Purification and Characterization of Transglutaminase from Japanese Oyster (*Crassostrea gigas*)

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A total of 73% transglutaminase (TGase) activity was detected in the gills and mantles of Japanese oysters (*Crassostrea gigas*), and TGase was purified by ammonium sulfate fractionation, followed by column chromatography. Two types of TGase with molecular weights of the 84 000 (TG-1) and 90 000 (TG-2) were obtained. The optimum pH was 8.0 for both TGases, and the optimum temperature for TG-1 and TG-2 was 40 and 25 °C, respectively. The activity of TG-1 increased with NaCl concentrations, whereas that of TG-2 was inhibited by NaCl. In the absence of NaCl, the activity of TG-1 increased with $CaCl_2$ concentrations up to 100 mM, but the concentration required to express full activity of TG-2 was 25 mM. This $CaCl_2$ concentration was lowered to 25 mM for TG-1 in the presence of 0.5 M NaCl, but not changed for TG-2. The ϵ-(γ-glutamyl)lysine was not detected in fresh oyster but was detected in processed oyster, suggesting the possibility that the intrinsic TGases react in the manufacturing process of oyster products.

Keywords: *Transglutaminase; oyster; purification*

INTRODUCTION

Transglutaminase (R-glutaminyl-peptide:amine γ-glutamyltransferase, EC 2.3.2.13, TGase), a Ca^{2+}-dependent enzyme, catalyzes an acyl transfer reaction between the γ-carboxyamide group of glutamine residues in peptide and various primary amines (Folk and Chung, 1973; Folk and Finlayson, 1977). When the ϵ-amino group of lysine in peptides acts as the acyl acceptor, an ϵ-(γ-glutamyl)lysine (ϵ-(γ-Glu)Lys) bond is formed intra- or intermolecularly, resulting in cross-linking and polymerization of proteins (Folk and Finlayson, 1977). TGases are widely distributed in various organisms, tissues, and body fluids, including vertebrates (Folk and Chung, 1973; Folk and Finlayson, 1977; Chung and Folk, 1972; Seitz et al., 1991; Abe et al., 1977; Kishi et al., 1991; Yasueda et al., 1994; Nakanishi et al., 1991), invertabrates (Tokunaga et al., 1993 ; Cariello et al., 1984), plants (Icekson and Apelbaum, 1987), and microorganisms (Ando et al., 1989 ; Klein et al., 1992).

TGase catalyzes cross-linking of protein and incorporation of primary amines into proteins, indicating that the enzymes have been utilized to modify the proteins (Whitaker, 1977). Some reports indicated that TGase could catalyze cross-linking among food proteins, such as casein, β-lactoglobulin, and soy bean proteins, which consequently improved their functionalities (Ikura et al., 1980; Nio et al., 1985). Ikura et al. (1981) reported TGase could incorporate some amino acids into proteins and improve their amino acid composition.

TGase products, ϵ-(γ-Glu)Lys bonds, are widely found in various biological materials and also in foods (Folk and Finalayson, 1977; Sakamoto et al., 1995). Recently, Seki et al. (1990) reported that fish muscle intrinsic TGase acts in the process of kamaboko (fish cake) and causes polymerization of the myosin heavy chain. Their studies suggest that TGase plays an important role in fish meat gelation.

Enzymic and structural properties of the TGase have been well characterized for mammalian origins (Ikura et al., 1988; Ichinose et al., 1986; Phillips et al., 1990; Nakanishi et al., 1991). The enzymes originating from marine organisms including fish, crustaceans, and echinoderms, for example, red sea bream liver (Yasueda et al., 1994, 1995), carp dorsal muscle (Kishi et al., 1991), limulus hemocyte (Tokunaga et al., 1993), lobster muscle (Myhrman and Bruner-Lorand, 1970), and sea urchin eggs (Cariello et al., 1984) have been reported. However, molluscous TGase has not been studied extensively.

To elucidate the properties of molluscous TGase, we attempted to obtain a purified enzyme from oyster. Oyster has been eaten raw and cooked for centuries; therefore, oyster is a friendly food material that has been associated with the eating pattern of humans. In this paper, we describe the purification and characterization of a TGase from Japanese oyster (*Crassostrea gigas*), which is distributed in large areas of the sea. In addition, the content of ϵ-(γ-Glu)Lys bonds was determined in fresh oyster and various oyster products and a discussion is given as to whether the oyster intrinsic TGase acts in the manufacturing process of oyster products.

