Purification and Characterization of Transglutaminase from Walleye Pollack Liver

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Transglutaminase (TGase, EC 2.3.2.13) from walleye pollack Theragra Chalcogramma liver was purified to electrophoretical homogeneity by Q-Sepharose and S-Sepharose chromatographies. The purified enzyme of 0.34 mg was obtained from 15 g of liver tissue and 591-fold purification was achieved from the liver extract. The molecular weight was estimated to be 77 kDa by SDS-polyacrylamide gel electrophoresis. The optimum pH and temperature for monodansyl cadaverine incorporation to N,N'-dimethylated casein were 9.0 and 50°C, respectively. The purified enzyme required Ca²⁺ above 3 mm for the maximum activity, and Sr²⁺ also fully activated the enzyme. The activity was inhibited by sulfhydryl reagent, suggesting this enzyme was a thiol enzyme, the same as mammalian TGases. By this purified TGase, the gelation of myosin B solution was catalyzed, possibly through the polymerization of myosin heavy chains.

Key words: walleye pollack, transglutaminase, liver, purification

Transglutaminase catalyzes an acyl transfer reaction between y-carboxyamide groups of glutamine residues in proteins, polypeptides and a variety of primary amines.1,2) When the e-amino group of lysine and lysyl residue acts as an acyl acceptor, e-(y-glutamyl)lysine crosslinks are formed in proteins.3) TGases are widely distributed in various organisms, including fish flesh.1-4)

It is well known that fish meat turns to a sticky paste when it is ground with NaCl (2-4%). In some fish, meat paste forms an elastic gel when it is incubated at appropriate temperatures (5-40°C). This setting phenomenon, called swari, is one of the important processes for manufacturing surimi-based products. It has been proposed that gelation of fish meat paste in the swari process is due to the network structures formed by myosin heavy chain. Recently, Seki et al.9) reported that intrinsic TGase, present in materials such as frozen surimi, reacts during the manufacturing process of kamaboko and contributes to the polymerization of the myosin heavy chain. In increases in the e-(y-glutamyl)lysine bond, which is a product of TGase reaction, in salted fish meat paste during setting have been reported.10,11) We have confirmed that the increases in the e-(y-glutamyl)lysine and gel strength were suppressed by the addition of EDTA or NH₄Cl, which are TGase inhibitors.11)

Numerous studies have been reported on this enzyme for mammalian origin such as guinea pig liver, however, there have been few studies on the enzymatic properties and structural characteristics of TGases of fish origin. Kishi et al.5) reported some properties of a crude enzyme from the dorsal muscle of carp. Fish TGases have been studied in regard to reactions with fish myosin B using crude TGase extracts.5,12-14) These studies have been designed to elucidate the mechanisms underlying gel formation in fish meat paste. In our previous studies,15) we succeeded in purifying the TGase from red sea bream Pagrus major liver, using ion-exchange and heparin affinity chromatographies and examined the properties of the purified enzyme, and reported molecular cloning of cDNA encoding the enzyme and characterized its entire primary structure.16)

To obtain further information on the properties of fish TGase, we used liver of walleye pollack the mince of which is usually used for making surimi based products. The present report describes the purification and characterization of a tissue-type TGase in walleye pollack liver, and compares the properties of pollack TGase with those of red sea bream liver TGase.

Materials and Methods

Samples and Chemicals

Walleye pollack Theragra chalcogramma liver was obtained from live pollack caught off the coast of Kushiro (Hokkaido, Japan). The liver was taken out and frozen in liquid nitrogen immediately and stored at −80°C until purification. After thawing at 4°C overnight, the liver was used to purify TGase. Monodansylcadaverine (MDC) and N,N'-dimethylated casein were purchased from Sigma (St. Louis, MO). Phenylmethysulfonyl fluoride (PMSF) and monoiodoacetic acid (MIA) were purchased from Wako Chemical (Osaka). N-ethylmaleimide (NEM), ethylene-

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diaminotetraacetic acid (EDTA) and dithiothreitol (DTT) were purchased from Nakalai tesque (Kyoto). Crude myosin B from frozen pollack surimi was prepared by the method of Takashi et al. with slight modification. The myosin B was finally dialyzed against 20 mM Tris-HCl (pH 7.5) containing 0.3 M NaCl.

