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Enzyme Responsible for Egg Envelope (Chorion) Hardening in Fish: Purification and Partial Characterization of Two Transglutaminases Associated with Their Substrate, Unfertilized Egg Chorion, of the Rainbow Trout, *Oncorhynchus mykiss*[1]

Chang-Rak Ha and Ichiro Iuchi[2]

Life Science Institute, Sophia University, Kioi-cho 7-1, Chiyoda-ku, Tokyo 102-8554

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From our previous studies, we have suggested that "egg envelope (chorion) hardening-enzyme" (first proposed by Zotin [*J. Embriol. Exp. Morph.* 6, 546-568 (1958)]) in the rainbow trout, *Oncorhynchus mykiss*, is transglutaminase (TGase), and that it coexists with its substrate, the unfertilized egg chorion, and forms ε-(γ-glutamyl)lysine cross-links between the chorion proteins. In the present study, we extracted the TGase activity from the isolated chorions by homogenization with isotonic saline (143 mM NaCl-10 mM Tris·HCl, pH 7.2) and fractionated the extract by Toyopearl HW55S gel filtration with the isotonic saline containing 5 mM $CaCl_2$ and 5 mM 2-mercaptoethanol (2-ME). One peak of TGase activity (P2) was obtained. When the eluates were dialyzed against 5 mM $CaCl_2$-5 mM 2-ME-10 mM Tris·HCl (pH 7.2), another peak of the activity (P1) appeared. P1 TGase activity, which becomes apparent in a medium of low ionic strength, is involved in acceleration of the chorion hardening after egg activation in fresh water, so-called water activation. We purified the two TGases, P1 and P2, by SP-Sepharose, Q-Sepharose, and TSK-gel column chromatography. The molecular mass of the native form of P1 TGase was estimated as 103 kDa by Toyopearl HW55S gel filtration and as 100 kDa by the TSK-gel filtration. SDS-PAGE analysis showed that it consisted of heterogeneous 86- and 76-kDa proteins. However, these proteins closely resembled each other in amino acid composition, which was characterized by high content of Thr, Gly, and Pro residues as compared with P2 TGase. In contrast, the P2 TGase was isolated as a homogeneous 76-kDa protein and characterized by high content of Glx (Glu/Gln) and His residues. Neither of the chorion TGases of rainbow trout, P1 and P2, was similar to the liver-type TGase of red sea bream or the tissue-type TGase of chum salmon in amino acid composition. Examination of susceptibility to various inhibitors, reactivation by $CaCl_2$, pH dependency, and activity of the polymerization of chorion proteins suggested that the P1 and P2 TGases were essentially similar to each other in enzymatic properties.

Key words: egg envelope hardening, *Oncorhynchus mykiss*, rainbow trout, transglutaminase, vitelline membrane.

The vitelline envelope or unfertilized egg envelope is converted into a fertilized egg envelope by the action of different enzymes in different animals: (i) protease [sea urchin (*1*), the fish, *Oryzias latipes* (*2*), *Xenopus laevis* (*3, 4*), mouse (*5, 6*)]; (ii) peroxidase [sea urchin (*7-9*), mouse (*10, 11*)], and (iii) transglutaminase [sea urchin (*12, 13*), rainbow trout (*14-17*), cod (*18*), *Oryzias latipes* (*19, 20*)]. In addition, a cortical granular lectin is known to participate in the conversion of *Xenopus laevis* egg (*21, 22*). The change of the vitelline envelope results in the hardening of the egg envelope.