MATERIALS AND METHODS

Materials. Live shelled Japanese oysters, *C. gigas*, were obtained from a fishery market in Tsukiji, Tokyo, Japan. Oyster products were obtained from local markets in Kawasaki, Japan. DEAE-Sephacel, Q-Sepharose FF, phenyl-Sepharose HP, heparin-Sepharose, Sephacryl S-200, and calibration kits for electrophoresis were purchased from Pharmacia (Uppsala, Sweden). Hydroxyapatite was purchased from Bio-Rad (Hercules, CA). *N*,*N*′-Dimethylated casein, monodansylcadaverine (MDC), porcine kidney prolidase, porcine kidney leucine aminopeptidase (type III-CP), and synthetic ϵ-(γ-Glu)Lys were from Sigma (St. Louis, MO). *Streptomyces griseus* protease (Pronase) and bovine pancreas carboxypeptidase A were obtained from Boehringer Mannheim Gmbh (Mannheim, Germany). Phenylmethanesulfonyl fluoride (PMSF), *o*-phthalaldehyde (OPA), 2-mercaptoethanol, and monoiodoacetic acid (IAA) were the products of Wako Chemical (Osaka, Japan). Dithiothreitol (DTT), ammonium sulfate, *N*-

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ethylmaleimide (NEM), and ethylenediaminetetraacetic acid (EDTA) were purchased from Nakarai Tesque (Kyoto, Japan). All other chemicals were of reagent grade. Myosin B was prepared from frozen surimi of Alaska pollack, *Theragra chalcogramma*, by the method of Takashi et al. (1970) with slight modification. Guinea pig liver TGase was purchased from Takara Shuzo Co. (Otsu, Japan).

TGase Activity. TGase activity was assayed in terms of the incorporation of MDC into *N*,*N*′-dimethylated casein by the method of Takagi et al. (1986) with slight modification. The assay mixture was comprised of 2.4 mL of 50 mM Tris-HCl (pH 8.5), 1 mg/mL *N*,*N*′-dimethylated casein, 15 μM MDC, 3 mM DTT, 10 mM $CaCl_2$, and an appropriate amount of an 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 solution. The fluorescence intensity was measured with excitation and emission wave lengths set at 350 and 480 nm, respectively. One unit of enzyme was defined as the amount that incorporated 1 nmol of MDC into *N*,*N*′-dimethylated casein per minute.

Purification of TGase. All following purification procedures were carried out below 4 °C.

Preparation of Crude TGase Extracts. An oyster with digestive diverticulum and adductor muscle removed was homogenized in a four volumes of 20 mM imidazole hydrochloride (pH 7.0) containing 10 mM NaCl, 5 mM EDTA, and 1 mM DTT (INED buffer). The homogenate was centrifuged at 10000*g* for 30 min, and the supernatant was filtered through a gauze. The filtered supernatant was further centrifuged at 100000*g* for 60 min, and supernatant was filtered through a cellulose acetate membrane (0.45 μm). The filtrate was referred to as the crude extract.

DEAE-Sephacel. The crude extract was brought to 80% saturation of ammonium sulfate, and the resulting precipitate was collected by centrifugation at 10000*g* for 30 min. The pellet was dialyzed overnight against INED buffer. The dialysate was applied to a DEAE-Sephacel column (5 × 18 cm), equilibrated with INED buffer. After washing the column with INED buffer, elution was carried out with a linear NaCl gradient from 10 to 500 mM.

Q-Sepharose FF. The TGase activity obtained in DEAE-Sephacel chromatography was dialyzed against INED buffer and then applied to a Q-Sepharose FF column (2.6 × 18 cm), equilibrated with INED buffer. After washing with the equilibration buffer, elution was carried out with a linear NaCl gradient from 10 to 500 mM. The TGase fraction was separated into two peaks (TG-1, TG-2), and these fractions were purified separately.

Purification of TG-1. *Phenyl-Sepharose HP.* The TGase fraction obtained in Q-Sepharose FF chromatography was brought to 20% of saturation with ammonium sulfate and applied to a phenyl-Sepharose HP column (2.6 × 10 cm), equilibrated with 20% ammonium sulfate containing INED buffer. After washing with the equilibration buffer, elution was carried out with a linear ammonium sulfate gradient from 20 to 0%.

Heparin Affinity. Partially purified TG-1 obtained in the phenyl-Sepharose HP chromatography was dialyzed against 10 mM Bis-Tris (pH 6.2) containing 10 mM NaCl, 1 mM EDTA, and 0.2 mM DTT (BEND buffer). The dialysate was applied to heparin-Sepharose column (volume 1 mL), equilibrated with BEND buffer. After washing with BEND buffer, elution was carried out with a linear NaCl gradient from 10 to 500 mM NaCl.

Sephacryl S-200. The concentrated sample obtained from heparin-affinity chromatography was applied to a Sephacryl S-200 column (2.6 × 60 cm), eluted with BEND buffer, and then used as purified TG-1.

Purification of TG-2. *Phenyl-Sepharose HP.* The partially purified TG-2 obtained in Q-Sepharose FF chromatography was applied to a phenyl-Sepharose HP chromatography column as described above.

Mono-Q. The TGase fraction obtained in phenyl-Sepharose HP chromatography was dialyzed against BEND buffer. The dialysate was chromatographed on a Mono-Q HR column (1.0 × 10 cm), equilibrated with BEND buffer, the activity eluted with a linear gradient of NaCl from 10 to 500 mM.

