PURIFICATION AND CHARACTERIZATION OF TRANSGLUTAMINASE FROM TROPICAL TILAPIA (*Oreochromis niloticus*)

Miss Anulak Worratao

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Technology

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การทำบริสุทธิ์และคุณลักษณะของเอนไซม์ทรานสกลูทามิเนสจากปลานิล (*Oreochromis niloticus*)

นางสาวอนุลักษณ์ วรเท่า

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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(Asst. Prof. Dr. Jirawat Yongsawatdigul)
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วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาปริมาณเอนไซม์ทรานสกลูทามิเนสจากกล้ามเนื้อปลานิลและศึกษาการเชื่อมโยงแอคโตมัยโอซินปลานิลโดยทรานสกลูทามิเนส นอกจากนี้ได้ศึกษาการทำเอนไซม์ให้บริสุทธิ์รวมทั้งศึกษาคุณสมบัติทางชีวเคมีของเอนไซม์ทรานสกลูทามิเนสบริสุทธิ์ จากผลการศึกษาพบว่ากล้ามเนื้อปลานิลมีปริมาณเอนไซม์ทรานสกลูทามิเนสเท่ากับ 60.8 ยูนิตต่อกรัมตัวอย่างซึ่งสูงกว่าปลา 11 ชนิดที่นำมาศึกษา ความเข้มข้นโซเดียมคลอไรด์ที่เหมาะสมต่อการเร่งปฏิกิริยาการเชื่อมโยงมัยโอซินเฮฟวีเชนของ crude เอนไซม์ทรานสกลูทามิเนสคือ 0.4 โมลาร์ แต่เมื่อใช้สารตั้งต้นโมโนแดนซิลคาดาเวอรีนและไดเมธิลเคซีนพบว่ากิจกรรมของเอนไซม์ลดลงเมื่อเพิ่มความเข้มข้นของโซเดียมคลอไรด์ ส่วนไดไธโอทริทอลไม่มีผลต่อกิจกรรมการเร่งปฏิกิริยาของ crude เอนไซม์ทั้งต่อสารตั้งต้นที่เป็นโมโนแดนซิลคาดาเวอรีนและไดเมธิลเคซีนหรือแอคโตมัยโอซิน มวลโมเลกุลของเอนไซม์บริสุทธิ์มีค่าประมาณ 85 กิโลดาลตันและค่าจุดไอโซอิเล็คตริคเท่ากับ 6.53 อุณหภูมิและพีเอชที่เหมาะสมของเอนไซม์บริสุทธิ์เท่ากับ 37-50 องศาเซลเซียสและ 7.5 ตามลำดับ ความเข้มข้นที่เหมาะสมของแคลเซียมคลอไรด์และไดไธโอทริทอลต่อการเร่งปฏิกิริยาเท่ากับ 1.25 และ 5 มิลลิโมลาร์ตามลำดับ เอนไซม์ทรานสกลูทามิเนสจากปลานิลถูกยับยั้งปฏิกิริยาเมื่อมีสารจับโลหะ โลหะหนักและสารที่ทำปฏิกิริยากับหมู่ซัลไฮดริลซึ่งแสดงว่าเอนไซม์ทรานสกลูทามิเนสจากปลานิลเป็นเอนไซม์ที่มีความจำเป็นต้องใช้แคลเซียมในการเร่งปฏิกิริยาและมีหมู่ซัลไฮดริลที่บริเวณเร่ง

สาขาวิชาเทคโนโลยีอาหาร	ลายมือชื่ออาจารย์ที่ปรึกษา…….
ปีการศึกษา 2545	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……..
ลายมือชื่อนักศึกษา……	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……..

ANULAK WORRATAO : PURIFICATION AND CHARACTERIZATION OF TRANSGLUTAMINASE FROM TROPICAL TILAPIA (*Oreochromis niloticus*)
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Objectives of this study were to investigate TGase activity of tropical tilapia muscle and to study the cross-linking of tilapia actomyosin by crude tilapia TGase. In addition, to purify as well as characterize tilapia TGase. Tilapia muscle contained TGase activity of 60.8 unit/g sample, which was the highest among other 11 freshwater fish studied. Crude TGase optimally catalyzed cross-linking of myosin heavy chain at 0.4 M NaCl. But its activity towards monodansylcadaverine (MDC) and N,N′-dimethylated casein (DMC) decreased as NaCl increased. Dithiothreitol (DTT) had no effect on crude TGase activity when either MDC and DMC or actomyosin was used as substrates. Molecular weight of purified tilapia TGase was estimated to be 85 kD with apparent pI of 6.53. The purified enzyme showed an optimal temperature at 37-50^{o}C and an optimum pH at 7.5. Calcium chloride and DTT increased TGase activity at the final concentration of 1.25 and 5 mM, respectively. The purified tilapia TGase was strongly inactivated by chelating agents, heavy metal ions, and sulfhydryl alkylating agents, suggesting that tilapia TGase is Ca^{2+}-dependent enzyme and possesses a thiol group at the active site.

School of Food Technology Advisor……...
Academic Year 2002 Co-advisor…….
Student……. Co-advisor……

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Anulak Worratao

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LIST OF ABBREVIATIONS

ABS	=	Absorbance
$BaCl_2$	=	Barium chloride
BME	=	β-Mercaptoethanol
BSA	=	Bovine serum albumin
^{O}C	=	Degree Celsius
$CaCl_2$	=	Calcium chloride
cm	=	Centimeter
CP	=	Cross-linked polymer
$CuCl_2$	=	Copper chloride
DEAE	=	Diethylaminoethyl
DMC	=	N,N′-Dimethylated casein
DTT	=	Dithiothreitol
EDTA	=	Ethylenediaminetetraacetic acid
EF	=	Enhancement factor
EGTA	=	Ethylene glycol-O,O′-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid
Fig.	=	Figure
g	=	Gram
h	=	Hour
IAA	=	Iodoacetic acid

LIST OF ABBREVIATIONS (Continued)

IEF	=	Isoelectric focusing
KCl	=	Potassium chloride
kD	=	Kilo dalton
L	=	Liter
$LiCl_2$	=	Lithium chloride
M	=	Molar
MDC	=	Monodansylcadaverine
mg	=	Milligram
$MgCl_2$	=	Magnesium chloride
MHC	=	Myosin heavy chain
min	=	Minute
$MnCl_2$	=	Manganese chloride
mL	=	Milliliter
mM	=	Millimolar
MW	=	Molecular weight
NaCl	=	Sodium chloride
NEM	=	N-Ethylmaleimide
ng	=	Nanogram
nm	=	Nanometer
PCMB	=	ρ-Chloromercuribenzoate
pI	=	Isoelectric point
PMSF	=	Phenyl methyl sulfonyl fluoride

LIST OF ABBREVIATIONS (Continued)

rpm	=	Revolution per minute
SDS	=	Sodium dodecyl sulfate
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH	=	Sulfhydryl
TEMED	=	N,N,N′,N′ -Tetramethyl-ethylenediamine
TGase, TG	=	Transglutaminase
TNBS	=	2,4,6-Trinitrobenzenesulfonic acid
Tris	=	Tris(hydroxyaminomethane)
Tris-HCl	=	Tris-hydrochloride
UV	=	Ultraviolet
V	=	Volt
×g	=	Gravitational acceleration
µg	=	Microgram
µL	=	Microliter
$ZnCl_2$	=	Zinc chloride

CHAPTER I

INTRODUCTION

Transglutaminase (TGase, protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) is an enzyme that catalyzes an acyl-transfer reaction between γ-carboxyamide groups of a glutamine residue and primary amino groups (Folk, 1980; Greenberg et al., 1991). When an ε-amino group of lysine acts as an acyl acceptor, protein molecules are covalently cross-linked, resulting in intra- and inter-molecular ε-(γ-glutamyl)lysine (GL) bonds (Folk, 1980; Greenberg et al., 1991). These bonds are stable and resistant to proteolysis (Joseph et al., 1994). Therefore, TGase has been widely study to improve functional properties of various food proteins (Jiang et al., 1992).

TGase has been used to improve the functionality and nutritional properties of food proteins (Ikura et al., 1985). TGase can enhance the gel strength of surimi (Sakamoto et al., 1995). Surimi is a Japanese term refering to fish paste produced by mincing and washing fish flesh. Surimi is used as a raw material for seafood analogs, such as imitation crab meat. Myosin is the major component responsible for surimi gelation (An et al., 1996). Surimi pastes can form very strong gels at low temperatures ($<40^{o}$C). Mostly these gels are form with hydrogen, hydrophobic, and disulfide bonds (Araki and Seki, 1993). TGase has the ability to covalently cross-link myosin heavy chain (MHC), resulting in the polymer GL bonds which can increase the gel strength (An et al., 1996). Cross-linking of myosin with endogenous TGase is associated with

a gel strength in surimi made with Alaska pollock (Sakamoto et al., 1995). Seki et al. (1990) isolated TGase from Alaska pollock and found that it could induce gelation of minced fish. It has been reported that strong gel-forming ability of sardine was due to the action of TGase (Tsukamasa et al., 1993). Jiang and Lee (1992) also reported that plasma factor XIII also had very strong MHC cross-linking. In addition, bovine TGase-enriched plasma fraction was used to enhance gel strength of Pacific whiting surimi (Seymour et al., 1997).

Tropical tilapia (*Oreochromis niloticus*) is an important freshwater species in Thailand. It was reported that tilapia surimi exhibited "setting" at 40^{o}C and these cross-links were likely to be related to an increased gel strength of minced fish (Klesk et al., 2000). However, knowledge of tilapia TGase's catalytic reaction is still limited. Understanding biochemical characteristics of TGase would lead to improve textural properties of tilapia mince and increase its use in various value-added products.

RESEARCH OBJECTIVES

Objectives of this study were :

(1) to investigate TGase activity in tilapia muscle,

(2) to study the cross-linking of tilapia actomyosin by crude tilapia TGase,

(3) to purify TGase from tilapia muscle, and

(4) to investigate biochemical properties of the purified TGase.

LITERATURE REVIEWS

TGases are found in different sources and known by different names, such as Factor XIIIa, Fibrinoligase (Folk, 1980). The presence of TGase is based on the

fact that the GL bond is taken place in the organisms (Folk, 1980). TGase can be categorized as follows:

1. **Keratinocyte TGase (TGase K).** This is a membrane bound TGase and found mainly in the keratinocytes, which are the principal cell type in mammalian epidermis and important for the formation of the cell envelope. The cDNA-cloning from human and rat keratinocytes revealed a protein composed of 817/813 and 824 amino acids, respectively, with a molecular weight (MW) of 89-90 kD (Phillips et al., 1990; Kim et al., 1991).

2. **Epidermal TGase (TGase E).** It is responsible for the later stages of cell envelope formation in the epidermis and the hair follicle. It is a monomer and zymogen that requires proteolytic activation. The cDNA-cloning from human and mouse epidermis revealed a protein of 692 amino acids with a MW of about 77 kD (Kim et al., 1993).

3. **Tissue TGase (TGase C).** A monomeric enzyme located in the cytoplasm. It highly concentrated in the liver and also found in other tissues. It modulates the regeneration of cells in tissue repair (Greenberg et al., 1991). Other tissue-type TGases were also found in fish muscle, including sardine, mackerels (Pacific, horse, and Atka), red sea bream, Ayu, carp, silver eel, white croaker, walleye pollock, chum salmon, and rainbow trout (An et al., 1996; Araki and Seki, 1993), as well as in surimi of these species. However, TGase activity are different depending on fish species (Araki and Seki, 1993).

4. **Plasma TGase (Factor XIII)**. It is also known as fibrinoligase, fibrin-stabilizing factor, and blood coagulation factor XIII. Factor XIII is a tetramer composed of two a-chains and two b-chains noncovalently associate. It is a zymogen

that activated by thrombin (to factor XIIIa) during blood coagulation (Lorand, 1986). Factor XIII also exists as a dimer of "a" subunits in platelets, placenta, uterus, and prostate.