The mechanism of the fertilization/egg activation-associated hardening of the egg envelope (chorion) in fish has been extensively investigated. Earlier studies have suggested the participation of Ca^{2+} ions (*23-25*), an acidic environment (*26*), and mucopolysaccharides in cortical alveoli (*27*), reduction/oxidation reactions (*28, 29*), and the formation of disulfide cross-links between chorion proteins (*28*) in the chorion hardening, but questions remain concerning the mechanism. In 1958, Zotin (*29*) proposed that the factor responsible for the hardening in some salmonid fishes is a "hardening enzyme." Further studies showed that the content of ε-(γ-glutamyl)lysine isopeptide in chorions increased after fertilization or egg activation (*14, 16*) and that the chorion hardening was inhibited by some amines such as cadaverine and monodansylcadaverine (*16, 18, 19*), suggesting that the hardening enzyme is transglutaminase (TGase). In addition, earlier studies showed that the hardening enzyme or the TGase was localized exclusively in its substrate, unfertilized egg

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.
[2] To whom correspondence should be addressed. E-mail: i-iuchi@hoffman.cc.sophia.ac.jp

chorion (*15, 17, 19, 30*). Thus, the chorion of fish egg is considered to be a functional structure in the sense that it contains both the enzyme and its substrate. Recently, we isolated a 76-kDa TGase from the unfertilized egg chorion of the rainbow trout, *Oncorhynchus mykiss*, and characterized it partially (*31*). In the present study, we found another TGase in the chorion whose activity becomes apparent only in a medium of low ionic strength, and which therefore seems to be responsible for physiological hardening of salmonid fish eggs on activation in water.

Transglutaminase (TGase) (protein-glutamine γ-glutamyltransferase; EC 2.3.2.13), which is responsible for ε-(γ-glutamyl)lysine isopeptide crosslink formation between proteins, is involved in blood clotting (coagulation factor XIIIa) (*32-34*), hair formation (*35*), keratin formation (*36*), and copulatory plug formation (*37*). It has also been found in liver (*38*), erythrocytes (*33*), and aortic endothelial cells (*39*), and it may play a role in apoptotic death of several types of cells (*40, 41*). In addition, *Limulus* hemocyte TGase has been reported to participate in blood coagulation (*42*), the annulin involved in morphogenetic activities of grasshopper embryonic cells to be homologous to TGase (*43*), and a mesenchymal TGase in early larva of *Ciona intestinalis* to participate in tunica formation (*44*). The fact that TGase is involved in the egg envelope hardening (*12, 14, 16-19, 31*) is a newly discovered function of this enzyme.

MATERIALS AND METHODS

Unfertilized eggs of the rainbow trout, *Oncorhynchus mykiss*, were collected at the hatcheries by artificial spawning. The eggs were kept in coelomic fluid at 0-4°C, transported to our laboratory, and stored in an ice-cold water bath. Within 2-3 days, they were used to isolate egg envelopes (chorions). The eggs showed no signs of activation, such as perivitelline space formation, or of degeneration, such as oil droplet assembly.

Estimation of Transglutaminase (TGase) Activity—TGase activity was determined essentially according to Lorand and Gotoh (*45*). The standard reaction mixture consisted of 0.5 mM monodansylcadaverine (MDC), 0.2% dimethylcasein, 5 mM 2-mercaptoethanol (2-ME), 5 mM $CaCl_2$, 50 mM Tris·HCl (pH 7.2), and various amounts of enzyme sample. After incubation at 10°C, the reaction was stopped by adding ice-cold trichloroacetic acid (TCA; final concentration, 5%). Incorporation of MDC into the TCA-insoluble dimethylcasein fraction thus precipitated was measured by fluorescence spectrophotometry (F-2000, Hitachi, Tokyo) with excitation at 355 nm and emission at 525 nm as described previously (*17*). Fluorescence of 1 nmol/ml of MDC was used as standard. One unit of activity was defined as the activity catalyzing the incorporation of 1 nmol of MDC into dimethylcasein per hour at 10°C (MIU; MDC incorporation unit per hour at 10°C; *31*).

Estimation of Protein Content—Protein content was measured essentially according to Smith *et al.* (*46*) with bovine serum albumin as standard (BCA Protein Assay Reagent, Pierce, Rockford), or monitored by absorbance at 280 nm throughout the purification steps.