Heparin-Affinity. The TGase fraction obtained in Mono-Q chromatography was dialyzed against BEND buffer. The dialysate was applied to a heparin-Sepharose column (vol. 1 mL), equilibrated with BEND buffer and the unadsorbed fraction with the TGase activity was collected.

Hydroxyapatite Chromatography. The TG-2 fraction obtained from heparin-affinity flow through solution was chromatographed onto an hydroxyapatite column (volume 5 mL), equilibrated with 10 mM sodium phospate (pH 7.2) containing 0.5 mM EDTA and 0.2 mM DTT. The TGase was eluted with a linear gradient of sodium phosphate from 10 to 400 mM. Finally, the TGase fraction was applied to a Sephadex G-25 column, equilibrated with BEND buffer to exchange to the dissolved buffer, and then used as purified TG-2.

Determination of Protein Concentration. Protein concentration was determined using the dye-binding method (Bradford, 1976). Bovine serum albumin was used as the standard protein.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis. SDS−PAGE was performed on a 5−20% gradient gel by the method of Laemmli (1970). 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 markers. Proteins were stained with Coomassie brillant blue R-250.

Effect of Inhibitors and Metal Ions. The purified enzymes were incubated with 1 mM each of IAA, NEM, and PMSF and various metal ions at 25 °C for 10 min, and the remaining activity was measured. The remaining activity was estimated as a percentage, taking untreated TGase activity as 100%.

Optimal pH. The activities of purified enzymes in 70 mM of sodium acetate buffer, pH 5−6, imidazole hydrochloride buffer, pH 6−8, and Tris-HCl buffer, pH 7−10 were measured at 37 °C for 30 min. Activity was estimated as a percentage, taking maximum pH activation as 100%.

Optimal Temperature. The activity of purified enzymes in 50 mM Tris-HCl buffer were measured at 5−60 °C for 30 min. Tris buffer was adjusted to pH 8.5 at each temperature using $-\Delta pK_a/°C = 0.031$. The activity was estimated as a percentage, taking maximum temperature activation as 100%.

Reaction with Myosin B. Myosin B solution (7.4 mg/mL) dissolved in 20 mM Tris-HCl (pH 7.5) containing 0.3 M NaCl and 10 mM $CaCl_2$ was incubated by stirring at 37 °C for up to 120 min with the appropriate amount of purified TG-1. An aliquot sample was taken and applied to SDS−PAGE to analyze the protein polymerization.

Determination of ϵ-(γ-Glu)Lys. The determination of ϵ-(γ-Glu)Lys content was performed according to the method of Griffin et al. (1982) with slight modification. Briefly, lyophilized sample was ground to a powder. Each sample (20−30 mg of protein) was digested exhaustively and successively by pronase (0.2 unit/mg of protein), prolidase (0.45 unit), leucine aminopeptidase (0.8 unit), and carboxypeptidase A (0.4 unit) to obtain a mixture of amino acids and ϵ-(γ-Glu)Lys in 0.1 M borate buffer (pH 8.0). The digested samples were preliminarily fractionated to remove interfering compounds by a reversed phase high-performance liquid chromatography (RP-HPLC) with an Inertsil ODS-2 column (G-L Sciences Co., Ltd., Tokyo, Japan). The fraction containing ϵ-(γ-Glu)Lys was collected and derivatized with OPA. The OPA-labeled ϵ-(γ-Glu)Lys was injected into a Zorbax BP-C8 column (G-L Sciences), fractionated by RP-HPLC, and detected using a fluorescence detector (excitation 334 nm, emission 440 nm). The amount of ϵ-(γ-Glu)Lys was determined with synthetic ϵ-(γ-Glu)Lys as standard.

RESULTS AND DISCUSSION

Distribution of TGase Activity in Oyster Organs. The distribution of TGase activity in an oyster was investigated. Oyster organs were separated into five parts: digestive diverticulum, peripheral part of the

Table 1. Distribution of TGase Activity in Oyster Tissues

tissue	specific act. (units/mg)	total act. (units)	distribution[a] (%)
digestive diverticulum	0.039	25.6	8.3
gill	0.600	119.6	38.7
mantle	0.310	106.8	34.5
adductor muscle	0.006	4.2	1.4
other[b]	0.160	53.1	17.2

[a] Expressed as relative values to the total TGase activity. [b] Peripheral part of digestive diverticulum.