5. **Microorganisms TGase (MTGase).** TGase has been found in a variant broth culture of microorganisms. Extracellular TGases were purified from cultural filtrate of *Streptoverticillium mobarense* (Gerber et al., 1994), from *Streptoverticillium* sp. (Ando et al., 1989) and from *Streptoverticillium ladakanum* (Tsai et al., 1996). Intracellular TGases were also found in *Bacillus subtilis* (Ramanujam and Hageman, 1990) and in *Physarum polycephalum* (Klein et al., 1992).

Molecular Characteristics of TGases

1. Factor XIII

Factor XIII is the best characterized TGase. It is a plasma protein that circulates in blood as a tetramer (MW 320 kD) and consists of two catalytic "a" subunits (MW 75 kD each) and two non-catalytic "b" subunits (MW 80 kD each). The "a" chain is folded into four sequential domains and the active site is located in the central core domain. The "b" subunit is thought to stabilize the "a" subunit.

The amino acid sequence of the factor XIII a-chain was established by cDNA cloning (Ichinose et al., 1990). The major protein consists of 730-731 amino acid residues with a MW of 83 kD. The cDNA sequence reveals the absence of a typical NH_2-terminal hydrophobic residue.

As for the amino acid sequence of the factor XIII b-chain was derived by sequencing cDNA clones (Bottenus et al., 1990). The major protein contains 641

amino acids with a MW of 80 kD. Purified factor XIII b-chains were reported to modulate activation of blood coagulation (Greenberg et al., 1991).

2. Tissue TGase

The amino acid sequences of tissue TGase were deduced from the cDNA sequences (Ikura et al., 1988). The major protein concists of 690 amino acids with a MW of 77 kD (Ikura et al., 1988). TGase contains 16-18 sulfhydryl groups and no disulfide bonds (Ikura et al., 1988). Active site of guinea pig liver TGase (GPTGase) is a sulfhydryl group at position 276. Pentapeptide sequence (Tyr-Gly-Gln-Cys-Trp) is conserved active site of TGase, therefore they are inhibited by sulfhydryl alkylating agents (NEM, PCMB, IAA) and heavy metal ions, such as Cu^{2+}, Hg^{2+}, Zn^{2+}, and Fe^{2+} (Jiang and Lee, 1992). It has been shown that the active site Cys is located near a hydrophobic region of TGase molecule (Ichinose et al., 1990).

3. Comparison molecular characteristic of human factor XIII, red sea bream TGase and MTGase

Red sea bream TGase shows 33% sequence homology to human factor XIII (Yasueda et al., 1995). It has also reported that the overall and active site structures of red sea bream TGase are similar to those of human factor XIII (Noguchi et al., 2001). They consists of four sequential domains named “β-sandwich,” “core,” “barrel 1,” and “barrel 2” (Yee et al., 1994). On the other hand, the structure of MTGase belongs to the α+β folding class, containing 11 α-helices and 8 β-strands. Therefore, the overall structure of MTGase is completely different from that of red sea bream TGase and factor XIII (Yee et al., 1994). In addition, red sea bream TGase and human factor

XIII required Ca^{2+} for catalytic activity, but MTGase does not require (Yasueda et al., 1995).

3.1 Active site stucture

The arrangements of the active sites, Cys64 in MTGase and Cys272 in red sea bream TGase, exist near the N terminus of the α-helices (α3 helix in MTGase). The catalytic triad of red sea bream TGase consists of Cys272, His332, and Asp355, while factor XIII consists of Cys314, His373, and Asp396. On the other hand, at the active site of MTGase is not showed the catalytic triad as "Cys-His-Asp(Asn)".

3.2 Activation process

The activity processes of red sea bream TGase and factor XIII are thought to be similar. The catalytic Cys residue is situated between a Tyr residue (Tyr515 in FTGase and Tyr560 in human factor XIII) and another Cys residue (Cys333 in FTGase and Cys374 in factor XIII). Tyr belongs to barrel 1 and its located covers the core. Therefore, Tyr must be removed (Noguchi et al., 2001). The activation process of FTGase is as follows (Noguchi et al., 2001). Firstly, calcium ion bound to a binding site of TGase molecule, resulting in a conformational change. Secondly, Tyr covering the catalytic Cys is removed. The acyl donor is binding with the Cys at the active site, resulting in an acyl-enzyme intermediate and ammonia was released. Finally, an acyl acceptor accesses the active site, resulting the GL bond. The activation process of human factor XIII is as same as FTGase but it needs thrombin to cleave the factor XIII to factor XIIIa in the presence of Ca^{2+}.

Substate specificity

TGase from different sources displays different substrate specificities (Folk, 1980). It was reported that the reactive Gln residues, which located at the surface of proteins, an N- or C-terminal Gln residue was considered as adverse for modification. The Gln placed between two positively charged residues or between two Pro residues can be considered as a discouraging feature (Grootjans et al., 1995). The presence of positively charged residues at 2 or 4 residues away from the Gln towards the N-terminal side do not affect the specificity of tissue TGase (Pastor et al., 1999). In addition, side chains adjacent to the Lys residue is also important (Grootjans et al., 1995). Residues containing aliphatic side chains are favorable but the reactivity does not increase proportionally to the length of the side chain (Grootjans et al., 1995). It was reported that Arg has positive effect, Ser has usually favorable effect and Asp has an adverse effect. Therefore, the enzyme prefers uncharged and basic polar residues and smaller aliphatic ones near the amine donor Lys residue (Grootjans et al., 1995).

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CHAPTER II

CROSS-LINKING OF ACTOMYOSIN BY CRUDE TILAPIA (*Oreochromis niloticus*) TRANSGLUTAMINASE

Anulak Worratao and Jirawat Yongsawatdigul

Running title: Cross-linking by transglutaminase

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ABSTRACT

Tilapia muscle (*Oreochromis niloticus*) contained the highest transglutaminase (TGase) activity of 60.8 units/g, while striped snake-head fish (*Channa striatus*) contained the lowest TGase activity of 2.7 units/g. Optimal temperature and pH of crude tilapia TGase were 50^{o}C and 7-7.5, respectively. Crude tilapia TGase had high thermal stability at 6 and 25^{o}C for 2 h. TGase activity towards actomyosin was highest at 0.4 M NaCl, but its activity towards monodansylcadaverine (MDC) decreased as NaCl increased. Dithiothreitol (DTT) had no effect on TGase activity regardless of substrate. Solubility and ε-amino group content of actomyosin were highest at 0.4 and 0.3-0.4 M NaCl, respectively. At optimum conditions, crude tilapia TGase displayed high activity for myosin cross-linking.

Key words: Transglutaminase, tilapia (*Oreochromis niloticus*), actomyosin, cross-linking

INTRODUCTION

Transglutaminase (TGase, protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) is an enzyme that catalyzes an acyl-transfer reaction between γ-carboxyamide groups of a glutamine residue and primary amino groups (Folk, 1980; Greenberg et al., 1991). When an ε-amino group of lysine acts as an acyl acceptor, protein molecules are covalently cross-linked, resulting in intra- and inter-molecular ε-(γ-glutamyl)lysine (GL) bonds. (Folk, 1980; Greenberg et al., 1991). TGase is widely found in living organisms, such as mammals, plants, microorganisms, and marine organisms including fish muscle (Ando et al., 1989; Icekson and Apelbaum, 1987; Wong et al., 1990; Yasueda et al., 1994).

TGase can catalyze *in vitro* cross-linking of carp, Alaska pollack, Atlantic croaker, and threadfin-bream myosin heavy chain (MHC) (Jiang et al., 2000; Joseph et al., 1994; Kishi et al., 1991; Seki et al., 1990). The enzyme is largely responsible for the "setting" phenomenon on fish protein gel, resulting in an increased covalent cross-linking of MHC and formation of an elastic gel. Optimum setting condition varies with fish species (Araki and Seki, 1993). Joseph et al. (1994) reported that the rate of MHC cross-linking induced by TGase was limited by the conformation of both TGase and substrate molecules. Protein solubility and exposure of ε-amino groups are important factors governing the catalytic reaction of TGase. Gel-forming ability of fish proteins can be enhanced through understanding the optimum condition for the catalytic reaction by TGase.

Tropical tilapia (*Oreochromis niloticus*) is an important freshwater species in Thailand. Annual catches were increased from 84,800 Metric tons in 1992 to 113,600

Metric tons in 1998 (Department of Fisheries, 2001). Most are sold raw and as frozen products with relatively low-value. Klesk et al. (2000) demonstrated that tilapia surimi exhibited "setting" at 40^{o}C. Its gel quality was comparable with that of Alaska pollack. However, knowledge of tilapia TGase's catalytic reaction is still limited. Understanding the role of TGase in MHC cross-linking would lead to a means to improve the textural properties of tilapia mince and increase its use in various value-added products. Hence, our objectives were to investigate TGase activity in tropical tilapia muscle and to study the cross-linking of tilapia actomyosin by crude tilapia TGase.

MATERIALS AND METHODS

Chemicals

Monodansylcadaverine (MDC), N,N′-dimethylated casein (DMC), β-mercaptoethanol (BME), Tris(hydroxyaminomethane), calcium chloride ($CaCl_2$), bovine serum albumin (BSA), and Folin Ciocalteu's phenol reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), dye reagent and Coomassie brilliant blue R-250 were purchased from Bio-Rad (Richmond, CA, USA). Dithiothreitol (DTT) was purchased from Fluka BioChemica (Buchs, Switzerland). Urea was purchased from Promega (Madison, WI, USA). All other reagents and chemicals were analytical grade.

Raw Materials

Frozen fish were used to determine TGase activity. Tropical tilapia (*Oreochromis niloticus*), giant gourami (*Osphronemus goramy*), rohu (*Labeo rohita*),

striped snake-head fish (*Channa striatus*), common silver barb (*Puntius gonionotus*), common carp (*Cyprinus carpio)*, silver carp (*Hypophthalmichthys molitrix*), giant catfish (*Clarias gariepinus*), and small scale mud carp (*Cirrhina microlepis*) were purchased from a fish farm at Nakhon Ratchasima and transferred to the laboratory within 30 min. Threadfin-bream (*Nemipterus hexodon*) was obtained from Andaman Surimi Industry Co., Samutsakorn and transferred in ice to the laboratory within 2.5 h. Grey featherback (*Notopterus notopterus*), and spotted featherback (*Notopterus chitala*) were purchased from a fish farm in Bangkok and transferred in ice to laboratory within 2 h. Fish were 8-12 months old, depending on species. Fish were gutted and skined upon arrival to the laboratory. Samples were vacuum-packed in a plastic bag and kept at –20^{o}C throughout the study.

Live tropical tilapia (*Oreochromis niloticus*) were used to prepare actomyosin and crude TGase. Live fish were purchased from the University Farm and transferred to the laboratory within 10 min. Fish were 8 months old with an average weight of 500 g each. The fish were killed upon arrival. Crude TGase and actomyosin were immediately extracted.

Preparation of Crude TGase

Whole muscle (ordinary and dark muscle) was homogenized with 4 volumes of extraction buffer (10 mM NaCl, 5 mM EDTA, 2 mM DTT, 10 mM Tris-HCl, pH 7.5). The homogenate was centrifuged at 16,000×g (Rotor F28/50, Sorvall RC 28S, Dupont Co., Newtown, CT., USA) for 20 min at 4^{o}C. Subsequently, the supernatant was centrifuged at 100,000×g (Rotor SW 40Ti, class S, Beckman Instruments Inc., Palo Alto, CA, USA) for 60 min at 4^{o}C. The supernatant was referred to as crude

TGase. The amount of protein in crude TGase was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin (BSA) as a standard.

Determination of TGase Activity

TGase activity was measured in term of the incorporation of MDC into DMC according to the procedure of Takagi et al. (1986) with a slight modification. The reaction mixture contained 1.25 mg/mL DMC, 18.75 μM MDC, 3.75 mM DTT, 6.25 mM $CaCl_2$, 62.5 mM Tris-HCl (pH 7.5), and 100 μL of crude tilapia TGase. The reaction mixture was incubated at 37^{o}C for 10 min, the time found to be in a linear range, and stopped by adding ethylenediaminetetracetic acid (EDTA) solution to a final concentration of 20 mM. The fluorescence intensity of MDC incorporated into DMC was measured using a Shimadzu fluorescence spectrofluorophotometer (RF-1501, Shimadzu Co., Kyoto, Japan) at an excitation and emission wavelengths of 350 and 480 nm, respectively. The control was performed as same as described above, except the EDTA was added in the reaction mixture before crude TGase. One unit of TGase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of MDC into DMC during 1 min of incubation at 37^{o}C. The enhancement factor (EF) indicating an increased fold of fluorescence intensity upon the incorporation of MDC into DMC was 1.93.

