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Purification and characterization of transglutaminase from Tropical tilapia (*Oreochromis niloticus*)

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Abstract

Transglutaminase (TGase) from Tropical tilapia (*Oreochromis niloticus*) was purified to electrophoretic homogeneity using successive chromatographies of DEAE-Sephacel, Sephacryl S-4 HR and HiTrap Heparin with a yield and purification-fold of 12.9% and 69.8, respectively. The molecular weight (MW) of the purified tilapia TGase was estimated to be 85 kDa using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The isoelectric point (pI) of tilapia TGase was 6.53. Optimal temperature and optimal pH of tilapia TGase were 37–50 °C and 7.5, respectively. Optimal concentrations of $CaCl_2$ and dithiothreitol (DTT) were at 1.25 and 5 mM, respectively. The activity of TGase towards monodansylcadaverine (MDC) decreased as the NaCl concentration increased. Chelating agents, ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-O,O′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (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 a thiol group at the active site.

Keywords: Transglutaminase; Tilapia (*Oreochromis niloticus*); Purification

1. 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 residues 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 acceptors (Folk, 1980; Greenberg, Birckbichler, & Rice, 1991). When acceptors are ε-amino groups of lysine residues, the formation of ε-(γ-glutamyl)lysine (GL) linkages occur both intra- and inter-molecularly (Folk, 1980; Greenberg et al., 1991). This introduces covalent cross-linkages between the ε-amino groups of lysine residues and the γ-carboxyamide group of a glutamine residue in a protein molecule (Folk, 1980; Greenberg et al., 1991). These bonds are stable and resistant to proteolysis (Joseph, Lanier, & Hamann, 1994). Therefore, TGase has been widely studied to improve functional properties of various food proteins (Jiang, Hsieh, & Chung, 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 & Cole, 1966; Icekson & Apelbaum, 1987; Kumazawa et al., 1997; Yasueda, Kumazawa, & Motoki, 1994). Seki et al. (1990) isolated TGase from pollock (*Theragra chalcogramma*) and found that it could induce the gelation of minced fish. Strong gel-forming ability of sardine resulted from the formation of the non-disulfide covalent bonds, catalyzed by endogenous TGase (Tsukamasa et al., 1993). Worratao and Yongsawatdigul (2003) demonstrated that

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Tropical tilapia (*Oreochromis niloticus*) muscle contained high TGase activity and that crude tilapia TGase catalyzed the cross-linking of myosin heavy chains (MHC). These cross-links were likely to be related to an increase in gel strength of minced tilapia. However, the purification of TGase from tilapia muscle has not yet been reported. 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 the biochemical properties of the purified TGase.

2. Materials and methods

2.1. Chemicals

Monodansylcadaverine (MDC), *N*,*N*′-dimethylated casein (DMC), β-mercaptoethanol (BME), Tris-(hydroxyaminomethane), calcium chloride ($CaCl_2$), bovine serum albumin (BSA), Folin and 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). Ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were purchased from Fluka BioChemica (Buchs, Switzerland). Dithiothreitol (DTT) and urea were purchased from Promega (Madison, Wis., USA). Sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250, standard protein kits for pI and SDS-PAGE were purchased from Bio-Rad (Richmond, Calif., USA). All other reagents and chemicals were of analytical grade.

2.2. Raw materials

Live Tropical tilapia (*O. niloticus*) were purchased from the Suranaree University of Technology Farm and transferred to the laboratory within 10 min of catching. The fish were killed, gutted and deskined upon arrival at the laboratory. Crude tilapia TGase was immediately extracted.

2.3. Preparation of crude TGase

Fish samples were homogenized with four 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, Kendro Laboratory Products, Newton, CT, USA) for 20 min at 4 °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 °C. The supernatant was used as crude TGase.

2.4. Determination of TGase activity

TGase activity was measured in terms of the incorporation of MDC into DMC according to the procedure of Takagi, Saito, Kikuchi, and Inada (1986) with a slight modification (Worratao & 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 °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 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 at 37 °C.