Isolation of Egg Envelope (Chorion)—Eggs were broken in isotonic saline (143 mM NaCl-10 mM Tris·HCl, pH 7.2) by gently passing them through a pipette with a sharp-edged tip (about 5 mm in diameter). Egg contents were removed by filtration through cloth, then chorions were washed several times with the same solution, and stored in a freezer (−30°C) until use.

Toyopearl HW55S Gel Filtration Column Chromatography—As reported previously (*16, 17*), TGase activity was localized exclusively in unfertilized egg chorions of rainbow trout. Therefore, we extracted the activity from the isolated chorions. About 3,100 isolated chorions (about 6 g in wet weight) were homogenized with about 14 ml of the isotonic saline. After centrifugation at 28,000×*g* for 30 min, the supernatant was concentrated by dialysis against Buffer A (143 mM NaCl-5 mM 2-ME-5 mM $CaCl_2$-10 mM Tris·HCl, pH 7.2) containing 2 M sucrose for 2 h. The presence of 5 mM $CaCl_2$ and 5 mM 2-ME is well known to stabilize TGases (*47*). After further dialysis against Buffer A overnight at 4°C, the material (about 2 ml) was applied to a Toyopearl HW55S column (1.6×94 cm, Tosoh, Tokyo) equilibrated with Buffer A, and fractionated with the same buffer at a flow rate of 12.5 ml/h. Fractions of 1.5 ml were collected, and their protein content were determined by absorbance at 280 nm. To estimate apparent molecular mass, yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and chymotrypsinogen A (25 kDa) (Sigma, St. Louis) were used as standard proteins.

Purification Procedures—We purified chorion TGases by essentially the same method as described previously (*31*). All the procedures were performed at 4°C.

Step1: About 32,000 isolated chorions (61 g in wet weight) were homogenized in about 122 ml of the isotonic saline, and the homogenate was centrifuged at 28,000×*g* for 30 min. The extract thus prepared was used as starting material for purification.

Step 2: The extract was dialyzed against Buffer B consisting of 5 mM $CaCl_2$, 5 mM 2-ME, and 10 mM Tris·HCl (pH 7.2) overnight at 4°C. A small amount of precipitate was frequently formed during the dialysis. After centrifugation at 28,000×*g* for 10 min, the supernatant was applied on an SP-Sepharose column (1.7×13.5 cm, Pharmacia, Uppsala) equilibrated with Buffer B and the run-off fraction was collected.

Step 3: The run-off fraction was applied on a Q-Sepharose column (2.0×4.5 cm, Pharmacia) equilibrated with Buffer B. The column was washed with about 60 ml of Buffer B, then elution was performed with a linear gradient of 0-500 mM NaCl in Buffer B, and fractions of 2.6 ml were collected. The elution profile was shown previously (*31*). The TGase activity-containing fractions eluted at the NaCl-concentration of 260-320 mM were pooled and subjected to the next step.

Step 4: The TGase fractions were dialyzed against 60% saturated ammonium sulfate in Buffer A at 4°C for about 16 h. After centrifugation at 28,000×*g* for 30 min, the precipitate was dissolved in about 0.95 ml of Buffer A, applied to a TSK-gel G3000SWXL column (0.78×30 cm, Tosoh) equilibrated with Buffer A, and fractionated by HPLC. The chromatographic profile comprised two major protein peaks, P1 and P2, as shown in Fig. 2A. Each peak was collected, concentrated with a concentrator (Centricon-50, Amicon, Berverly), and rechromatographed by the same method. Typical profiles are shown in Fig. 2, B and C. To estimate apparent molecular mass, the TSK-gel filtration column was previously calibrated with standard

proteins such as bovine serum albumin (monomer, 66 kDa; dimer, 132 kDa) and chymotrypsinogen A (25 kDa) (Sigma).