Optimal Temperature

The reaction mixtures contained 1.25 mg/mL DMC, 18.75 μM MDC, 3.75 mM DTT, 6.25 mM $CaCl_2$, 62.5 mM Tris-HCl (pH 7.5) were pre-incubated at various temperatures (0, 25, 37, 40, 45, 50, 55, 60, 65, and 70^{o}C) for 5 min. The reaction was

initiated by adding 20 μL of crude TGase and incubated at the respective temperature for 10 min. The reaction was stopped by adding EDTA solution to a final concentration of 20 mM. TGase activity was assayed as described previously.

Optimal pH

The reaction mixtures contained 1.25 mg/mL DMC, 18.75 μM MDC, 3.75 mM DTT, 6.25 mM $CaCl_2$, and various buffers (pH 4-6.5, using 100 mM acetate buffer; pH 7-7.5, using 50 mM Tris-HCl; pH 8-9, using 50 mM borate buffer) were pre-incubated at 37^{o}C for 5 min. The reaction was initiated by adding 20 μL of crude TGase and incubated at 37^{o}C for 10 min. The reaction was stopped by adding EDTA solution to a final concentration of 20 mM. TGase activity was assayed as described previously.

Thermal Stability

Crude TGase suspended in extraction buffer was incubated at various temperatures (6-50^{o}C) for 1 min – 24 h. At each determined time interval, the enzyme was cooled immediately in iced water for 15 min. Subsequently, it was centrifuged at 5,000×g for 10 min at 4^{o}C. Remaining activity of the supernatants were measured at 37^{o}C as described previously. Relative activity was calculated as the percentage of activity remaining after incubation at various temperatures, taking the initial TGase activity of a sample without incubation as 100%.

Preparation of Actomyosin

Actomyosin was prepared according to the method of Ogawa et al. (1999) with a slight modification. Tilapia whole muscle was homogenized in 5 volumes of 50 mM NaCl, 20 mM Tris-HCl, pH 7.5. The homogenate was centrifuged at 8,000×g (Rotor PK 121R, ACCEL, MI, Italy) for 5 min. The resultant pellet was homogenized in 10 volumes of 0.5 M NaCl, 20 mM Tris-HCl, pH 7.5, and the homogenate was centrifuged at 8,000×g (Rotor PK 121R, ACCEL, MI, Italy) for 5 min. The supernatant was filtered through triple-layer cheesecloth. The supernatant was diluted with 3 volumes of 20 mM Tris-HCl (pH 7.5) with constant agitating. The pellet was collected by centrifugation at 8,000×g (Rotor PK 121R, ACCEL, MI, Italy) for 10 min and then suspended in 5 volumes of 50 mM NaCl, 20 mM Tris-HCl, pH 7.5. The washed pellet was collected by centrifugation at 8,000×g (Rotor PK 121R, ACCEL, MI, Italy) for 20 min. All procedures were carried out at 4^{o}C.

Effect of DTT on TGase Activity

The effect of DTT on TGase activity using MDC and DMC as substrates was determined as described above. The final concentrations of DTT in the reaction mixtures were 0.01, 1.01, 3.01, 5.01, 7.01, and 10.01 mM.

The effect of DTT on actomyosin cross-linking was done by solubilizing actomyosin in 0.5 M NaCl at 4^{o}C for 30 min. The amount of protein in the supernatant was measured by the dye-binding method (Bradford, 1976) using BSA as a standard. The reaction mixtures contained 20 mM Tris-HCl (pH 7.5), 2.5 mM $CaCl_2$, 0.5 M NaCl, 10 mg of actomyosin and various concentrations of DTT (0-10 mM). The mixture was pre-incubated at 37^{o}C for 5 min. The reaction was initiated by

adding 1 unit of crude TGase. The mixtures were incubated at 37°C for 2 h and then terminated by heating at 90°C for 5 min. SDS-PAGE of all samples were analysed.

Effect of NaCl on TGase activity

The effect of NaCl on TGase activity using MDC and DMC as substrates was determined as described above. The final concentrations of NaCl in the reaction mixture were 0, 0.15, 0.3, 0.4, 0.5, 0.6, and 1.0 M.

In the presence of TGase, the reaction mixture contained 20 mM Tris-HCl (pH 7.5), 2.5 mM $CaCl_2$, 10 mg of actomyosin and various concentrations of NaCl (0-1 M). The mixture was pre-incubated at 37°C for 5 min. The reaction was initiated by adding 1 unit of crude TGase into the mixture. The mixture was incubated at 37°C for 2 h and then terminated by heating at 90°C for 5 min. Samples were used for SDS-PAGE analyses. At each NaCl concentration, the reaction mixture in the absence of crude TGase was used as a control.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples (0.5 mL) were solubilized with 2.0 mL of solubilizing buffer (4% SDS, 7.5% BME, 8 M urea, 60 mM phosphate buffer, pH 7.0). Raw actomyosin (0.5 mL) was solubilized in 2.5 mL of solubilizing buffer. All samples were heated at 90°C for 3 min followed by mechanical shaking overnight at room temperature. Subsequently, the samples were centrifuged at 9,000×g (Rotor PK 121R, ACCEL, MI, Italy) for 30 min. Due to the presence of urea, protein content was measured by the dye-binding method (Bradford, 1976) using BSA as a standard. Samples were

boiled for 5 min in a sample buffer containing 20 mM sodium phosphate buffer (pH 7.0), 0.2% SDS, 0.2% BME, and 40% glycerol.

SDS-PAGE was carried out according to the procedure of Weber and Osborne (1969) using 4% acrylamide (w/v) and 0.25% (w/v) agarose (Cooper, 1977). Applied protein was 20 μg/lane. Electrophoresis was performed at a constant voltage of 60 V. Gels were stained with 0.125% Coomassie brilliant blue R-250 and destained in a solution containing 25% methanol and 10% acetic acid.

Actomyosin Solubility and ε–Amino Group Content

Solubility and ε-amino group content of actomyosin were elucidated by solubilizing 2 g of actomyosin in 18 mL of 20 mM Tris-HCl (pH 7.5) at various concentrations of NaCl (0, 0.15, 0.3, 0.4, 0.5, 0.6, and 1.0 M). The mixtures were stirred for 30 min and brought to a volume of 25 mL using the same buffer. Supernatants were collected by centrifugation at 9,000×g for 30 min at 4^{o}C. Protein content of the supernatant was determined by the Lowry method (Lowry et al., 1951) using BSA as a standard. The ε-amino group content was measured according to Fields (1972). The final concentration of 2,4,6-trinitrobenzenesulfonic acid (TNBS) was 7 mM. Absorbance at 420 nm was measured. The concentration of the ε-amino group was calculated as μmole/mL using $\varepsilon = 13{,}500\ M^{-1}cm^{-1}$ (Fields, 1972).

RESULTS AND DISCUSSION

TGase Activity in Fish Muscle

All fish samples analyzed were freshwater species, except for threadfin bream. TGase activities of these samples ranged from 2.7 to 60.8 units/g (Table 2.1). Tilapia showed the highest activity of 60.8 units/g, while striped snake-head fish contained the lowest TGase activity of 2.7 units/g. TGases are found in fish muscle including sardine, mackerels (Pacific, horse, and Atka), red sea bream, ayu, carp, silver eel, white croaker, walleye pollock, chum salmon, and rainbow trout as well as in surimi of these species (An et al., 1996; Araki and Seki, 1993; Kishi et al., 1991). The TGase activity of tilapia found in our study was greater than those reported in carp and white croaker muscle, 1.1-2.4 unit/g, which were assayed at 25^{o}C (Araki and Seki, 1993). Tilapia contained higher TGase than threadfin-bream, an important marine species for surimi production in Thailand. It is postulated that tilapia might be a potential raw material to produce an elastic protein gel. Klesk et al. (2000) reported that tilapia surimi exhibited "setting" at 40^{o}C, yielding high shear stress. Purification and characterization of TGase from tropical tilapia muscle is under investigation at our laboratory.

Optimum Temperature and pH

The optimal temperature of crude TGase for the catalytic reaction of MDC was 50^{o}C (Fig. 2.1), while the optimal pH was 7-7.5 (Fig. 2.2). The optimum pH found in this study was lower than those of guinea pig (Ikura et al., 1988), rat liver (Wong et al., 1990), Japanese oyster (*Crassostrea gigas*) (Kumazawa et al., 1997), red sea bream liver (Yasueda et al., 1994), and carp and scallop (Nozawa et al., 1997),

Table 2.1. TGase activity of various fish species [a].

Species	Activity (units/g of muscle)[b]
Tilapia (*Oreochromis niloticus*)	60.8 ± 0.3
Common carp (*Cyprinus carpio)*	18.0 ± 0.7
Giant catfish (*Clarias gariepinus*)	14.6 ± 0.8
Rohu (*Labeo rohita*)	11.4 ± 0.4
Spotted featherback (*Notopterus chitala*)	10.8 ± 0.3
Small scale mud carp (*Cirrhina microlepis*)	10.5 ± 0.2
Threadfin-bream (*Nemipterus hexodon*)	10.3 ± 0.2
Grey featherback (*Notopterus notopterus*)	7.1 ± 0.2
Giant gourami (*Osphronemus goramy*)	5.4 ± 0.4
Common silver barb (*Puntius gonionotus*)	3.9 ± 0.6
Silver carp (*Hypophthalmichthys molitrix*)	3.3 ± 1.0
Striped snake-head fish (*Channa striatus*)	2.7 ± 0.1

[a]Assayed at 37^{o}C and pH 7.5

[b]Activity is reported as mean ± S.D. (n=6)

which had pH optimums of 8.0-9.5. The optimal temperature of crude TGase was lower than that of red sea bream liver (55^{o}C) (Yasueda et al., 1994), but higher than that of Japanese oyster TG-1 and TG-2, which exhibited maximum activity at 40 and 25^{o}C, respectively (Kumazawa et al., 1997).

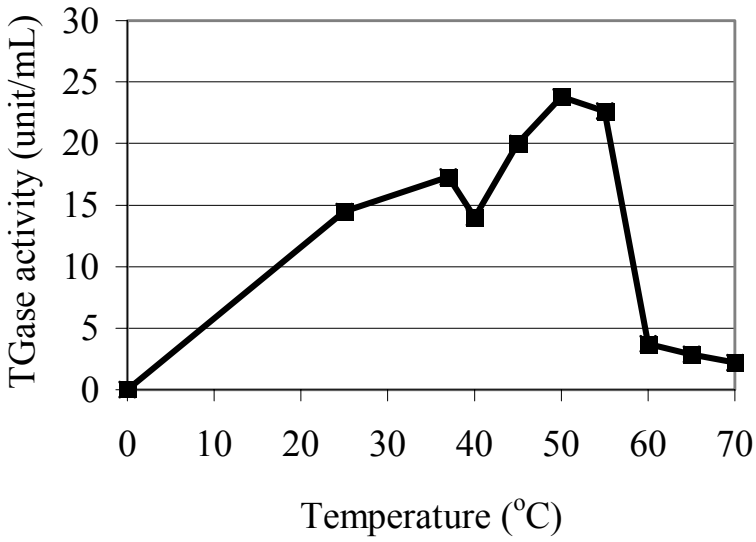

Fig. 2.1.Effect of temperature on crude tilapia TGase activity.