Table 2. Summary of the Purification of TG-1 from Oyster[a]

procedure	total protein (mg)	total act. (units)	specific act. (units/mg)	yield (%)	purification (*x*-fold)
crude extract	8200	2550	0.311	100	1
precipitate[b]	5824	2900	0.498	114	1.6
DEAE-Sepharose	1371	2427	1.77	95.4	5.7
Q-Sepharose	307	629.4	2.05	24.7	6.6
phenyl-Sepharose	43	460.1	10.70	18.0	34.4
heparin-Sepharose	3.6	370.8	103.0	14.5	331.3
Sephacryl S-200	0.34	53.2	156.6	2.09	503.5

[a] Oyster with adductor muscle and digestive diverticular removed was used. [b] An 80% saturated ammonium sulfate fractionation.

digestive diverticulum, gill, mantle, and adductor muscle. TGase activity in the crude extracts prepared from each organ were measured (Table 1). All the organs assayed contained TGase activity in a range from 0.006 to 0.6 unit/mg of protein. Among these organs, the gill demonstrated the highest specific activity. Combined TGase activity in the gill and mantle reached ~73% of total activity; on the other hand, significantly lower activity was detected in the aductor muscle and digestive diverticulum.

Purification of TGase from Oyster. As described above, the activity was low in digestive diverticulum and adductor muscle; therefore, these parts were removed from the oyster for TGase purification. A total of 290 g of wet weight oyster without the digestive diverticulum and adductor muscle was obtained. The protein fraction obtained by 80% saturated ammonium sulfate fractionation from the crude extract was applied to DEAE-Sephacel column chromatography. All of the activity was adsorbed on the resin and eluted with 0.15 M NaCl as a single peak (data not shown). The TGase fraction was subsequently applied to Q-Sepharose FF column chromatography. During the elution with NaCl gradient, we found that two peaks with TGase activity appeared (data not shown). The two fractions were separately collected and designated as TG-1 and TG-2, respectively. The first fraction (TG-1) was purified by phenyl-Sepharose, heparin-Sepharose, and Sephacryl S-200 chromatography. The second fraction (TG-2) was purified Mono-Q, heparin-Sepharose, and hydroxyapatite chromatography. From the activity and protein elution profiles of the Sephacryl S-200 and hydroxyapatite chromatographies, the enzyme activity peak coincided with the protein peak (data not shown), indicating that the TGases have been purified. The result of purification was summarized in Tables 2 and 3. From these procedures, 0.34 and 0.24mg of purified TG-1 and TG-2, respectively, were obtained from 290g of oyster. The specific activity and final yield for TG-1 and TG-2 were 156.6 units/mg, 2.1%, 110.6 units/mg and 1.0%, respectively.

Mammalian endogenous tissue-type TGases, such as guinea pig liver and rat liver TGases, have been purified

Table 3. Summary of the Purification of TG-2 from Oyster[a]

procedure	total protein (mg)	total act. (units)	specific act. (units/mg)	yield (%)	purification (*x*-fold)
Q-Sepharose	337	1331	3.95	52.1	12.7
Phenyl-Sepharose	59	672.6	11.4	26.5	36.8
Mono-Q	17	460.7	27.1	18.1	87.1
heparin-Sepharose	1.4	59.5	42.5	2.3	136.8
hydroxyapatite	0.24	26.5	110.6	1.0	355.6

[a] Purification was separately performed after the Q-Sepharose step in Table 2.

by anion exchange chromatography, protamine extracts, and gel filtration (Connellan et al., 1971; Abe et al., 1977). Brookhart et al. (1983) reported purification of guinea pig liver TGase by phenylalanine affinity chromatography. Ikura et al. (1985) reported one-step purification of guinea pig liver TGase by immobilized antibody affinity chromatography. In general, anion exchange and gel filtration chromatographies are effective for the purification of mammalian tissue-type TGase. As for TGases from marine organisms, limulus hemocyte, was purified by using zinc-chelating Sepharose (Tokunaga et al., 1993). In a previous report, we performed the purification of red sea bream liver TGase by anion−cation exchange chromatography and heparin affinity chromatography (Yasueda et al., 1994). In our present results, for both TGases, after purification by phenyl-Sepharose, the buffer pH was lowered to 6.2 from 7.0 to facilitate the removal of other contaminant proteins. The activity of TG-1 was adsorbed on heparin-Sepharose at pH 6.2, but TG-2 was not adsorbed under the same conditions. The cause of the difference for heparin affinity is unclear, but heparin affinity chromatography was effective in the purification of both the oyster TGases.

SDS−PAGE Homogeneity and Molecular Weight. The purified enzymes obtained from the last chromatography were subjected to SDS−PAGE (Figure 1). Both TGases showed a single protein band. According to the calibration curve for the standard proteins, the molecular weights of the purified enzymes were estimated to be about 84 000 (TG-1) and 90 000 (TG-2) on SDS−PAGE. As a control, guinea pig liver TGase was also applied on the same gel, indicating the molecular size of both oyster TGases was larger than that of guinea pig liver TGase, whose molecular size is 76 600 (Ikura et al., 1988). In marine organisms, fish (red sea brem and Alaska pollack liver) TGase and crustacean (limulus hemocyte) TGase have been reported of having a molecular weight of 78 000 (Yasueda et al., 1994), 77 000 (Kumazawa et al., 1996), and 86 000 (Tokunaga et al., 1993), respectively, and these TGases were monomeric proteins. In our result, the molecular weights of oyster TGases were similar to that of limulus hemocyte TGase. When the crude TGase extract was subjected to gel filtration using Superdex 200, the activity eluted as a single peak at the position around 100 000, suggesting oyster TGases were in the monomeric form composed of a single polypeptide chain, but exact molecular weight determination requires futher study. It is noteworthy that oyster has two types of TGase, whose molecular sizes are different. To our knowledge, this is the first report that purification of mollusc TGase shows a single band on SDS−PAGE and two types of TGase are present in the mollusc tissue.

