linking. In addition, conformational changes of protein substrates induced by either Ca^{2+} or thermal treatment should also be concerned in order to obtain the desirable textural improvement of surimi-based products.

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