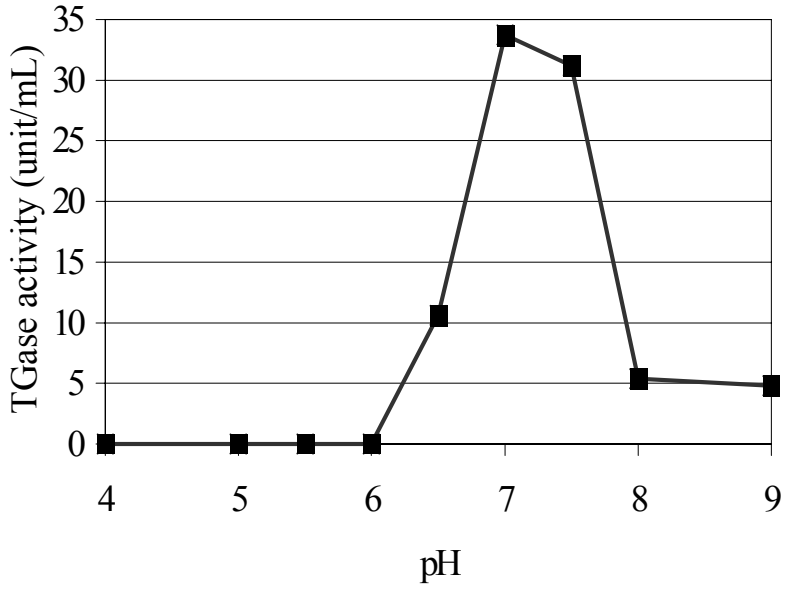

Fig. 2.2.Effect of pH on crude tilapia TGase activity at 37°C.

Thermal Stability

Crude tilapia TGase was more stable at 25°C than at 6, 37, and 50°C for up to 30 min (Fig. 2.3A and B). We speculated that the high thermal stability of TGase was due to the high habitat temperature of tropical tilapia. At 24 h incubation, however, remaining TGase activity at 6 and 25°C was 66 and 21%, respectively. Therefore, purification of TGase should be carried out below 6°C. Remaining TGase activity after holding the enzyme at 50°C for 10 min was 6% (Fig. 2.3B). Thermal stability of crude tilapia TGase at 50°C is similar to that reported by Kumazawa et al. (1996), who observed the rapid loss of Walleye pollock TGase activity within a few minutes at 50°C.

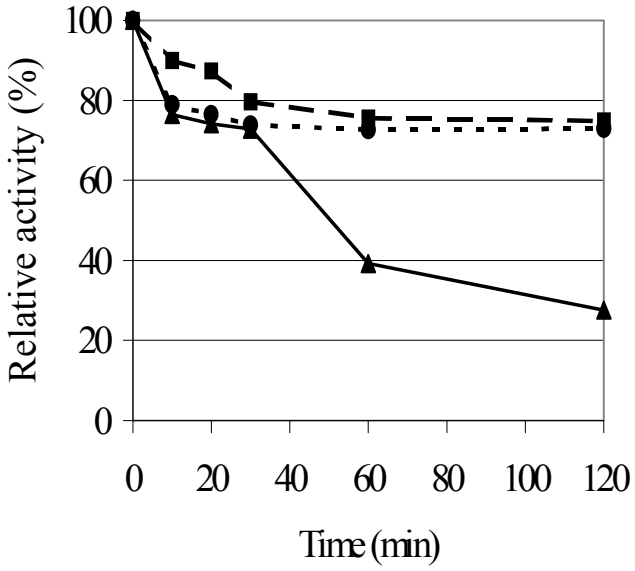

Fig. 2.3A. Thermal stability of crude tilapia TGase at various temperatures: (●) 6°C, (■) 25°C, (▲) 37°C.

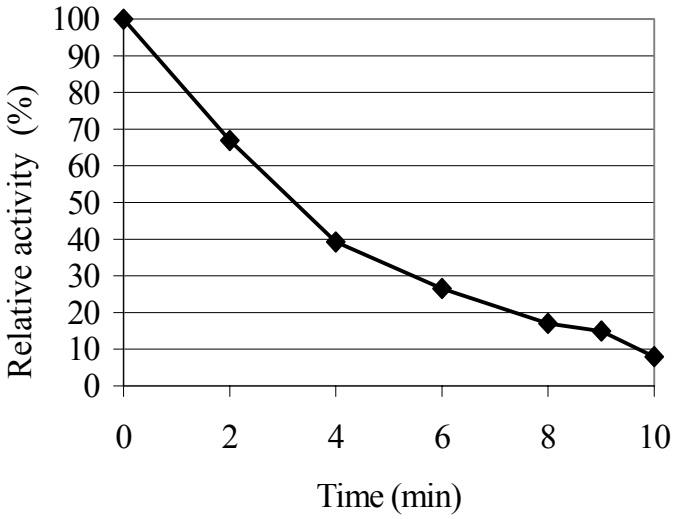

Fig. 2.3B. Thermal stability of crude tilapia TGase at 50^{o}C.

Effect of DTT on TGase Activity

Since crude tilapia TGase was not stable at its optimum temperature, 50^{o}C, (Fig. 2.3B) we examined the catalytic reaction of TGase at 37^{o}C. Crude tilapia TGase induced cross-linking of MHC, resulting in a cross-linked polymer (CP) (Fig. 2.4). In the presence of crude tilapia TGase, CP was observed in the samples with (Lane 1-10, Fig. 2.4) and without DTT (Lane 0, Fig. 2.4). In the absence of crude tilapia TGase, CP was not detected when actomyosin was incubated without NaCl at 37^{o}C for 2 h (lane 2h, Fig. 2.4). Therefore, NaCl is a prerequisite for cross-linking of actomyosin by TGase.

In addition, a decrease of MHC at various DTT concentrations was comparable (Lane 1-10, Fig. 2.4), indicating that crude tilapia TGase did not required additional DTT for catalyzing MHC cross-linking. These results were in agreement

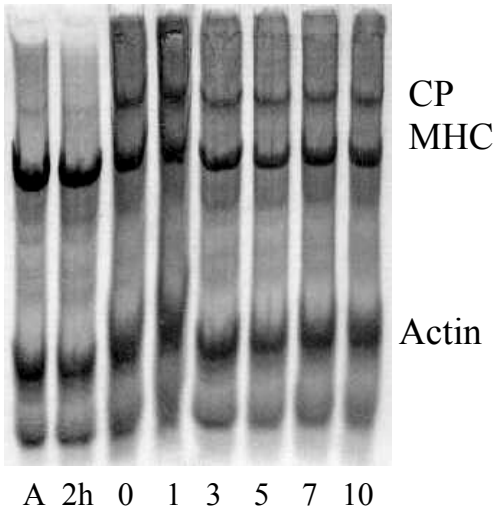

Fig. 2.4. Changes in SDS-PAGE patterns during incubation at various DTT concentrations of 10 mg actomyosin with 1 unit crude tilapia TGase. Lane A: actomyosin (Control sample, no incubation and without TGase), lane 2h: actomyosin incubated at 37^{o}C for 2 h (without TGase). Numbers 0-10 indicate concentration of added DTT in reaction mixture. Abbreviation: MHC, myosin heavy chain; CP, cross-linked polymer.

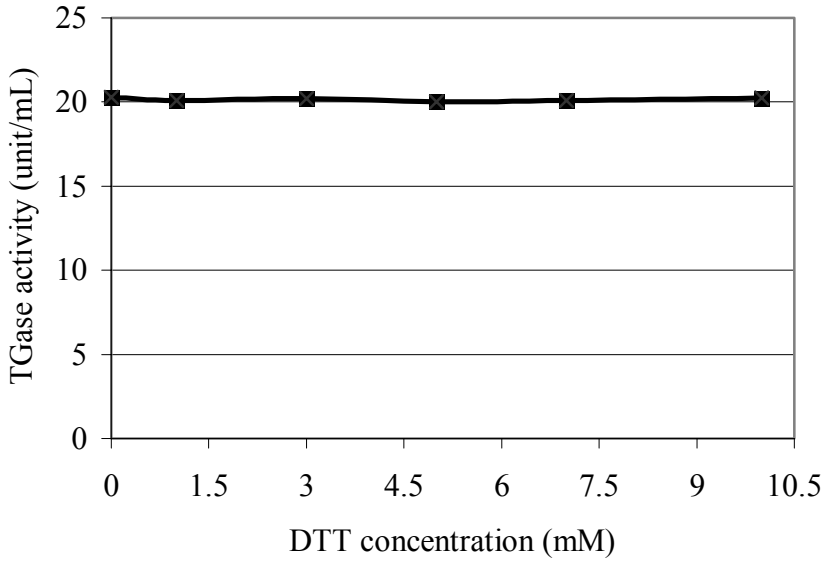

Fig. 2.5. Effect of DTT concentration on TGase activity assayed using MDC and DMC as substrates.

with those used MDC and DMC as substrates (Fig. 2.5). It is known that TGases possess sulfhydryl group at the active site and DTT was found to enhance TGase activity (Folk, 1980). Our results suggested that crude tilapia TGase did not required additional DTT in the assay buffer for the full expression of the activity. DTT concentration in the extraction buffer (2 mM) might be adequate to protect sulfhydryl group at an active site from oxidation. Tokunaga et al. (1993) also found that DTT had little effect on purified limulus hemocyte TGase.

Effect of NaCl on TGase Activity

MHC content decreased when the concentration of NaCl increased from 0 to 0.4 M (Fig. 2.6). A decrease of MHC at 0.4 M NaCl was the highest in the presence of TGase; CP also appeared at the top of the gel. The formation of MHC polymers catalyzed by TGase involved non-disulfide covalent bond, which was not solubilized by urea, SDS, and BME (Jiang et al., 2000; Kim et al., 1993). The MHC content of samples without added crude tilapia TGase also decreased as NaCl increased (Fig. 2.6). This might be due to the actomyosin preparation did not completely eliminate endogenous TGase. Therefore, NaCl is an important factor for catalyzing the cross-linking of actomyosin by tilapia TGase. The MHC of samples with added crude tilapia TGase decreased to a greater extent than those without TGase. It should be pointed out that the MHC content did not reduce further as the concentration of NaCl increased to greater than 0.4 M (Fig. 2.6). NaCl at high concentration (0.5-1 M) might cause conformation changes of the TGase molecule, resulting in a decreased MHC cross-linking. The activity of TGase with MDC and DMC suggested the adverse effect of NaCl on the enzyme structure (Fig. 2.7). The result was similar to the

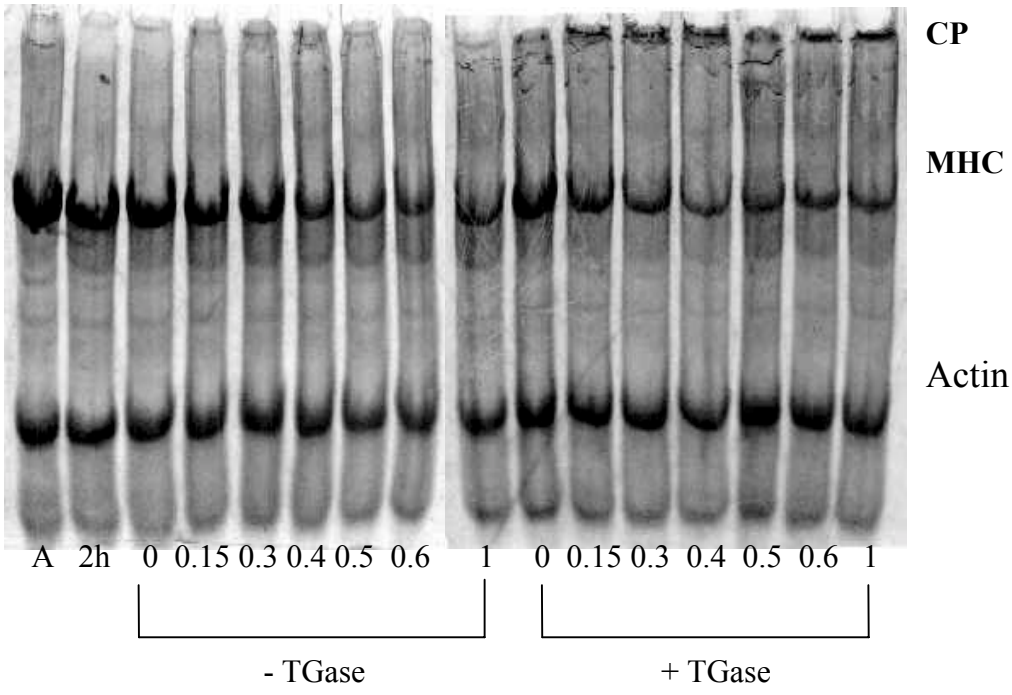

Fig. 2.6. Changes in SDS-PAGE patterns during incubation at various NaCl concentrations of 10 mg actomyosin with 1 unit crude tilapia TGase. Lane A: actomyosin (Control sample, no incubation and without TGase), lane 2h: actomyosin incubated at 37^{o}C for 2 h (without TGase). Numbers 0-1 indicate concentration of NaCl in reaction mixture. Abbreviation: MHC, myosin heavy chain; CP, cross-linked polymer.

activity of limulus hemocyte TGase (Tokunaga et al., 1993), Japanese oyster TGase (Kumazawa et al., 1997) and carp dorsal muscle TGase (Kishi et al., 1991), which also decreased with increasing NaCl. However, NaCl significantly activated TGases from scallop, squid, and shrimp (Nozawa et al., 1997). It should be noted that the cross-linking of MHC by tilapia TGase requires NaCl to unfold actomyosin but NaCl at high concentration can inactivate TGase activity.