Effect of Inhibitors. The effects of enzyme inhibitors on the TGases were investigated, and the results

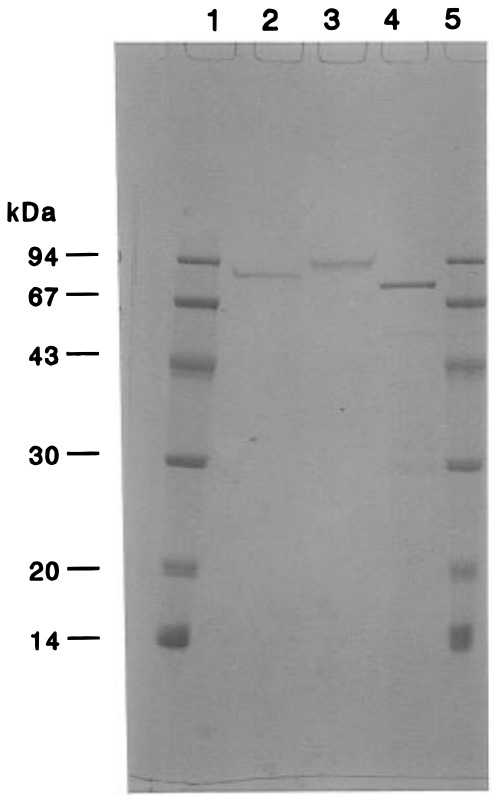

Figure 1. Sodium dodecyl sulfate gel electrophoresis profile of the purified enzymes. A 5−20% gradient gel was used: lanes 1 and 5, protein molecule weight standards; lane 2, TG-1 from oyster; lane 3, TG-2 from oyster; lane 4, guinea pig liver.

Table 4. Influence of Inhibitors on Purified Enzymes

	remaining act.[a] (%)	
inhibitors	TG-1	TG-2
control	100	100
NEM[b]	11.8	10.8
IAA[b]	0	0
PMSF[b]	96.4	102

[a] The remaining activity was measured after incubating with 1 mM of inhibitor at 25 °C for 10 min. [b] NEM, *N*-ethylmaleimide; IAA, iodoacetic acid; PMSF; phenylmethanesulfonyl fluoride.

are summarized in Table 4. Both TGases were markedly inhibited by NEM, as well as IAA, but the enzymes were insensitive to PMSF. As for divalent metal ions, the homologous series elements of Ca^{2+} in the periodic table, such as Ba^{2+}, Sr^{2+}, and Mg^{2+}, hardly inhibited the activity (data not shown). Both TGases, however, were significantly inhibited by Cu^{2+}, Zn^{2+} ,and Pb^{2+} (data not shown), which have strong affinity to sulfhydryl groups in cysteine residues. These results suggest both oyster TGases possess (a) sulfhydryl group(s) in the active site, similarly to other TGases.

Optimal pH. The optimal pH for the incorporation of MDC into *N*,*N*′-dimethylated casein was 8.0 for both the purified enzymes (Figure 2). The result was similar to that of guinea pig liver TGase under the same assay conditions (data not shown). These pH values were lower than that for red sea bream liver TGase, whose optimal pH was 9−9.5 (Yasueda et al., 1994).

Optimal Temperature. The optimal temperatures of TG-1 and TG-2 activity, for a reaction time of 30 min at pH 8.5, were 40 and 25 °C, respectively (Figure 3). The relative activity of TG-2 was more than 40% at 10 °C, but the relative activity of TG-1 was 20% at the same temperature (10 °C). In contrast, the activity of TG-2

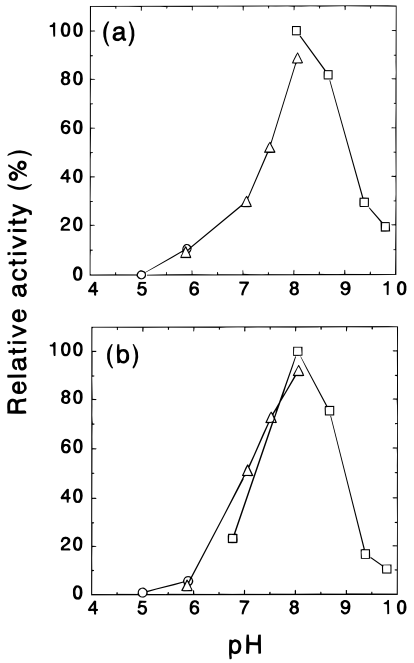

Figure 2. Effect of pH on the activity of the purified enzymes: (a) TG-1; (b) TG-2. (○) Sodium acetate buffer; (△), imidazole hydrochloride buffer; (□), Tris-HCl.