2.5. Purification of tilapia TGase

2.5.1. 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 two 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 cut-off (MWCO) 30 kDa (Centripep Model YM, Millipore, Billerica, Mass., USA).

2.5.2. Sephacryl S-200 HR

One milliliter of the concentrated TGase obtained from the DEAE-Sephacel column was applied to a Sephacryl S-4 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 an ultrafiltration membrane with a MWCO cut-off 30 kDa (Centriprep Model YM, Millipore, Billerica, Mass., USA).

2.5.3. HiTrap Heparin

One milliliter of the concentrated TGase obtained from the Sephacryl S-200 was further loaded onto a 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 MWCO 30 kDa (Centriprep Model YM 30, Millipore, Billerica, Mass., USA) and used as purified TGase. All purification procedures were carried out at 4 °C.

2.6. Protein determination

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

2.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze protein samples during the purification. 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 Laemmli (Laemmli, 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, Michael, and Stuart (1996). Gels were stained with silver nitrate, and developed in 0.005% acetic acid and 0.019% formaldehyde. The reaction was stopped by adding 1% acetic acid. To dertermine MW of TGase, myosin (220 kDa), β-galactosidase (116.25 kDa), phosphorylase B (97.4 kDa), serum albumin (66.2 kD), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa) were used as standards.

2.8. Isoelectric point

The isoelectric point (pI) was determined using a Pharmacia PhastSystem electrophoresis unit (Pharmacia, Uppsala, Sweden). One microliter of the purified tilapia TGase was applied to the 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).

2.9. Biochemical characteristics of tilapia TGase

The optimal temperature was tested using the reaction mixtures as described previously. The samples were pre-incubated and assayed at 0, 25, 37, 40, 45, 50, 55, 60, 65, and 70 °C. The reactions were carried out at 62.5 mM Tris–HCl (pH 7.5), 6.25 mM $CaCl_2$, and 3.75 mM DTT.

Optimal pH was determined using 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. The reactions were carried out at 6.25 mM $CaCl_2$, and 3.75 mM DTT.

The effect of $CaCl_2$ was investigated at 3.75 mM DTT. Effect of DTT was studied at 1.25 mM $CaCl_2$. The effect of NaCl was tested at final concentrations of 0–1.5 M in the presence of 1.25 mM $CaCl_2$. In addition, the effect of KCl was elucidated at the final concentration of 1 M. The effect of metal ions including $SrCl_2$, $MgCl_2$, $BaCl_2$ was investigated at 10 mM in the absence 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, IAA, NEM, PMSF, and PCMB. The relative activity was calculated as the percentage of activity remaining after incubation with various reagents, taking the TGase activity of a sample without inhibitor as 100%.

3. Results and discussion

3.1. Purification of Tropical tilapia TGase

The initial effective purification step was chromatographed on DEAE-Sephacel. Protein profiles and TGase activities (Fig. 1) indicated that TGase was bound to the

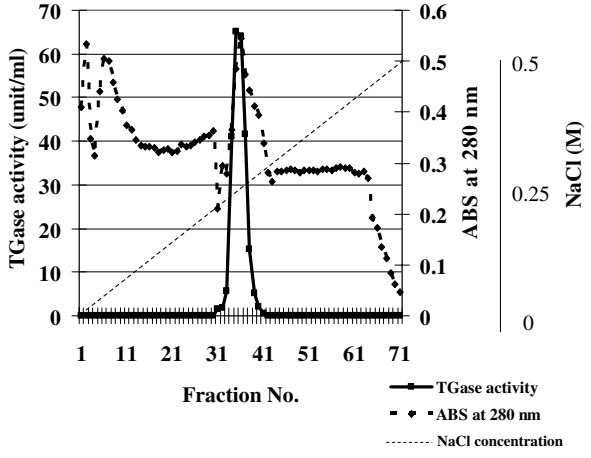

Fig. 1. Chromatogram of Tropical tilapia TGase on DEAE-Sephacel column.