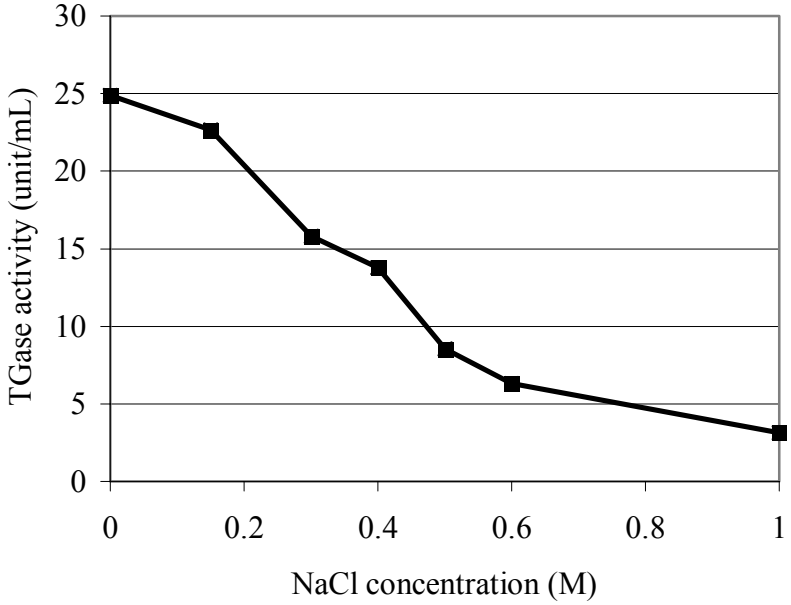

Fig. 2.7. Effect of NaCl concentration on TGase activity assayed using MDC and DMC as substrates.

Effect of NaCl on Actomyosin Solubility and ε-Amino Group Content

Solubility of actomyosin was highest at 0.4 M NaCl (Fig. 2.8), but ε-amino group content of actomyosin was highest at 0.3-0.4 M NaCl (Fig. 2.9). However, MHC cross-linking was highest at 0.4 M NaCl (Fig. 2.6). It is known that NaCl partially unfolds actomyosin, exposing more glutamine and lysine residues, substrates for TGase (Toyoda et al., 1992). Thus, a greater accessibility of ε-lysine residues by TGase could result in a higher degree of TGase-catalyzed MHC cross-linking. However, a lower MHC cross-linking at 0.3 M NaCl could be explained by the lower actomyosin solubility. Kurth and Rogers (1984) reported that the structure of the substrate was more important for the TGase reaction than their absolute lysine and glutamine contents. The extent of MHC cross-linking by crude tilapia TGase seemed

to depend on the conformation of both myosin and TGase. Since optimum NaCl for MHC cross-linking was 0.4 M NaCl, it was likely that actomyosin might exhibit more favorable conformation for MHC cross-linking by TGase at this condition. Therefore, actomyosin solubility appeared to be an important factor governing cross-linking of MHC.

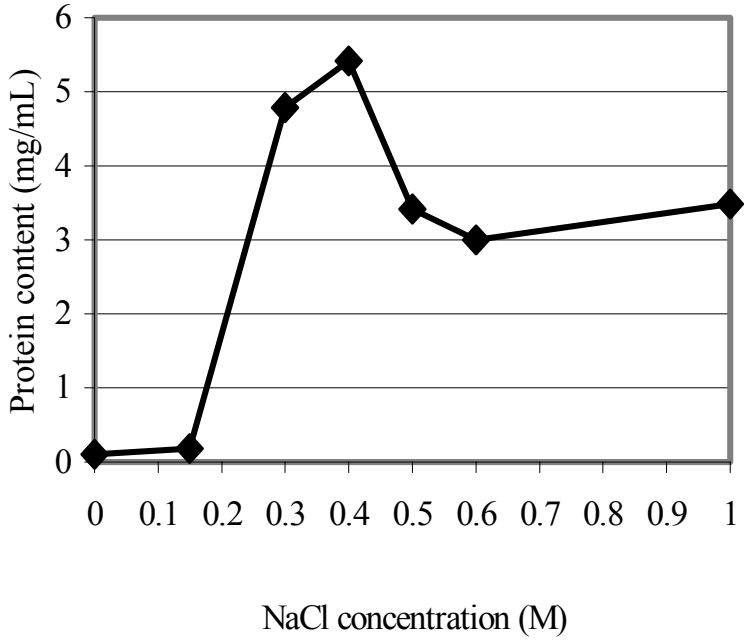

Fig. 2.8. Effect of NaCl concentration on solubility of actomyosin.

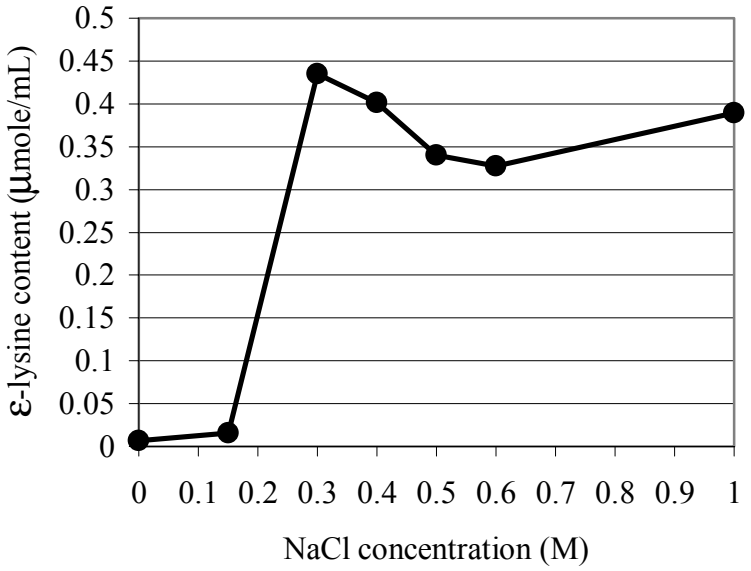

Fig. 2.9. Effect of NaCl concentration on ε-lysine content.

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CHAPTER III

PURIFICATION AND CHARACTERIZATION OF TRANSGLUTAMINASE FROM TROPICAL TILAPIA (*Oreochromis niloticus*)

Anulak Worratao and Jirawat Yongsawatdigul

ABSTRACT

Transglutaminase (TGase) from tropical tilapia (*Oreochromis niloticus*) was purified to electrophoretic homogeneity using successive chromatographies of DEAE-Sephacel, Sephacryl S-200 HR and HiTrap Heparin with a yield and purification-fold of 12.9% and 69.8 fold, respectively. Molecular weight of the purified tilapia TGase was estimated to be 85 kD using SDS-PAGE. pI of tilapia TGase was at 6.53. Optimal temperature and pH of tilapia TGase were at 37-50^{o}C and 7.5, respectively. Optimum concentration of $CaCl_2$ and DTT were at 1.25 and 5 mM, respectively. TGase activity towards MDC decreased as NaCl increased. Chelating agents, EDTA and EGTA, inhibited TGase activity. Tilapia TGase was strongly inactivated by ρ-chloromercuribenzoic acid (PCMB), N-ethylmaleimide (NEM), iodoacetamide (IAA), Cu^{2+}, and Zn^{2+}, suggesting the thiol group at the active site.

Key words: Transglutaminase, tilapia (*Oreochromis niloticus*), purification

INTRODUCTION

Transglutaminase is a transferase whose systematic name is protein-glutamine γ-glutamyltransferase (TGase; EC 2.3.2.13). The enzyme catalyzes the acyl-transfer reaction in which the γ-carboxyamide groups of glutamine residue in proteins, peptides and various primary amines, act as acyl donors and primary amino groups including ε-amino groups of lysine residues either as peptide-proteins bound or free lysine, act as the acyl acceptor (Folk, 1980; Greenberg et al., 1991). When acceptors are ε-amino group of lysine residues, the formation of ε-(γ-glutamyl)lysine (GL) linkages occur both intra- and inter-molecular of proteins (Folk, 1980; Greenberg et al., 1991). This introduces covalent cross-linkages between ε-amino group of lysine residue and the γ-carboxyamide group of a glutamine residue in protein molecule (Folk, 1980; Greenberg et al., 1991). These bonds are stable and resistant to proteolysis (Joseph et al., 1994). Therefore, TGase has been widely study to improve functional properties of various food proteins (Jiang et al., 2000).

TGases have been purified and characterized from various living organisms, such as mammals, plants, microorganisms, and marine organisms including fishes (Ando et al., 1989; Folk and Cole, 1966; Icekson and Apelbaum, 1987; Kumazawa et al., 1997; Yasueda et al., 1994). Seki et al. (1990) isolated TGase from Alaska pollock and found that it could induce the gelation of minced fish. Tsukamasa et al. (1993) further found the strong gel-forming ability of sardine was due to the formation of the non-disulfide covalent bond, which was resulted from the catalytic activity of TGase. Worratao and Yongsawatdigul (2003) demonstrated that tropical tilapia (*Oreochromis niloticus*) muscle contained high TGase activity and crude tilapia TGase catalyzed the

cross-linking of myosin heavy chains (MHC). These cross-links were likely to be related to an increased gel strength of minced fish. However, the purification of TGase from tilapia muscle has not yet been purified. In order to clarify the biochemical characteristics of tilapia TGase, we attempted to obtain the purified enzyme. Our objectives were to purify TGase from tropical tilapia muscle and to investigate biochemical properties of the purified TGase.

MATERIALS AND METHODS

Chemicals

Monodansylcadaverine (MDC), N,N′-dimethylated casein (DMC), β-mercaptoethanol (BME), Tris(hydroxyaminomethane), calcium chloride ($CaCl_2$), bovine serum albumin (BSA), Folin & Ciocalteu's phenol reagent, iodoacetic acid (IAA), N-ethylmaleimide (NEM), ρ-chloromercuribenzoate (PCMB), and phenyl methyl sulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co., Ltd (St. Louis, MO., USA). DEAE-Sephacel, Sephacryl S-200 HR, and HiTrap Heparin were purchased from Pharmacia (Uppsala, Sweden). Dithiothreitol (DTT) and urea were purchased from Promega (Madison, WI, USA). Sodium dodecyl sulfate (SDS), Coomassie brilliant blue R-250, standard protein kits for pI and SDS-PAGE were purchased from Bio-Rad (Richmond, CA, USA). All other reagents and chemicals were of analytical grade.

Raw Materials

Live tropical tilapia (*Oreochromis niloticus*) were purchased from the Suranaree University of Technology Farm and transferred to the laboratory within 10 min after catch. Fish were killed, gutted and skined upon arrival to the laboratory. Crude tilapia TGase was immediately extracted.

Preparation of crude TGase

Fish samples were homogenized with 4 volumes of extraction buffer (10 mM NaCl, 5 mM EDTA, 2 mM DTT, 10 mM Tris-HCl, pH 7.5). The homogenate was centrifuged at 16,000×g (Rotor F28/50, Sorvall RC 28S, Dupont Co., Nowtown, CT., USA) for 20 min at 4^{o}C. Subsequently, the supernatant was centrifuged at 100,000×g (Rotor SW 40Ti, class S, Beckman Instruments Inc., Palo Alto, CA, USA) for 60 min at 4^{o}C. The supernatant was used as crude TGase.