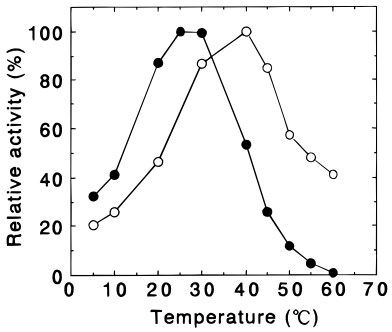

Figure 3. Effect of temperature on the activity of the purified enzymes: (○), TG-1; (●), TG-2.

decreased to 12% in 50 °C and disappeared at 60 °C; however, the activity of TG-1 was 60 and 40% at 50 and 60 °C, respectively. Therefore, there was a tendency for TG-1 to be more active at a high temperature, while TG-2 was more active at a low temperature. Both of these optimal temperatures were lower compared to the red sea bream liver TGase, which had maximum activity at 55 °C (Yasueda et al., 1994). The reason for optimal temperature difference is unclear; one reason for the difference may relate to the thermostability of these TGases. This difference in optimal temperature between TG-1 and TG-2 suggests independent roles for each in oyster tissue.

Effects of NaCl on TGase Activity. The effect of NaCl on the purified TGases was investigated. The activity was measured in assay mixtures containing various concentrations of NaCl up to 1 M. As shown in Figure 4, the specific activity of TG-1 increased as a function of NaCl concentration and was 3-fold higher in the presence of more than 0.6 M NaCl, compared to the activity at the lower amount of (0.08 mM) NaCl. On the other hand, no remarkable changes were observed in the specific activity of TG-2 up to 0.2 M NaCl, but the activity decreased at higher concentration. The activity hardly changed, when red sea bream liver TGase and guinea pig liver TGase were used in the same investigation (data not shown). Kishi et al. (1991)

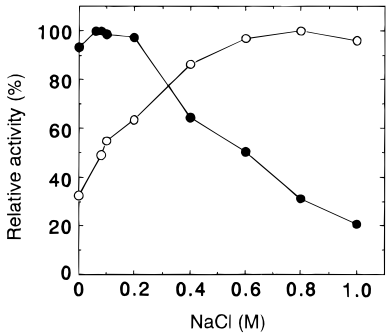

Figure 4. Effect of NaCl on the activity of purified enzymes. The relative activity was expressed as a percentage taking maximal NaCl activation of each enzyme as 100%: (○), TG-1; (●), TG-2.

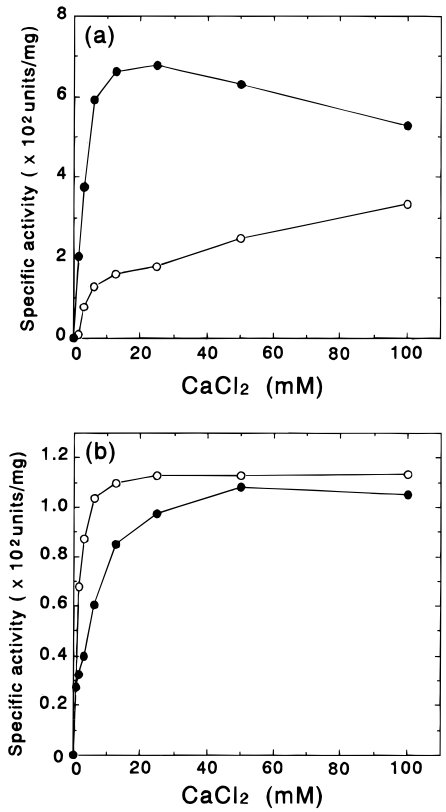

Figure 5. Effect of $CaCl_2$ on the activity of the purified enzymes: (a) TG-1; (b) TG-2. (○) Without NaCl; (●) with 0.5 M NaCl.

reported that NaCl had no effect on the activity of TGase from carp dorsal muscle, using acetylated casein and MDC as substrates. It has been reported that limulus hemocyte TGase was inhibited by NaCl (Tokunaga et al., 1993). We assume that the cause of these changes in the specific activity by NaCl may be some structural changes in the enzyme, not in the substrates (*N*,*N*′-dimethylated casein and MDC). The reason for the activation or inhibition in oyster TGases by NaCl is left unexplained.

Effects of Ca^{2+} on TGase Activity. The effect of Ca^{2+} on the activity of the purified TGases was investigated. $CaCl_2$ was added in the assay mixuture from 0 to 100 mM with or without 0.5 M NaCl. As shown in Figure 5, the enzymes showed no activity in the absence of Ca^{2+}. The activity of TG-1 increased with high concentrations of Ca^{2+} up to 100 mM (Figure 5a) without NaCl, but the curve changed abruptly and a maximum activity was observed at 25 mM Ca^{2+} in the presence of 0.5M NaCl. The maximum activity of TG-2 was observed over 25 mM Ca^{2+} in the absence of NaCl, and the curve was suppressed by NaCl (Figure 5b).