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially by the method of Laemmli (*48*) using 7.5% gel. Protein samples for SDS-PAGE were boiled for 5 min in 1% SDS-100 mM phosphate buffer (pH 6.8) with or without 3.1% 2-ME. To estimate molecular mass, SDS molecular weight marker kit (MW-SDS-200, Sigma) was used, comprising the following standard proteins: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa). Gels were stained with Coomassie Brilliant Blue R250 (Sigma).

Amino Acid Analysis—Purified TGase was hydrolyzed in 6 N HCl containing 2% thioglycolic acid for 22 h at 110°C. Amino acid analysis was performed with a Hitachi amino acid analyzer L-8500 at Toray Research Center (Tokyo). In some cases, we analyzed the amino acid composition of a protein band blotted on PVDF (polyvinylidene difluoride) membrane (Immobilon-P^{SQ}, Millipore Corporation, Bedford) essentially by the method of Sugiyama *et al.* (*49*).

Examination of Enzymatic Properties—To examine the effect of EDTA, the enzyme sample was dialyzed against 5 mM 2-ME-10 mM Tris·HCl (pH 7.2). The activity was estimated using the reaction mixture consisting of 3.7 MIU of enzyme, 0.5 mM MDC, 0.2% dimethylcasein, 5 mM 2-ME, 50 mM Tris·HCl (pH 7.2), and various concentrations of EDTA. To examine the effect of iodoacetamide, 2-ME was removed from the enzyme sample by dialysis against 5 mM $CaCl_2$-10 mM Tris·HCl (pH 7.2). The activity was estimated using the reaction mixture containing 4.7 MIU of enzyme, 0.5 mM MDC, 0.2% dimethylcasein, 5 mM $CaCl_2$, 50 mM Tris·HCl (pH 7.2), and various concentrations of iodoacetamide. In addition, we examined the effect of hydroxylamine, cadaverine, spermidine, ATP, and GTP. Solutions of these reagents of pH 7.2 were added to the standard reaction mixture.

Ca^{2+} requirement was examined with purified sample that had been dialyzed against 1 mM EDTA-10 mM Tris·HCl (pH 7.2). TGase activity of the dialyzed sample was measured in the reaction mixture consisting of 0.5 mM MDC, 0.2% dimethylcasein, 5 mM 2-ME, 0.01 mM EDTA, 50 mM Tris·HCl (pH 7.2), and various amounts of $CaCl_2$.

The pH dependency was examined using various buffers. The 50 mM Tris·HCl buffer (pH 7.2) in the standard reaction mixture was replaced by 50 mM acetate buffer (pH 4.0-6.0), 50 mM imidazole buffer (pH 6.0-7.5), 50 mM Tris·HCl buffer (pH 7.2-9.0), or 50 mM glycine buffer (pH 9.0-11.5).

Incorporation of MDC into Chorion and Polymerization of Chorion Subunits Catalyzed by Purified TGase—Chorions isolated from unfertilized eggs were incubated in a solution of 143 mM NaCl-100 mM EDTA (pH 7.2) at 60°C for 20 min in order to inactivate endogenous TGase (*17*). The chorions thus prepared were washed several times with the same solution and finally with 10 mM Tris·HCl (pH 7.2), and homogenized with 10 mM Tris·HCl buffer (pH 7.2). The chorion homogenate was mixed with the purified TGase in a solution of 0.5 mM MDC, 5 mM $CaCl_2$, 5 mM 2-ME, and 50 mM Tris·HCl (pH 7.2) and incubated at 10°C. Incorporation of MDC into chorion proteins was determined by the same method as described in "Estimation of Transglutaminase (TGase) Activity."

Polymerization of chorion subunits was examined essentially according to Ha and Iuchi (*17, 31*). The chorion homogenate and the purified TGase were mixed in a solution of 5 mM $CaCl_2$, 5 mM 2-ME, and 10 mM Tris·HCl (pH 7.2) and incubated at 10°C for various times. The reaction was stopped by adding EDTA (final concentration, 200 mM), and the reaction mixture was centrifuged at $2,000 \times g$ for 15 min. The chorion precipitate was boiled in 12 M urea-100 mM sodium phosphate (pH 6.8). Unhardened chorions can be solubilized with this solution (*16*). The chorion sample thus prepared was subjected to SDS-PAGE.