Determination of TGase activity

TGase activity was measured in term of the incorporation of MDC into DMC according to the procedure of Takagi et al. (1986) with a slight modification (Worratao and Yongsawatdigul, 2003). The reaction mixture contained 1.25 mg/mL DMC, 18.75 μM MDC, 3.75 mM DTT, 6.25 mM $CaCl_2$, 62.5 mM Tris-HCl (pH 7.5), and 20 μL of tilapia TGase. The reaction was performed at 37^{o}C for 10 min and stopped by adding EDTA solution at a final concentration of 20 mM. Fluorescence intensity of MDC incorporated into DMC was measured with a Shimadzu fluorescence spectrofluorophotometer (RF-1501, Shimadzu, Kyoto, Japan) at an excitation and emission wavelengths of 350 and 480 nm, respectively. One unit of

TGase activity was defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of MDC into DMC during 1 min of incubation at 37^{o}C.

Purification of tilapia TGase

DEAE-Sephacel. The crude TGase was applied onto a DEAE-Sephacel column (2.5×27 cm) equilibrated with 10 mM NaCl, 5 mM EDTA, 2 mM DTT, 10 mM Tris-HCl, pH 7.5 (buffer A). A flow rate of 60 mL/h was maintained. After being washed with 2 bed volumes of buffer A, the bound components were eluted with a linear gradient of 0-1 M NaCl. Fractions of 6 mL were collected using a fraction collector (Model 2110, Bio-Rad, Richmond, CA, USA) and assayed for TGase activity. Fractions possessing TGase activity were pooled, and concentrated into 3 mL by ultrafiltration with the molecular weight (MW) cut-off 30 kD (Centripep Model YM 30, Amicon, Inc., MA, USA).

Sephacryl S-200 HR. The 1.0 mL of the concentrated TGase obtained from DEAE-Sephacel column was applied to Sephacryl S-200 column (1.5×60 cm), equilibrated with buffer A containing 0.1 M NaCl. A constant flow rate of 120 mL/h was performed. Fractions of 6 mL were collected and used for TGase activity measurement. Fractions that contained TGase activity were combined, and concentrated into 1.5 mL by ultrafiltration membrane with the MW cut-off 30 kD (Centricon Model YM 30, Amicon, Inc., MA, USA).

HiTrap Heparin. One mL of the concentrated TGase obtained from Sephacryl S-200 was further loaded to HiTrap Heparin column (5 mL), equilibrated with the buffer A. A flow rate of 60 mL/h was maintained. TGase was eluted with a linear gradient of 0-1 M NaCl. Fractions of 5 mL were collected and assayed for

TGase activity. Fractions containing TGase activity were concentrated by ultrafiltration with the MW cut-off 30 kD (Centricon YM 30, Amicon, Inc., MA, USA) and used as purified TGase. All purification procedures were carried out at 4^{o}C.

Protein determination

The amount of protein was determined by Lowry method (Lowry et al., 1951) using bovine serum albumin (BSA) as a standard. In the case of chromatographic separations, protein concentration was monitored by measuring UV absorption at 280 nm.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to analyze protein samples during the purification procedure. Samples obtained from each purification step were boiled for 5 min in a sample buffer containing 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% BME. SDS-PAGE was performed according to the method of Leammli (Leammli, 1970) using stacking and separating gels of 4% (w/v) and 10% (w/v) polyacrylamide, respectively. Applied protein was 1.8 μg/lane. Electrophoresis was performed at a constant voltage of 120 V. Silver staining was applied according to Daniel et al. (1996). Gels were stained with silver nitrate, then developed by 0.005% acetic acid and 0.019% formaldehyde. The reaction was stopped by 1% acetic acid. To dertermine molecular weight of TGase, myosin (220 kD), β-galactosidase (116.25 kD), phosphorylase B (97.4 kD), serum albumin (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), trypsin inhibitor (21.5 kD), lysozyme (14.4 kD), and aprotinin (6.5 kD) were used as standards.

Isoelectric point

Isoelectric point (pI) was performed using a Pharmacia PhastSystem electrophoresis unit (Pharmacia, Uppsala, Sweden). One microliter of the purified tilapia TGase was applied on PhastGel 3/9 IEF gels as described by the manufacturer. Protein bands were visualized by silver staining in the development unit of the PhastSystem and following the instruction given by Pharmacia for IEF analysis. Calibration proteins for pI measurements were cytochrome c (pI 9.6), lentil lectin (7.8, 8.0, 8.2), human hemoglobin C (7.5), human hemoglobin A (7.1), equine myoglobin (7.0), human carbonic anhydrase (6.5), bovine carbonic anhydrase (6.0), β-lactoglobulin B (5.10), and phycocyanin (4.45, 4.65, 4.75).

Biochemical characteristics of tilapia TGase

Optimal temperature was tested using the reaction mixtures as described previously. Samples were pre-incubated and assayed at various temperatuers, 0, 25,37, 40, 45, 50, 55, 60, 65, and 70^{o}C. It was carried out at 62.5 mM Tris-HCl (pH 7.5), 6.25 mM $CaCl_2$ and 3.75 mM DTT.

Optimal pH was carried out as described above with various buffers, pH 4-6.5, using 100 mM acetate buffer; pH 7-7.5, using 50 mM Tris-HCl; pH 8-9, using 50 mM borate buffer. It was carried out at 6.25 mM $CaCl_2$ and 3.75 mM DTT.

Effect of $CaCl_2$ was carried out as described above at various concentrations of $CaCl_2$ (0- 5 mM) and at 3.75 mM DTT. Effect of DTT was carried out at 0-20 mM and at 1.25 mM $CaCl_2$.

Effect of NaCl was tested at various final concentration of 0-1.5 M in the presence of 1.25 mM $CaCl_2$. In addition, effect of KCl was tested with the final

concentration of 1 M. Effect of metal ions including $SrCl_2$, $MgCl_2$, $BaCl_2$ was investigated at 10 mM in the absences of $CaCl_2$, but the effect of $MnCl_2$ was investigated at 1 mM. Effect of $CuCl_2$ and $ZnCl_2$ were investigated at 10 mM in the presence of 1.25 mM $CaCl_2$.

The effect of inhibitors on the tilapia TGase were tested using EDTA, EGTA, iodoacetic acid (IAA), N-ethylmaleimide (NEM), phenyl methyl sulfonyl fluoride (PMSF), and ρ-chloromercuribenzoate (PCMB). Relative activity was calculated as the percentage of activity remaining after incubation with various reagents, taking the TGase activity of a sample without reagent as 100%.

RESULTS AND DISCUSSION

Purification of TGase from tropical tilapia

Purification of tilapia TGase was achieved by successive chromatographies on DEAE-Sephacel, Sephacryl S-200 HR and HiTrap Heparin, respectively. The elution profiles of these chromatographies are shown in Fig. 3.1-3.3 and TGase activities at each purification procedures are summarized in Table 3.1.

Table 3.1. Summary of purification of tropical tilapia TGase.

Purification step	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Purification (fold)	Yield (%)
Crude extract	1450.53	514.36	2.82	1	100
DEAE-Sephacel	435.42	12.84	33.91	12.03	30.02
Sephacryl S-200	248.45	9.94	24.99	8.86	17.13
Hitrap Heparin	187.21	0.95	197.06	69.82	12.91

The initial effective purification step was chromatographed on DEAE-Sephacel. Protein profiles and TGase activities (Fig. 3.1) indicated TGase was bound to DEAE-Sephacel. TGase was eluted as a single peak between 0.15 to 0.2 M NaCl. It is shown that DEAE-Sephacel and ultrafiltration could eliminate other proteins from tilapia TGase as shown on SDS-PAGE (Fig. 3.4). Yield obtained from DEAE-Sephacel was 30% with a purification fold of 12 (Table 1). It was reported that ion-exchange chromatography, such as DEAE-Sepharose, Q-Sepharose, and SP-Sepharose, can be used to purify other TGases and showed lower purification fold than our study (Kumazawa et al., 1997; Ha and Iuchi, 1997).

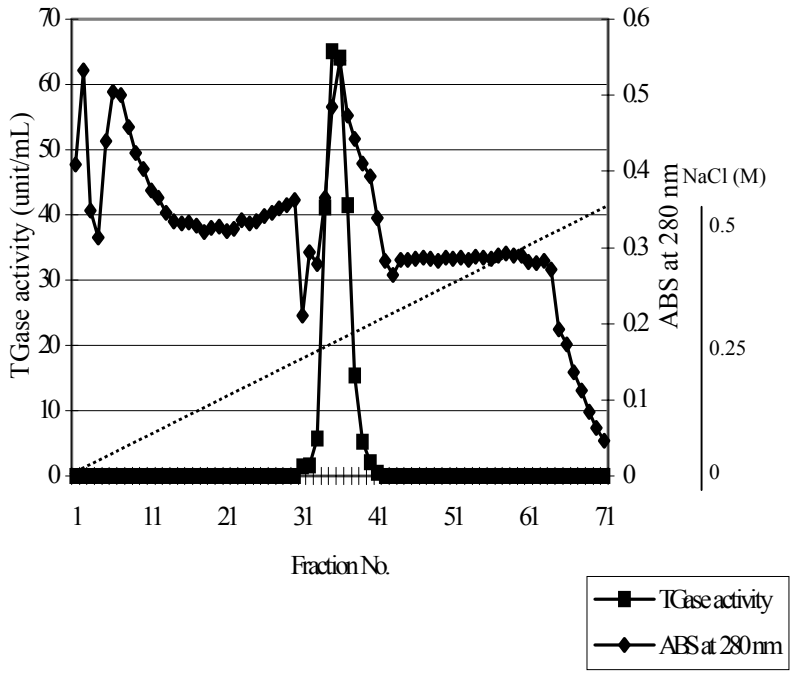

Fig. 3.1. Chromatogram of tropical tilapia TGase on DEAE-Sephacel column.

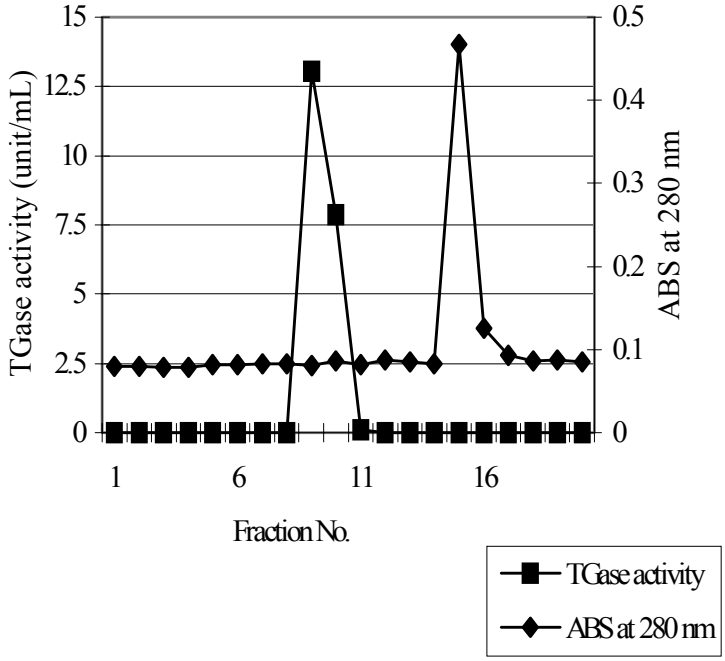

Fig. 3.2. Chromatogram of tropical tilapia TGase on Sephacryl S-200 HR column.