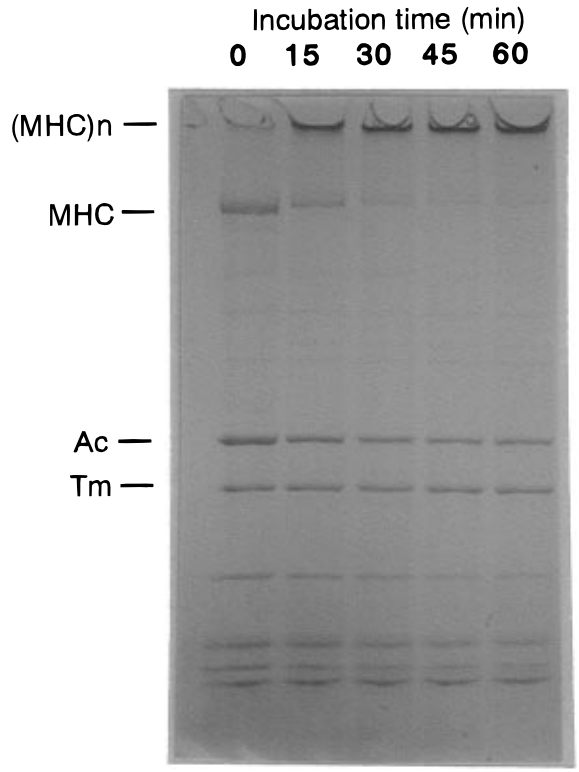

Figure 6. SDS−PAGE profiles of myosin B prepared from frozen pollack surimi during incubation with TG-1. Myosin B solution (7.4 mg/mL) dissolved in 20 mM Tris-HCl (pH 7.5) containing 0.3 M NaCl and 10 mM $CaCl_2$ was incubated with stirring at 37 °C with 20 μg of TG-1. An aliquot sample was taken and applied to SDS−PAGE using a 5−20% gradient gel. The positions of polymerized myosin heavy chain, myosin heavy chain, actin, and tropomyosin are indicated as $(MHC)_n$, MHC, Ac, and Tm, respectively.

The purified enzymes of red sea bream liver (Yasueda et al., 1994), limulus hemocyte (Tokunaga et al., 1993), and guinea pig liver (Ando et al., 1989) required 0.5, 8, and 10 mM Ca^{2+}, respectively, to express its full activity. Thus, the sensitivity of oyster TGases for Ca^{2+} was the lower than that of other purified TGases. It is interesting that the concentration of Ca^{2+} needed to express its full TG-1 activity was related to the concentration of NaCl. Such a tendency with guinea pig liver TGase was not observed under the same conditions (data not shown). These unique properties may be attributable to some structural changes in oyster TG-1 in the presence of NaCl, although more detailed studies are required.

Reaction with Myosin B and Formation of ε-(γ-Glu)Lys. Crude myosin B solution (7.4 mg/mL) prepared from frozen Alaska pollack surimi dissolved in 20 mM Tris-HCl (pH 7.5) containing 0.3 M NaCl, 25 mM $CaCl_2$, and 5 mM DTT was incubated with stirring with 20 μg of TG-1 for 37 °C. An aliquot sample was taken out and subjected to SDS−PAGE. As shown in Figure 6, monomeric myosin heavy chain decreased with incubation time and high molecular weight components were observed at the top of the gel, suggesting that the polymerization of myosin heavy chain occurred by the addition of purified enzyme. These phenomena were similar to polymerization of myosin heavy chain during the setting process of kamaboko (Numakura et al., 1985). These results suggest that TGase from oyster

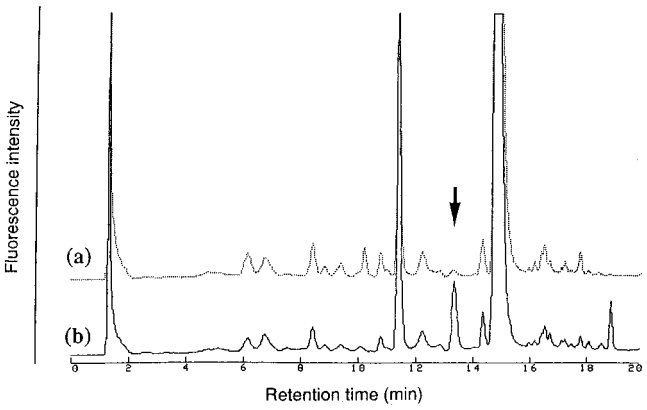

Figure 7. HPLC profiles of the proteolytic digest of the myosin B. The digest was labeled with OPA, and the detection was done with excitation at 334 nm and emission at 440 nm. An arrow indicates the position of ϵ-(γ-Glu)Lys: (a) intact myosin B; (b) myosin B incubated with TG-1 for 30 min.