RESULTS

Identification of Two Transglutaminases (TGases) in Extracts from Unfertilized Egg Chorions—As reported previously (*16, 17*), the activity of TGase responsible for

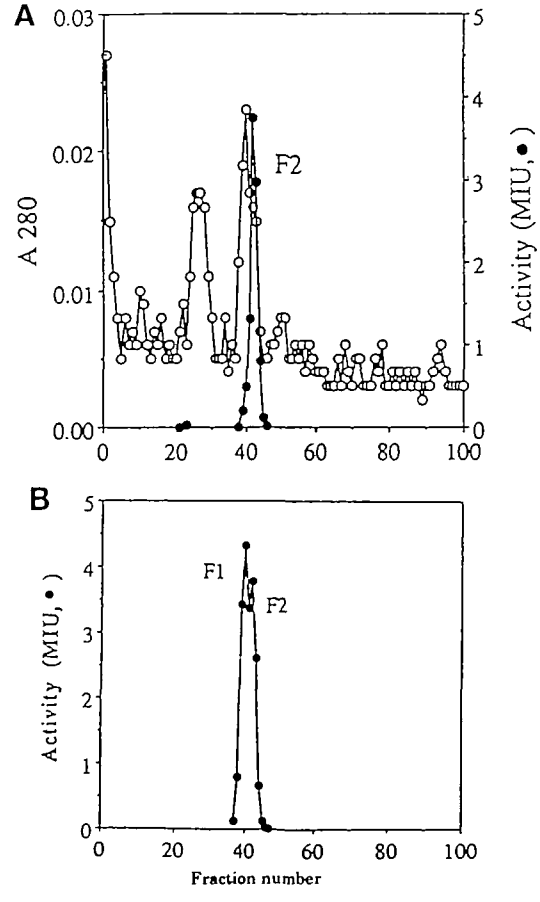

Fig. 1. Two chorion transglutaminases in unfertilized egg of rainbow trout. A: Crude extract prepared from unfertilized egg chorion was loaded on a Toyopearl HW55S column (1.6×94 cm) equilibrated with Buffer A and eluted with the same solution. Fractions of 1.5 ml were collected. ○ and ● indicate protein content measured by absorbance at 280 nm and TGase activity of each 1.5-ml fraction expressed in MIU, respectively. B: Fractions 37 to 47 in A were each dialyzed against Buffer B overnight at 4°C, and then assayed for TGase activity. ● indicates TGase activity expressed in MIU. Two peaks of activity were obtained and tentatively named F1 and F2. Further analyses showed that F1 and F2 corresponded to P1 and P2 in Fig. 2, respectively.

ε-(γ-glutamyl)lysine crosslink formation between proteins was localized exclusively in the chorion of unfertilized eggs of rainbow trout, *Oncorhynchus mykiss*. We homogenized unfertilized egg chorions with isotonic saline and centrifuged the homogenate at 28,000 × *g* for 30 min. The supernatant was concentrated by dialysis against 2 M sucrose in Buffer A for 2 h, then dialyzed further against Buffer A, and subjected to Toyopearl HW55S gel filtration column chromatography using Buffer A. Only one peak of TGase activity was obtained as shown in Fig. 1A. When fractions numbers 37 to 47 were dialyzed against Buffer B overnight at 4°C, two peaks of activity were detected and tentatively named F1 and F2, as shown in Fig. 1B. TGase activity of peak F1 appeared exclusively in Buffer B, a medium of low ionic strength, whereas the activity of F2 was not affected by the dialysis (Fig. 1, A and B). Molecular masses of F1 and F2 estimated by Toyopearl HW55S gel filtration were 103 and 83 kDa, respectively.