Table 1
Purification result of Tropical tilapia TGase

Purification step	Total activity(unit)	Total protein (mg)	Specific activity (unit/mg)	Purification (fold)	Yield (%)
Crude extract	1451	514	2.82	1	100
DEAE-Sephacel	435	12.8	33.9	12.0	30.0
Sephacryl S-200	248	9.94	25.0	8.86	17.1
Hitrap Heparin	187	0.95	197	69.8	12.9

DEAE-Sephacel. TGase was eluted as a single peak between 0.15 and 0.2 M NaCl. It was shown that DEAE-Sephacel and ultrafiltration could eliminate other proteins from tilapia TGase as shown on SDS-PAGE (Fig. 4). The yield obtained from DEAE-Sephacel was 30% with a purification-fold of 12 (Table 1). Ion-exchange chromatography, such as DEAE-Sepharose, Q-Sepharose, and SP-Sepharose, have been used to purify other TGases and showed lower purification fold than our study (Ha & Iuchi, 1997; Kumazawa et al., 1997).

In Sephacryl S-200 HR chromatography, TGase activity was eluted as a single peak at fraction 10 and 11 (Fig. 2). The yield and purification-fold at this step were 17.1% and 8, respectively (Table 1). Although purification-fold in this step was not increased, other small MW compounds were removed, yielding three major protein bands on SDS-PAGE (Fig. 4). Sephacryl S-200 was used to purify TGase from red sea bream (*Pagrus major*) (Yasueda et al., 1994), and Japanese oyster (*Crassostrea gigas*) (Kumazawa et al., 1997).

The final step of purification was affinity chromatography on HiTrap Heparin chromatography. Heparin is a highly sulfated glycosaminoglycan with the ability to bind coagulation enzymes, including TGase (Daniel, Rozycki, & Edelstein, 1996). The protein profile and TGase activity indicated that a single peak of TGase was eluted out at 0.04 M NaCl (Fig. 3). HiTrap Heparin has been used to purify TGase from red sea bream 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,

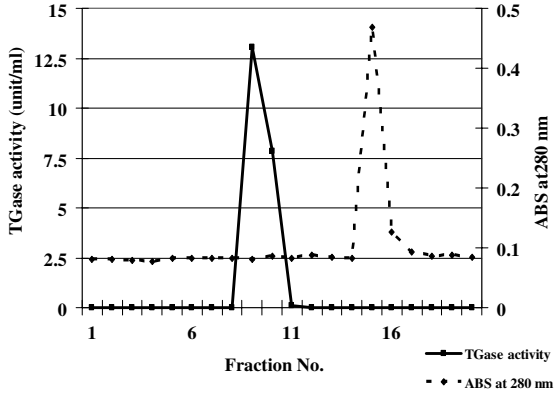

Fig. 2. Chromatogram of Tropical tilapia TGase on Sephacryl S-200 HR column.

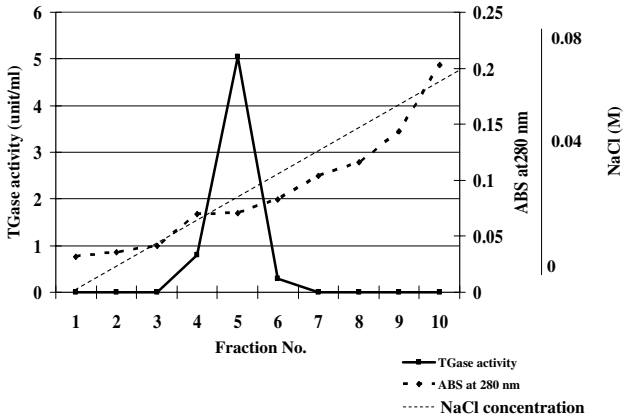

Fig. 3. Chromatogram of Tropical tilapia TGase on HiTrap Heparin column.