Table 5. ϵ-(γ-Glutamyl)lysine Contents in Fresh and Processed Oysters

oyster sample	ϵ-(γ-glutamyl)lysine (nmol/g of wet weight)	oyster sample	ϵ-(γ-glutamyl)lysine (nmol/g of wet weight)
fresh	nd[b]	smoked[a]	46.8
pickled[a]	19.3	baked[a]	130

[a] Pickled, pickled in soy sauce and boiled; baked, salted and baked. [b] Not detected.

can catalyze the cross-linking of the myosin heavy chain, resulting in the formation of gel to fish meat sol. The amount of actin also decreased slightly, but other proteins scarcely changed during the incubation. The protein that acts with TGase mainly was related to the myosin heavy chain. After a 30 min incubation, an aliquot sample was subjected to ϵ-(γ-Glu)Lys analysis (Figure 7). The peak of ϵ-(γ-Glu)Lys was observed, but no peak in the sample without the incubation was observed. Thus, the myosin heavy chain was preferentially polymerized by TG-1, resulting in formation of covalent ϵ-(γ-Glu)Lys bonds.

Recently, intrinsic TGases contained in fish surimi reacted during the manufacturing process of kamaboko (Seki et al., 1990), resulting in formation of myosin heavy chain polymers and ϵ-(γ-Glu)Lys (Tsukamasa et al., 1993). Polymerization of the myosin heavy chain by the red sea bream (Yasueda et al., 1994) and guinea pig liver TGases (Joseph et al., 1994) and plasma factor XIIIa (Jiang et al., 1992) have been reported. In our results, polymerization of myosin heavy chain was also confirmed and suggests that oyster TGases can improve surimi gel.

Quantitative Analysis of ϵ-(γ-Glu)Lys in Oyster Products. The contents of ϵ-(γ-Glu)Lys in fresh oyster and various processed oyster products were determined. The results are summarized in Table 5. ϵ-(γ-Glu)Lys was not detected in fresh oyster, but we presume that undetectable amounts exist rather than the ϵ-(γ-Glu)-Lys not being present in the oyster flesh. On the other hand, all the oyster products contained ϵ-(γ-Glu)Lys ranging from 19.3 to 130 nmol/g of wet weight product. Baked oyster had the largest amount. We reported the quantitative analysis of ϵ-(γ-Glu)Lys in 127 foods obtained from local markets. From the results, we found that processed foods had relatively higher contents of ϵ-(γ-Glu)Lys than raw materials (Sakamoto et al., 1995). ϵ-(γ-Glu)Lys was formed during mild heating (10−40 °C) during the setting of salted fish meat paste, called suwari, and this is an example of an increase of ϵ-(γ-Glu)Lys during the manufacturing process (Kimura et al., 1991; Tsukamasa et al., 1993; Kumazawa et al., 1995). Suwari is an important process, with a significant influence on surimi products, which changes fish meat sol to elastic gel. We reported the correlation between ϵ-(γ-Glu)Lys contents in surimi products and its rheological properties (Seguro et al., 1995a), suggesting some relation between ϵ-(γ-Glu)Lys content and the rheological properties of oyster products.

From a nutritional point of view, lysine is an important amino acid in protein-based foods, but the ϵ-amino group of lysine, known as an active group, is sometimes modified with another acyl group. Some of the ϵ-amino group modified lysines have decreased its bioavailability. Fink et al. (1980) reported that ϵ-(γ-Glu)Lys was digested into lysine and 5-oxo-L-proline by γ-glutamylamine cyclotransferase. Recently, we reported enzymic cleavage of the ϵ-(γ-Glu)Lys bond and liberation of free lysine and glutamic acid by γ-glutamyltranspeptidase from bovine kidney (Seguro et al., 1995b). These studies imply that ϵ-(γ-Glu)Lys would be digested and lysine, as released by enzymic reaction, may possibly be utilized in the animals. Actually, we studied bioavailability of the ϵ-(γ-Glu)Lys moiety in TGase-treated cross-linked casein in rats. As a result, ϵ-(γ-Glu)Lys was cleaved and lysine in the dipeptide utilized normally (Seguro et al., 1996).

The cross-links were widely distributed in various organism tissues (Folk and Finlayson., 1977). ϵ-(γ-Glu)-Lys was also formed in proteins during heating and increased with the severity of heating (4−27 h, 121 °C) (Asquith and Otterburn, 1971). These phenomena are independent of TGase activity.

As for ϵ-(γ-Glu)Lys in oyster products, we did not confirm whether ϵ-(γ-Glu)Lys detected in oyster products was formed through catalytic action of intrinsic TGases or by heat treatment, but we assume that enzymatic formation may contribute to a larger extent. In smoked oyster, ϵ-(γ-Glu)Lys cross-links increased during mild heating (37 °C); however, these findings will be reported elsewhere.

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