TGase Activity Assay

TGase activity was measured in terms of the incorporation of MDC into N,N'-dimethylated casein according to the method of Takagi et al. with slight modification. The reaction mixture comprised 2.4 ml of 50 mM Tris-HCl (pH 8.5), 1.0 mg/ml N,N'-dimethylated casein, 15 μM MDC, 3 mM DTT, 10 mM CaCl₂ and appropriate enzyme solution. After incubation at 37°C for 20-30 min, the reaction was stopped by the addition of 0.1 ml of 0.5 M EDTA. The intensity of fluorescence of MDC incorporated into N,N'-dimethylated casein was measured with excitation and emission wavelengths set at 350 and 480 nm, respectively. One unit of the enzyme was defined as the amount that incorporated 1 nmol of MDC into N,N'-dimethylated casein per min at 37°C.

Purification of TGase

All the following purification procedures were carried out below 4°C.

Preparation of crude extract

Pollack liver (15 g) was minced and homogenized in a two-fold volume of 20 mM Tris-HCl (pH 8.3) containing 10 mM NaCl, 5 mM EDTA and 1 mM DTT (TEND buffer). The homogenate was centrifuged at 10,000×g for 10 min, and the supernatant was filtered through a gauze. Then, the filtered supernatant was further centrifuged at 100,000×g for 60 min and the supernatant was filtered through a cellulose acetate membrane (0.45 μm, GL Sciences, Tokyo) to remove cell debris. The filtrate (24 ml) was referred to as crude extract.

Step I. First Q-Sepharose chromatography

Crude extract was applied to a Q-Sepharose HP column (1.6×10 cm, Pharmacia, Uppsala) equilibrated with TEND buffer. After loading, the column was washed with TEND buffer to eliminate the unabsorbed proteins and then the protein was eluted with a linear gradient (200 ml) of 10-500 mM NaCl in the TEND buffer. The eluent flow rate was 0.5 ml/min and fractions of 2 ml were collected.

Step II. 2nd Q-Sepharose chromatography

Fractions (10 ml) containing TGase activity from the 1st Q-Sepharose chromatography were dialyzed against the TEND buffer and then the dialyze was loaded on the Q-Sepharose HP column (1.6×10 cm). Then elution was done with a linear gradient (60 ml) of 10-300 mM NaCl in TEND buffer. The eluent flow rate was 0.5 ml/min and fractions of 2 ml were collected.

Step III S-Sepharose chromatography

The combined fractions (9.5 ml) containing TGase activity were dialyzed against 20 mM sodium acetate (pH 6.45) containing 50 mM NaCl, 2 mM EDTA and 0.5 mM DTT. Then the dialyze was loaded on a S-Sepharose HP column (1.6×10 cm, Pharmacia) equilibrated with the same buffer and washed to eliminate unabsorbed proteins. Then elution was done with a linear gradient (56 ml) of 50-500 mM NaCl in the same buffer. The eluent flow rate was 0.5 ml/min and fractions of 1.5 ml were collected. The fractions containing TGase activity were used as purified enzyme.

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a 5–20% gradient gel by the method of Laemmli. Phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and α-lactalbumin (14,400) were used as molecular weight markers. Proteins were stained with Coomassie Brilliant Blue R-250.

Determination of Protein Concentration

Protein concentration was determined using the protein-dye binding method. The bovine serum albumin was used as a standard protein.

Reaction with Myosin B

One ml of myosin B prepared from frozen pollack surimi (7.1 mg/ml) in 20 mM Tris-HCl (pH 7.5) containing 0.3 M NaCl and 10 mM CaCl₂ was incubated with appropriate amounts of purified TGase at 37°C for up to 60 min. The degree of gelation was judged by test tube inversion. The degree of protein polymerization was analyzed by SDS-PAGE.