have also been applied to purify microbial TGase (*Streptoverticillium ladakanum*) (Tsai, Lin, & Jiang, 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 1). Purification fold was lower than that from red sea bream TGase (195-fold) (Yasueda et al., 1994), muscle of scallop (*Patinopecten yessoensis*) TGase (101.9-fold) (Nozawa & Seki, 2001), but higher than that of the gill of squid (*Todarodes pacificus*) TGase (14.1-fold) (Nozawa, Cho, & Seki, 2001), and rainbow trout (*Oncorhynchus mykiss*) TGase (5.4-fold) (Ha & Iuchi, 1997). The final recovery yield of the purified tilapia TGase was 12.9% (Table 1.), which was similar to that from squid (Nozawa et al., 2001), red sea bream (Yasueda et al., 1994) and muscle of scallop (Nozawa & Seki, 2001).

3.2. Molecular weight and isoelectric point

A single protein band on SDS-PAGE was observed for the purified enzyme (Fig. 4) with an apparent MW of 85 kDa. The MW of tilapia TGase was slightly greater than that of other tissue TGases, including guinea pig liver (76.6 kDa), human placenta (76.6 kDa), and rat liver (80 kDa) (Abe, Chung, Diaugustion, & Folk, 1977; De Backer-Royer, Traore, & Meunier, 1992; Ikura et al., 1988). The MW of tilapia TGase was similar to that of other fish, such as ordinary carp (*Cyprinus carpio*) (80 kDa), and pollock (85 kDa) (Kishi,

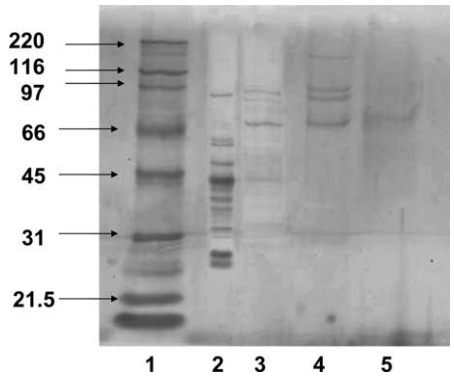

Fig. 4. SDS-PAGE of samples obtained from each purification step: lane 1, standard MW; lane 2, crude tilapia TGase; lane 3, DEAE-Sephacel; lane 4, Sephacryl S-200 HR; lane 5, HiTrap Heparin (purified TGase).

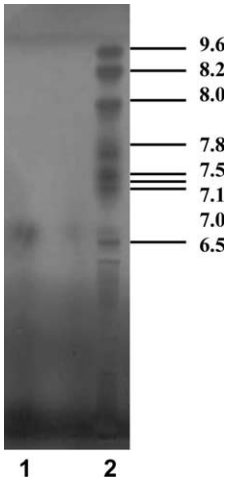

Fig. 5. pI of purified tilapia TGase: lane 1, purified TGase; lane 2, standard pI.

Nozawa, & Seki, 1991; Seki et al., 1990). Therefore, we speculate that tilapia TGase is the monomeric form composed of a single polypeptide chain similar to TGase from other marine organisms (Kumazawa, Nakanishi, Yasueda, & Motoki, 1996; Tokunaga et al., 1993; Yasueda et al., 1994).

The pI of tilapia TGase was about 6.53 (Fig. 5), while the pI of other tissue TGases, such as guinea pig liver TGase, rabbit liver TGase, human placenta TGase, was in the range of 4.5–5.4 (Abe et al., 1977; De Backer-Royer et al., 1992; Folk & Cole, 1966). In addition, the pI of microbial TGase was about 7.2–8.9. (Ando et al., 1989; Tsai et al., 1996).