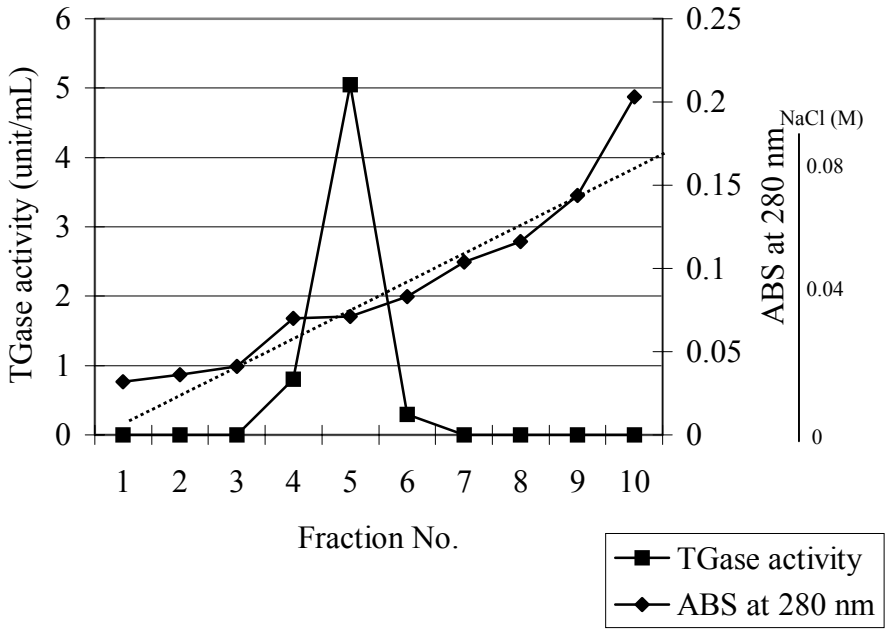

Fig. 3.3. Chromatogram of tropical tilapia TGase on HiTrap Heparin column.

In Sephacryl S-200 HR chromatography, TGase activity was eluted as a single peak at a fraction number 10 and 11 (Fig. 3.2). Yield and purification-fold at this step were 17.1% and 8 folds, respectively (Table 3.1). Although purification fold in this step was not increased but other small MW compounds were removed, yielding three major protein bands on SDS-PAGE (Fig. 3.4). Sephacryl S-200 has been used to purify TGase from red sea bream (Yasueda et al., 1994), Japanese oyster (TG-1; Kumazawa et al., 1997) and also showed the increased purification fold of 503.5 (Kumazawa et al., 1997).

The final step of purification was affinity chromatography on HiTrap Heparin chromatography. Heparin is a highly sulfate glycosaminoglycan with the ability to bind coagulation enzymes, including TGase (Daniel et al., 1996). Protein profile and TGase activity indicated that a single peak of TGase was eluted out at 0.04 M NaCl (Fig. 3.3). HiTrap Heparin has been used to purify TGase from red sea bream (*Pagrus major*) with a purification fold of 195 (Yasueda et al., 1994). Heparin affinity chromatography was also used to purify TGase from Japanese oyster (Kumazawa et al., 1997). Other affinity media, such as Blue Sepharose Fast Flow, were also used to purify microbial TGase (*Streptoverticillium ladakanum*) and showed a purification fold and yield of 26.3 fold and 63%, respectively (Tsai et al., 1996).

Tilapia TGase was purified to electrophoretic homogeneity with a specific activity of 196.9 unit/mg protein and purification fold of 69.8 (Table 3.1). Purification fold was lower than that from red sea bream TGase (195-fold) (Yasueda et al., 1994), scallop muscle TGase (101.9-fold) (Nozawa et al., 2001b), but higher than that from squid gill TGase (14.1-fold) (Nozawa et al., 2001a), and rainbow trout TGase (5.4-fold) (Ha and Iuchi, 1997). Final recovery yield of purified tilapia TGase was 12.9%

(Table 3.1.), which was similar to that from squid gill TGase (12.6%) (Nozawa et al., 2001a), red sea bream TGase (14%) (Yasueda et al., 1994) and scallop muscle TGase (16.6%) (Nozawa et al., 2001b).

Molecular Weight (MW) and Isoelectric point (pI)

Purified tilapia TGase revealed a single protein band on SDS-PAGE (Fig. 3.4) with an apparent MW of 85 kD. MW of tilapia TGase was slightly greater than that of other tissue TGases, guinea pig liver (76.6 kD), human placenta (76.6 kD), and rat liver (80 kD) (Abe et al., 1977; De Backer-Royer et al., 1992; Ikura et al., 1988). However, MW of tilapia TGase was similar to other fish TGases, such as ordinary carp (80 kD), and Alaska pollock (85 kD)(Kishi et al., 1991; Seki, et al., 1990).

The pI of tilapia TGase was about 6.53 (Fig. 3.5), which was similar to pI of other tissue TGases, such as guinea pig liver TGase (Folk and Cole, 1966), rabbit liver TGase (Abe et al., 1977), human placenta TGase (De Backer-Royer et al., 1992), whose the pI was at 4.5-5.4. pI of tilapia TGase was lower than that of microbial TGase (Ando et al., 1989; Tsai et al., 1996), which had the pI value at 7.2-8.9.

Optimal temperature

Optimal temperature of the purified tilapia TGase for the catalytic reaction of MDC was in the range of 37-50^{o}C (Fig. 3.6), while crude tilapia TGase was at 50^{o}C (Worratao and Yongsawatdigul, 2003). The purified tilapia TGase exhibited the broader temperature optimum than crude extract. No TGase activity was detected at 70^{o}C (Fig. 3.6). The results was similar to those of scallop TGase (Nozawa et al., 2001b), Japanese oyster TG-1 (Kumazawa et al., 1997) and walleye pollock liver

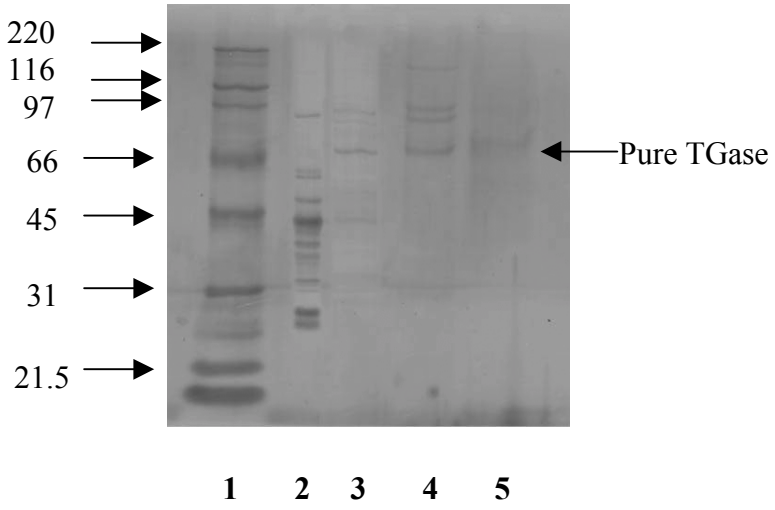

Fig. 3.4. SDS-PAGE of samples obtained at each purification step. Lane 1, standard molecular weight; lane 2, crude TGase; lane 3, DEAE-Sephacel; lane 4, Sephacryl S-200 HR; lane 5, HiTrap Heparin (purified TGase).

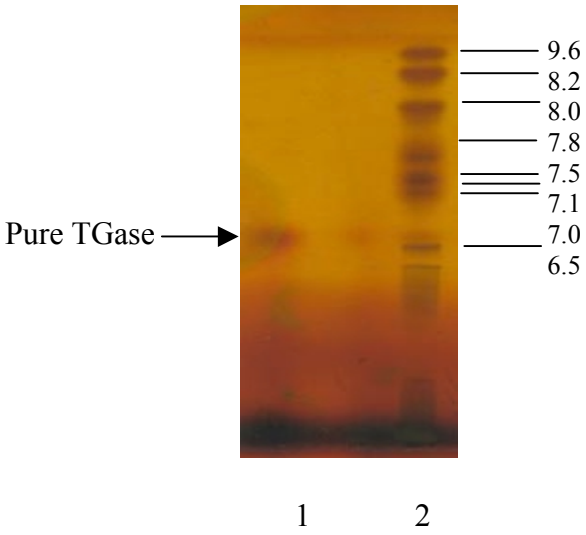

Fig. 3.5. pI of purified tilapia TGase. Lane 1, purified TGase; lane 2, standard pI.

TGase (Kumazawa et al., 1996), which had optimal temperature at 35-50^{o}C. The optimum temperature of tilapia TGase was slightly lower than that of red sea bream liver TGase (Yasueda et al., 1994), which had the optimal temperature at 55-60^{o}C. Japanese oyster TG-2 (Kumazawa et al., 1997), exhibited the optimal temperature at 25^{o}C. The difference of optimal temperature could depend on the habitat temperature of fish.

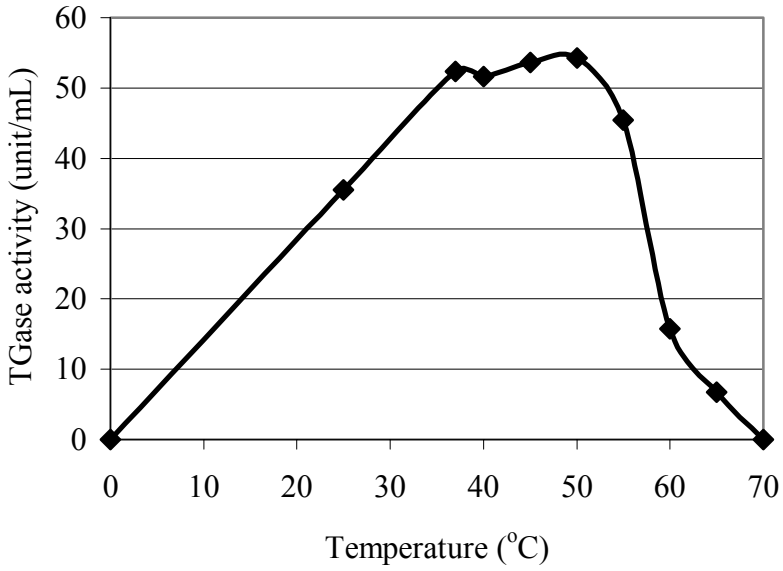

Fig. 3.6. Temperature profile of tilapia TGase.

Optimal pH

Purified tilapia TGase showed the optimal pH was at 7.5 (Fig. 3.7). This results was similar to that of crude tilapia TGase (7-7.5) (Worratao and Yongsawatdigul, 2003). The optimum pH was lower than that of other TGases which showed the pH optimum at the basic range of 8.0-9.5 (Yasueda et al., 1994; Kumazawa et al., 1996; 1997; Nozawa et al., 1997; 2001b). The difference of pH

optimum might depend on the substrate used in the assay (Nozawa et al., 1997). However, tilapia showed the high activity in the pH range of 7-9 (Fig. 3.7). Therefore, tilapia TGase had the broad pH range than other fish TGase. Then, it can be used to improve the functional property of various food products.

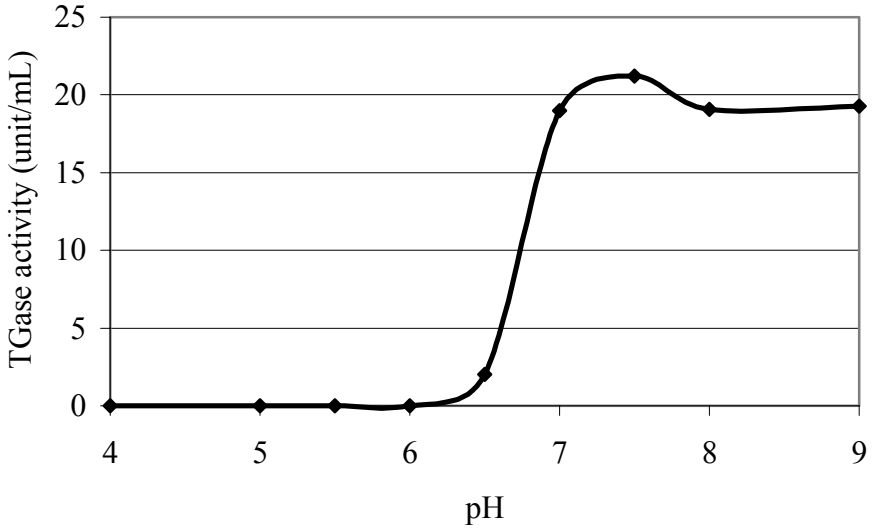

Fig. 3.7. pH profile of tilapia TGase at 37°C.