Results

Thermostability of Crude TGase

At first, the thermostability of TGase in the crude extract was examined (Fig. 1). The TGase activity scarcely decreased in the 2 and 20°C incubations for 120 min, but about 80% activity was lost in the 37°C incubation for 1 hr, and almost of all the activity was lost at 50°C within a
few minutes. In addition, over 80% of the initial TGase activity remained for one month in iced water (below 2°C). From these results, purification procedures should be carried out below 20°C. Thus, in this study the purification temperature was determined to be 4°C.

Purification of TGase

The crude extract (24 ml) prepared from 15 g of pollack liver was applied on a Q-Sepharose HP column. TGase activity was not detected in the through fraction of Q-Sepharose, indicating that all the TGase activity was adsorbed on the resin. The TGase activity was eluted as a single peak from 0.1 M NaCl (Fig. 2(a)) and the active 10 ml fraction was obtained. The elution profile of Q-Sepharose rechromatography is shown in Fig. 2(b). The TGase activity was eluted as a single peak and 9.5 ml of combined fraction was obtained. The active fraction was dialyzed against the start buffer for S-Sepharose and then applied to S-Sepharose HP column chromatography. As shown in Fig. 2(c), all the TGase activity was adsorbed on the S-Sepharose resin and eluted from 0.2 M NaCl as a single peak coinciding with the protein peak. The purification and yields of the enzyme are summarized in Table 1. The purified enzyme of 0.34 mg was obtained from 15 g of liver. In this method, the activity yield and purification were 23.7% and 591-fold, respectively.

Homogeneity of the Enzyme and Molecular Weight

The TGase active fractions from the last purification step gave a single band on SDS-PAGE (Fig. 3). The molecular weight of the purified enzyme was estimated to

![Fig. 2. Purification of TGase from walleye pollack liver.](image)

(a) Chromatography of the crude extract on Q-Sepharose HP using NaCl gradient. (b) Chromatography of the TGase fraction on Q-Sepharose HP using NaCl gradient. (c) Chromatography of the TGase fraction purified by Q-Sepharose on S-Sepharose HP using NaCl gradient. ○, Absorbance at 280 nm; ●, TGase activity; ..., NaCl concentration.

![Fig. 3. SDS-polyacrylamide gel electrophoresis of TGase fractions.](image)

Each sample was put on 5-20% polyacrylamide gel for SDS-PAGE analysis. Lanes 1, 9, protein molecule weight standards (kDa); Lane 2, the crude extract; Lane 3, the first Q-Sepharose HP fraction; Lane 4, the second Q-Sepharose HP fraction; Lanes 5-8, the S-Sepharose HP fractions 37-40, respectively.
be about 77 kDa on SDS-PAGE and is 60 kDa on a Super-
dex 200 gel filtration, suggesting that pollack liver TGase is a
monomeric protein (data not shown).

Metal ion Dependency of TGase Activity
The dependency on metal ions (Ca$^{2+}$, Sr$^{2+}$, Ba$^{2+}$ or
Mg$^{2+}$) of the activity of purified TGase was examined
(Fig. 4). In the absence of the metal ions, the TGase
showed no activity. However, the activity rose with the in-
crease in the concentration of Ca$^{2+}$ and Sr$^{2+}$, which fully
activated the enzyme above the concentrations of 3 mM
and 5 mM, respectively. The maximum TGase activities
were not changed up to 10 mM. Each Ba$^{2+}$ and Mg$^{2+}$,
homologous series elements of Ca$^{2+}$ in the periodic table,
scarcely activated the enzyme.