3.3. Optimal temperature and pH

The optimal temperature of the purified tilapia TGase for the catalytic reaction of MDC ranged from 37 to 50 °C (Fig. 6), while that of crude tilapia TGase was at 50 °C (Worratao & Yongsawatdigul, 2003). No

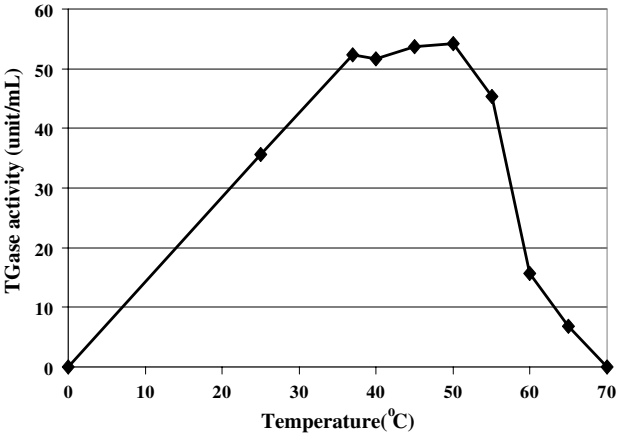

Fig. 6. Temperature profile of the purified tilapia TGase.

TGase activity was detected at 70 °C (Fig. 6). The optimal temperature of the purified TGase varied with sources. TGase from scallop, Japanese oyster, and pollock liver exhibited the optimum activity between 35 and 50 °C (Kumazawa et al., 1996, 1997; Nozawa & Seki, 2001). The optimal temperature of red sea bream liver TGase was between 55 and 60 °C (Yasueda et al., 1994).

Klesk, Yongsawatdigul, Park, Viratchakul, and Virulhakul (2000) found that tilapia surimi exhibited "setting" at 40 °C but not at lower temperatures (5 and 25 °C). The setting phenomenon is known to be catalyzed by endogenous TGase (Joseph et al., 1994). Tilapia TGase would catalyze the cross-linking of muscle proteins to a greater extent at 40 °C than at a lower temperature, leading to more covalent cross-links. Purified tilapia TGase showed the optimal pH at 7.5 and slightly decreased between pH 8 and 9 (Fig. 7). The optimal pH was similar to that of crude tilapia TGase (7–7.5) (Worratao & Yongsawatdigul, 2003). TGases from other fish species showed the pH optima between 8.0 and 9.5 (Kumazawa et al., 1996, 1997; Nozawa, Mamegoshi,

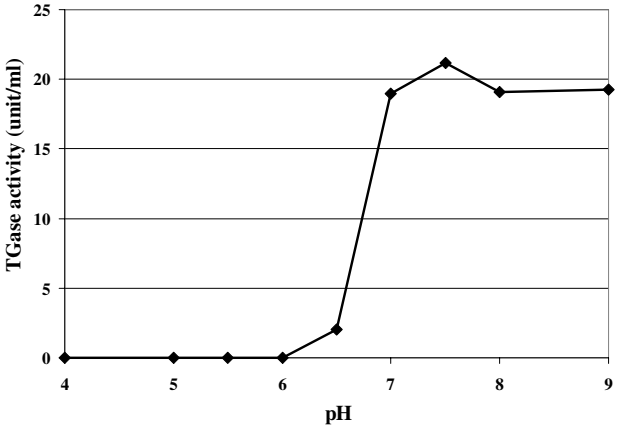

Fig. 7. pH profile of the purified tilapia TGase at 37 °C.

& Seki, 1997; Nozawa & Seki, 2001; Yasueda et al., 1994). It should be noted that tilapia TGase has a broader pH range than TGases from other sources.

3.4. Effect of $CaCl_2$ and DTT

Purified tilapia TGase showed an absolute requirement for calcium ions to catalyze MDC incorporation (Fig. 8), which is a typical characteristic of mammalian and fish TGases (Yongsawatdigul, Worratao, & Park, 2002). TGase activity increased with Ca^{2+} concentration and reached a maximum at 1.25 mM (Fig. 8). Optimal Ca^{2+} concentrations for TGase from red sea bream liver, Japanese oyster, scallop, and pollock liver were at 0.5, 25, 10, and 3 mM, respectively (Kumazawa et al., 1996, 1997; Nozawa et al., 2001; Yasueda et al., 1994). It was postulated that the calcium ion induced the conformational changes of the enzyme, which consequently exposed the cysteine located at the active site to a substrate (Jiang & Lee, 1992). Noguchi et al. (2001) reported that the calcium ion bound to a binding site of red sea bream TGase molecule, resulting in conformational changes. Subsequently, Tyr covering the catalytic Cys was removed. Then, the acyl donor bind with the Cys at the active site, forming an acyl-enzyme intermediate.