Effect of $CaCl_2$

Purified tilapia TGase showed an absolute requirement of calcium ions to catalyze the MDC incorporation (Fig. 3.8) similar to endogenous threadfin bream TGase (Yongsawatdigul et al., 2002). It showed no activity in the absence of Ca^{2+}, suggesting that tilapia TGase is Ca^{2+}-dependent enzyme. TGase activity increased with Ca^{2+} concentration and reached the maximum at 1.25 mM (Fig. 3.8). Optimum Ca^{2+} for TGase participation in the reaction was at 0.5 mM for red sea bream liver TGase (Yasueda et al., 1994), 25 mM for Japanese oyster TGase (Kumazawa et al., 1997), 10 mM for scallop TGase (Nozawa et al., 2001b), and 3 mM for walleye

pollock liver TGase (Kumazawa et al., 1996). It was postulated that calcium ion induced the conformational changes of TGase, which consequently exposed the cysteine located at the active site to substrate (Jiang and Lee, 1992). Noguchi et al. (2001) reported that calcium ion bound to a binding site of red sea bream TGase molecule, resulting in a conformational changes. Subsequently, Tyr covering the catalytic Cys is removed. Then, the acyl donor binds with the Cys at the active site, resulting in an acyl-enzyme intermediate.

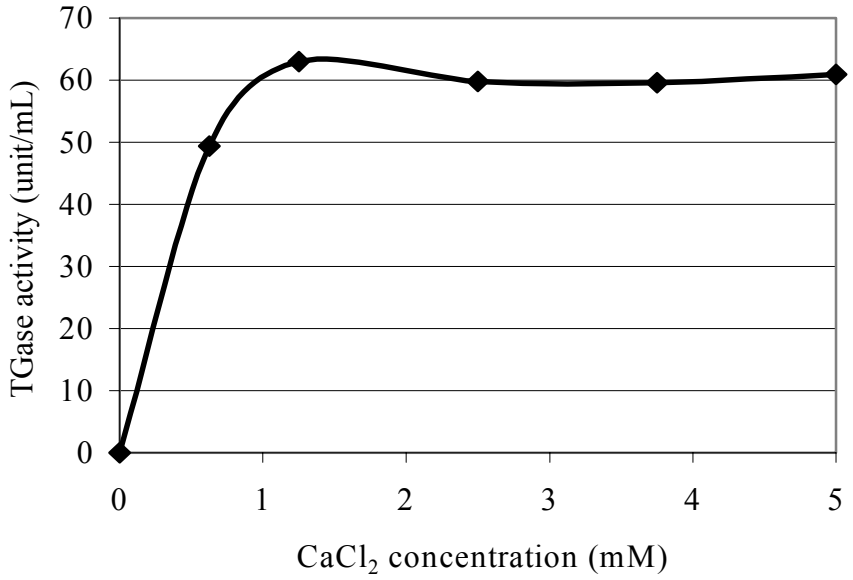

Fig. 3.8. Effect of $CaCl_2$ on tilapia TGase activity.

Effect of DTT

DTT had an effect on the purified tilapia TGase (Fig. 3.9). The concentration of DTT less than 5 mM was found to enhance tilapia TGase activity (Fig. 3.9). On the other hand, activity decreased when concentration of DTT increased more than 5 mM.

However, Tokunaga et al. (1993) reported that DTT showed little effect on the limulus hemocyte TGase.

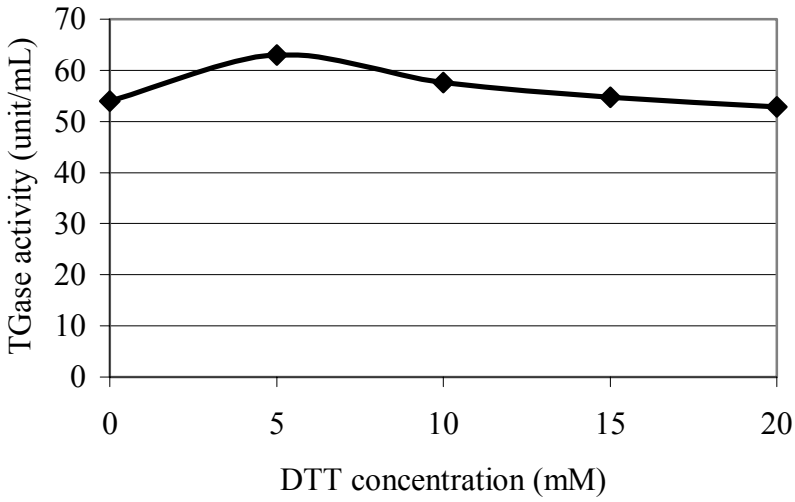

Fig. 3.9. Effect of DTT on tilapia TGase activity.

Effect of NaCl on TGase activity

TGase activity of purified tilapia decreased with increasing NaCl (Fig. 3.10) similar to that of crude tilapia TGase (Worratao and Yongsawatdigul, 2003). In addition, LiCl and KCl (Table 3.2) were also decreased tilapia TGase activity similar to limulus hemocyte TGase (Tokunaga et al., 1993). Sensitivity of TGase to NaCl was also reported in other TGases including those from carp dorsal muscle (Kishi et al., 1991), and Japanese oyster (Kumazawa et al., 1997). We speculated that these reagents at high concentration might cause conformation changes of the TGase molecule, resulting in a decrease of TGase activity. However, NaCl significantly increased TGase activity from marine source, such as scallop, botan shrimp, and squid (Nozawa et al., 1997). These suggest that the physiological function of marine TGase is closely related to their surrounding. However, purified tilapia TGase also showed

high activity at 0.4 M NaCl similar to crude tilapia TGase (Worratao and Yongsawatdigul, 2003). Then purified TGase can be used to improve the textural properties of minced tilapia.

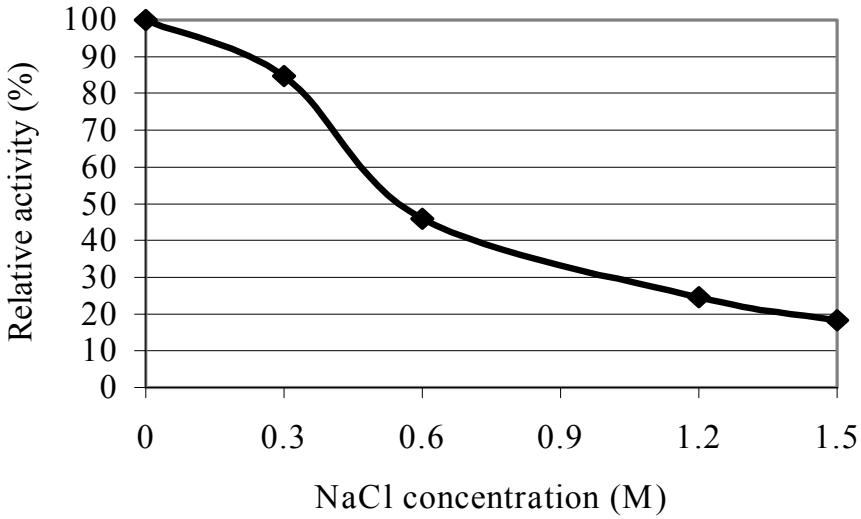

Fig. 3.10. Effect of NaCl on tilapia TGase activity.

Effect of metal ions on TGase activity

Tilapia TGase was inhibited by Ba^{2+}, Mg^{2+}, and Mn^{2+} (Table 3.2). It was reported that these metal ions scarcely activated red sea bream TGase (Yasueda at al., 1994) and walleye pollock liver TGase (Kumazawa et al., 1996). It was speculated that Mg^{2+}, and Mn^{2+} are too small to induce the conformational changes of TGase molecule (Yasueda et al., 1994).

Sr^{2+} reduced tilapia TGase activity to 55% (Table 3.2). It was reported that carp muscle TGase was not fully activated by Sr^{2+} (Kishi et al., 1991). Red sea bream TGase (Yasueda at al., 1994), and walleye pollock liver TGase (Kumazawa et al., 1996) showed full activity in the presence of Sr^{2+}. It was speculated that Sr^{2+} which is

efficiently bulky could induce TGase molecule, resulting the high specificity of cation-binding and then TGase could exert the full activity (Yasueda et al., 1994).

Table 3.2. Effects of various reagents on tilapia TGase activity.

Reagents	Concentrations	Relative activity (%)
Control		100
Sr^{2+}	10 mM	55.06
Mn^{2+}	10 mM	19.4
Cu^{2+}	10 mM	0
Zn^{2+}	10 mM	0
Ba^{2+}	10 mM	0
Mg^{2+}	1 mM	0
Li^{+}	10 mM	0
Na^{+}	1M	42.68
K^{+}	1M	38.31
EDTA	10 mM	0
EGTA	10 mM	0
IAA	10 mM	0
NEM	10 mM	0
PCMB	10 mM	0
PMSF	10 mM	0

Tilapia TGase was significantly inhibited by Cu^{2+} and Zn^{2+}, similar to other TGases (Jiang and Lee, 1992; Tokunaga et al., 1993, Tsai et al., 1996, Nozawa et al., 2001a,b). It is well known that Zn^{2+}, and Cu^{2+} had strong affinity towards sulfhydryl groups at the active site (Nozawa et al., 1997). In addition, these reagents might react with the functional residues at the active center and, therefore, inhibit activity. These

results indicated that tilapia TGases could possess thiol group at the active site, similarly to other tissue-type TGases (Nozawa et al., 2001a,b).

Effect of inhibitors on TGase activity

Chelating agents (EDTA, and EGTA) was inhibited tilapia TGase activity to 0% (Table 3.2) similar to limulus hemocyte TGase (Tokunaga et al., 1993), and rainbow trout TGase (Ha and Iuchi, 1998). Therefore, it is confirmed that tilapia TGase is a Ca^{2+}-dependent enzyme. In addition, tilapia TGase was completely inhibited by NEM, IAA, and PCMB. It was reported that Japanese oyster TGase (Kumazawa et al., 1997), red sea bream TGase (Yasueda et al., 1994), scallop TGase (Nozawa et al., 2001b), limulus hemocyte TGase (Tokunaga et al., 1993), and rainbow trout TGase (Ha and Iuchi, 1998) were also inhibited by these reagents. These reagents were sulfhydryl alkylating agents which react with the thiol group. It is likely that tilapia TGase contain a thiol group at the active site similar to other TGases.

PMSF was also inhibited tilapia TGase activity to 0% (Table 3.2). It was reported that MTGase from *S. ladakanum* was sensitive to PMSF (Tsai et al., 1996). However, PMSF did not inhibit TGase from red sea bream (Yasueda et al., 1994), Japanese oyster (Kumazawa et al., 1997), walleye pollock liver (Kumazawa et al., 1996), and *S. mobarense* (Ando et al., 1989). Generally, PMSF irreversibly inactivates a serine proteinase. It reacts with the hydroxyl group of seryl residue at the active site (Neurath, 1989). In addition, PMSF also react with the histidine residue at the catalytic triad (Neurath, 1989). However, it was also reported that TGase molecule contains histidine residue at the active site (Yee et al., 1994). Therefore, PMSF might

react with the histidine that elaborated to the catalytic triad, resulting the decreased of TGase activity (Noguchi et al., 2001).

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CHAPTER IV

CONCLUSIONS

Tilapia muscle contained higher TGase activity than other freshwater fish studied. Crude tilapia TGase catalyzed the cross-linking of tilapia MHC, resulting in a polymer of MHC. For this reason, catalytic reaction of the purified tilapia TGase was studied. The purified enzyme had the estimated MW of 85 kD and pI of 6.53. Tilapia TGase required $CaCl_2$ for activation similar to other fish TGases. The purified tilapia TGase showed the broad range of optimum temperature (37-50^{o}C) and pH (7-9). Maximum activity of the purified enzyme was found at 5 mM DTT. Its activity was inhibited by sulfhydryl alkylating agents, indication the presence of thiol group at the active site.

CURRICULUM VITAE

NAME Anulak Worratao
DATE OF BIRTH August 4, 1976 at Roi-Et, Thailand
EDUCATION 1999-2002 Master degree program, School of Food Technology, Suranaree University of Technology (SUT), Thailand
1995-1998 B.Sc. Food Technology, SUT, Thailand

EXPERIENCES: 2000-2001
1. Teaching Assistant, School of Food Technology, SUT (Food quality control, Food analysis, and Food analysis laboratory Food chemistry, Food chemistry laboratory, and Bakery product technology)
2. Research Assistant, School of Food Technology, SUT (Proteinase and transglutaminase in freshwater species)

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