Optimum pH and Temperature for TGase Activity
The effect of various pH conditions on TGase activity
was determined. The activity increased remarkably from
neutral or mildly alkaline and the maximum activity was
observed at pH 9.0 (Fig. 5(a)). The effect of temperature
on TGase activity was examined in Tris buffer adjusted to
pH 8.5 at each temperature. The optimum temperature
was around 50°C (Fig. 5(b)).

Thermostability of Purified TGase
The thermostability of the purified TGase was examined
at 2°C or 40°C with or without CaCl$_2$ (Fig. 6). During incu-
bation at 40°C, the activity decreased with incubation
time. In the presence of Ca$^{2+}$, TGase activity could not be
detected after incubation for 10 min, however, 50% of the
activity remained in the absence of Ca$^{2+}$. This suggested
that the TGase in the active form is less stable than in the
inactive form. At 2°C, the activity was scarcely changed
during incubation for 20 min, regardless of Ca$^{2+}$ concen-
tration.

Fig. 4. Effect of metal ions on the activity of purified TGase.
TGase activity was assayed as described in Materials and Methods
except for metal ions. The relative activity was expressed as a per-
centage taking the maximum activity in the presence of Ca$^{2+}$ as 100%.
●, Ca$^{2+}$; ○, Sr$^{2+}$; △, Mg$^{2+}$; ▽, Ba$^{2+}$.

Fig. 5. Effect of pH (a) and temperature (b) on the activity of purified
TGase.
The following 75 mM buffers were used: △, Sodium acetate buffer
(pH 4–6); ○, Tris-HCl buffer (pH 6.8–8.5); ▽, Sodium borate
buffer (pH 9–10).

Fig. 6. Effect of Ca$^{2+}$ on thermostability of purified TGase.
TGase was incubated in the presence (closed symbol) or absence
(open symbol) of Ca$^{2+}$ at 2°C (triangles) or 40°C (circles) for up to
20 min, and the remaining activity was measured as relative values
against the activity prior to the incubation. (●, △), 50 mM Tris-HCl
(pH 8.0) containing 50 mM NaCl, 10 mM CaCl$_2$, 2 mM EDTA, 0.5
mM DTT; (○, ▽), 50 mM Tris-HCl (pH 8.0) containing 50 mM NaCl,
2 mM EDTA, 0.5 mM DTT.
Effect of Enzyme Inhibitors and Metal Ions on TGase Activity

The purified TGase was incubated at 25°C for 10 min with 1 mM enzyme inhibitor or metal ion, and the remaining activity of the enzyme was measured. The effects of various enzyme inhibitors and metal ions on the activity are summarized in Table 2. The TGase activity was strongly inhibited by MIA and NEM, known as thiol-enzyme inhibitors. In contrast, PMSF, a serine enzyme inhibitor, did not inhibit the enzyme activity strongly. As for metal ions, the enzyme activity was inhibited in the presence of Cu²⁺ and Zn²⁺, which have a strong affinity toward SH groups.

Reaction with Myosin B

Myosin B from pollack surimi was incubated with 7.6 units of the purified TGase at 37°C by standing for 60 min. The reaction resulted in the formation of the gel (Fig. 7(a)). When the solution was incubated with stirring, gelation was not observed. An aliquot sample was taken out from the reaction solution and subjected to SDS-PAGE to analyze the change in constituent proteins. As a function of reaction time, the amount of monomeric myosin heavy chain decreased and high molecular weight components were observed at the top of the gel, suggesting that myosin heavy chain was polymerized by the addition of the purified enzyme (Fig. 7(b)). In addition, a slight decrease in actin content was observed with increase in the incubation time, whereas other proteins hardly changed during the incubation. These results indicate that TGase catalyzes the crosslinking reaction of myosin heavy chain exclusively. The amounts of myosin heavy chain and actin were not changed in the absence of either TGase or Ca²⁺ (data not shown).