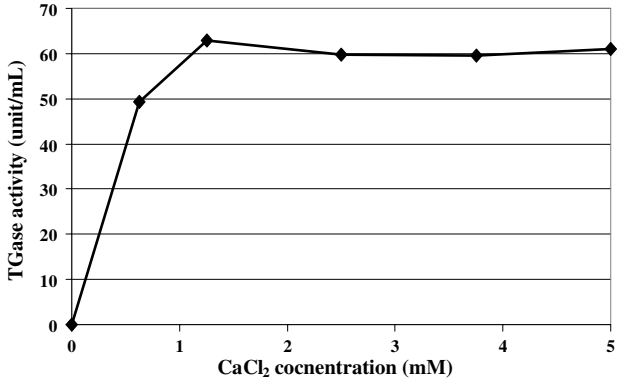

Fig. 8. Effect of $CaCl_2$ on the purified tilapia TGase activity.

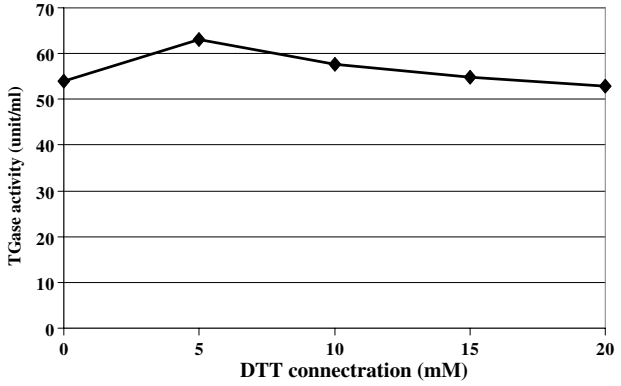

Fig. 9. Effect of DTT on the purified tilapia TGase activity.

DTT had no effect on activity of the purified tilapia TGase (Fig. 9). Similarly, the activity of crude tilapia TGase was not affected by DTT (Worratao & Yongsawatdigul, 2003). DTT was found to enhance TGase activity because it helped to maintain a "reduced state" of the sulfhydryl group at the active site (Folk, 1980). The activity of the purified TGase decreased at concentration >5 mM. Tokunaga et al. (1993) reported that DTT showed little effect on the limulus hemocyte (*Tachypleus tridentatus*) TGase which was similar to our observation from the tilapia TGase.

3.5. Effect of salts on TGase activity

The TGase activity of purified tilapia decreased with increasing NaCl concentration (Fig. 10) similar to that of crude tilapia TGase (Worratao & Yongsawatdigul, 2003). In addition, LiCl and KCl (Table 2) decreased tilapia TGase activity. Sensitivity of TGase to NaCl

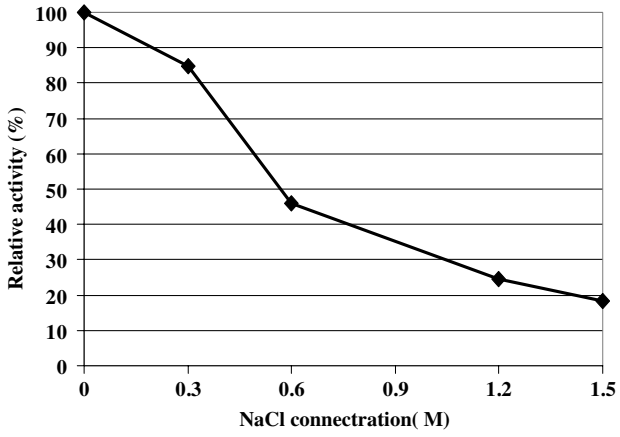

Fig. 10. Effect of NaCl on tilapia TGase activity.