Discussion

In a previous study, we reported the purification of red sea bream (Pagrus major) liver TGase using ion-exchange and heparin affinity chromatographies. In this study, however, pollack liver TGase could be purified only by ion-exchange chromatographies. We assume, therefore, that a combination of cation- and anion-exchange chromatographic methods is significantly effective for the purification of fish TGase. In the present study, that the enzyme was well purified by one cation-exchange chromatography (S-Sepharose) is particularly noteworthy in the purification (from 8.8-fold to 144-fold by this step, Table 1).

Table 2. Effect of inhibitors and metal ions on enzyme stability

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Remaining activity (%)</th>
<th>Metal ions</th>
<th>Remaining activity (%)</th>
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<td>Mg</td>
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<td></td>
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</table>

* Remaining activity was measured after incubating the enzyme solution at 25°C for 10 min with each 1 mM metal ions or inhibitors.

The molecular weight of pollack liver TGase was estimated to be 77 kDa from SDS-PAGE mobility under the reduced condition. This is similar to that of guinea pig liver (76.6 kDa), red sea bream liver TGase (78 kDa) and partially purified carp muscle TGase (80 kDa). In contrast, the molecular weight of Ca²⁺-independent microbial TGase was one half (38 kDa) that of the tissue-type TGase of these vertebrates. The concentration of Ca²⁺ to express full TGase activity was 3 mM. Thus, this enzyme requires higher Ca²⁺ than red sea bream liver TGase, which required 0.5 mM Ca²⁺ for its maximum activation. Kishi et al. reported that partially purified TGase from carp muscle required 5 mM Ca²⁺ for the full activation, however, Sr²⁺ activated TGase to 35% of the full activity over 10 mM. On the other hand, in our results, Sr²⁺ fully activated pollack liver TGase over 5 mM to the same extent as Ca²⁺. From these differences in Ca²⁺ and Sr²⁺ sensitivity for enzyme activation, the molecular tertiary structures of the TGase can be clarified.

When MDC and N,N'-dimethylated casein were used as substrates, the optimum pH of the pollack TGase was around 9.0 and it was similar to that of the purified red sea bream liver TGase. In contrast, the optimum pH of guinea pig liver TGase was demonstrated to be pH 8.0 under the same assay conditions (data not shown), thus, it is suggested that fish TGases tend to have optimum pHs in the basic region more than mammalian liver TGase. The optimum temperature of the pollack TGase was about 50°C and the activity decreased subsequently beyond this temperature. The optimum temperature was slightly lower compared to the optimum temperature of red sea bream liver TGase, whose maximum activity was at 55°C. It is
well known that the thermostability of myofibrillar proteins corresponds with the environmental temperature at which fish live.\(^2\) Thus, in walleye pollack, living in cold seas, myosin Ca\(^{2+}\)-ATPase shows significant instability compared to that in red sea bream, which lives in mild seas.\(^2\) Therefore, the fact that the pollack TGase has a lower optimum temperature than red sea bream suggests some relation with the environmental conditions. In addition, the thermostability of the pollack TGase was slightly smaller compared to that of the red sea bream liver TGase (data not shown). The pollack TGase was inhibited by thiol-enzyme inhibitors, suggesting this enzyme has (a) cysteine residue(s) in the active site and could be classified as an SH-enzyme like other TGases, such as guinea pig liver TGase,\(^1\) microbial TGase\(^9\) and blood coagulation factor XIII.\(^1\)

When myosin B from pollack surimi was used as protein substrate, myosin heavy chain preferentially reacted and was polymerized by the purified TGase. This phenomenon was similar to the polymerization of myosin heavy chain during the setting process.\(^2\) In addition, gelation of myosin B could be observed by using the crude TGase extract instead of the purified TGase (data not shown). These results suggested that TGase from pollack liver can catalyze not only MDC incorporation, but also the crosslinking of myosin heavy chain, resulting in the formation of gel from fish meat sol. The total amount of enzyme activity to form actomyosin gel was similar in pollack and guinea pig liver TGase (data not shown). It is interesting to investigate the structural difference and protein substrate specificity from other TGases, for instance guinea pig liver, fish and microbial TGase.

**References**