Table 2
Effects of various reagents on tilapia TGase activity

Reagent	Concentration	Relative activity (%)
Control		100
Sr^{2+}	10 mM	55.1
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^{+}	1 M	42.7
K^{+}	1 M	38.3
EDTA	10 mM	0
EGTA	10 mM	0
IAA	10 mM	0
NEM	10 mM	0
PCMB	10 mM	0
PMSF	10 mM	0

was also reported in other TGases, including those from carp dorsal muscle (Kishi et al., 1991) and from Japanese oyster (Kumazawa et al., 1997). High concentration of NaCl could induce conformational changes in the enzyme molecule, resulting in a decrease of TGase activity (Kishi et al., 1991; Kumazawa et al., 1997). However, NaCl significantly increased TGase activity from marine species, such as scallop, botan shrimp (*Pandalus nipponensis*), and squid (Nozawa et al., 1997). These suggested that the optimal conditions for enzyme activity are closely related to the environmental habitat of the aquatic species. Since tilapia is a freshwater species, tilapia TGase would physiologically function at relatively low NaCl. For this reason, the activity of tilapia TGase decreased at high NaCl concentration.

3.6. Effect of metal ions on TGase activity

Tilapia TGase was inhibited by Ba^{2+}, Mg^{2+}, and Mn^{2+} (Table 2). These metal ions scarcely activated red sea bream TGase (Yasueda et al., 1994) or pollock liver TGase (Kumazawa et al., 1996). It was speculated that the concentrations of Mg^{2+}, and Mn^{2+} are too low to induce a proper conformational changes for the catalytic reaction of TGase molecule (Yasueda et al., 1994).

Sr^{2+} reduced tilapia TGase activity to 55% (Table 2). A similar result was reported in carp muscle TGase (Kishi et al., 1991). The binding of Sr^{2+} to TGase molecule induced the exposure of sulfhydryl active site, leading to partial activity. However, red sea bream TGase and pollock liver TGase showed full activity in the presence of Sr^{2+}(Kumazawa et al., 1996; Yasueda et al., 1994). Tilapia TGase was completely inhibited by Cu^{2+} and Zn^{2+}, similar to TGase from other marine species (Jiang & Lee, 1992; Nozawa et al., 2001; Nozawa & Seki, 2001; Tokunaga et al., 1993; Tsai et al., 1996). It is well known that Zn^{2+} and Cu^{2+} have strong affinity towards sulfhydryl groups at the active site of TGase (Nozawa et al., 1997). These results indicate that tilapia TGases could possess thiol group at the active site, similar to other tissue-type TGases (Nozawa et al., 2001; Nozawa & Seki, 2001).

3.7. Effect of inhibitors on TGase activity

Chelating agents (EDTA and EGTA) completely inhibited tilapia TGase activity, confirming that tilapia TGase is a Ca^{2+}-dependent enzyme (Table 2). In addition, tilapia TGase was completely inhibited by NEM, IAA, and PCMB. TGase from other aquatic animals were also inhibited by these components (Ha & Iuchi, 1998; Kumazawa et al., 1997; Nozawa & Seki, 2001; Tokunaga et al., 1993; Yasueda et al., 1994). These reagents are sulfhydryl alkylating agents which react with a thiol group. These results support the view that tilapia TGase contains a thiol group at the active site.

PMSF also inhibited tilapia TGase activity (Table 2). TGase from *S. ladakanum* was also 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), pollock liver (Kumazawa et al., 1996), or *S. mobarense* (Ando et al., 1989). Generally, PMSF irreversibly inactivates a serine proteinase. It reacts with the hydroxyl group of the seryl residue at the active site (Neurath, 1989). In addition, PMSF also reacts with the histidine residue at the catalytic triad of serine proteinase (Neurath, 1989). It was reported that TGase molecule from human blood contains histidine residue at the active site (Yee et al., 1994). Therefore, PMSF might react with the histidine that was involved in the catalytic triad, resulting in a decreased activity (Noguchi et al., 2001).

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