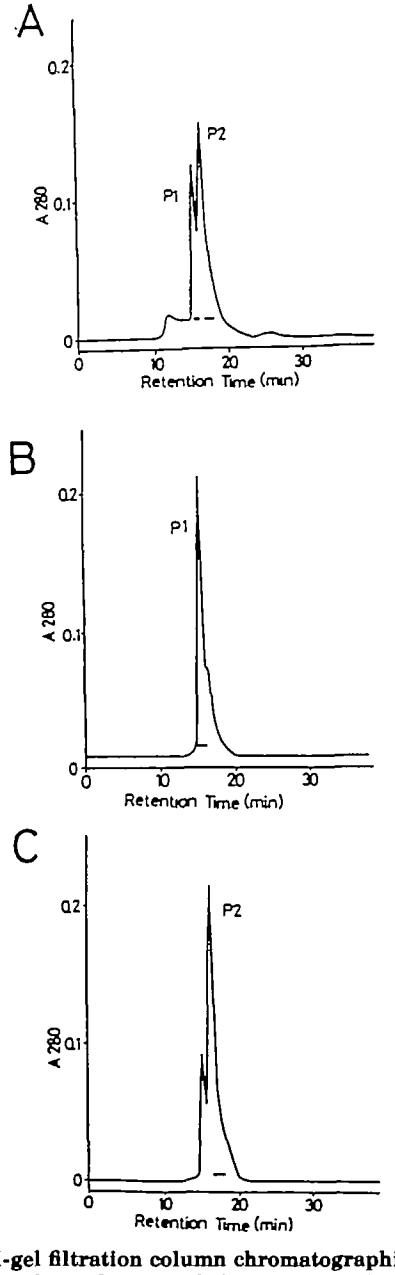

Fig. 2. **TSK-gel filtration column chromatographic pattern at the final step of purification of chorion transglutaminase.** A: TGase-containing fractions from step 3 were subjected to HPLC on a TSK-gel (G3000WXL) column. Protein content was monitored by absorbance at 280 nm. Two protein peaks were obtained and tentatively named P1 and P2. B and C: Fractions of P1 and P2 (bars in A) were each collected and rechromatographed by the same method. The patterns of P1 and P2 are shown in B and C, respectively.

Purification of Two TGases—The TSK-gel filtration at the final step of purification gave two protein peaks, tentatively designated P1 and P2, as shown in Fig. 2A. Repeated chromatography using the same column and the same buffer yielded symmetrical peaks of P1 and P2. P1 showed little activity in the elution buffer, Buffer A (Table I and Fig. 3), which consisted of the isotonic saline for rainbow trout egg supplemented with 5 mM $CaCl_2$ and 5 mM 2-ME as stabilizers (*47*). However, when dialyzed against low ionic strength Buffer B overnight at 4°C, P1 exhibited a very high activity (Fig. 3). In contrast, P2 showed a high enzymatic activity in both the elution buffer and the low ionic strength buffer. Molecular masses of P1 and P2 were estimated as 100 and 54 kDa, respectively. The findings showed that F1 and F2 separated by Toyopearl HW55S gel filtration corresponded to P1 and P2 separated

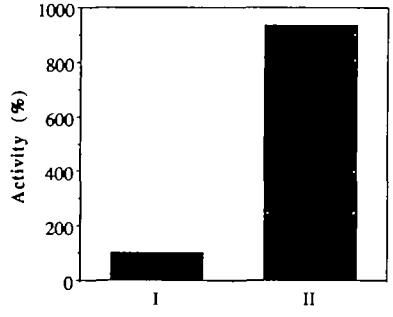

Fig. 3. **Transglutaminase activity of the purified P1.** I indicates the activity of P1 in Buffer A on elution from the TSK-gel filtration column. II indicates the activity of P1 after overnight dialysis against Buffer B at 4°C.

TABLE I. **Summary of purification of chorion transglutaminases P1 and P2.**

	Total activity (MIU)	Protein (mg)	Specific activity (MIU/mg protein)	(Fold)	Yield (%)
Crude extract	10,900	15.7	690	1	100
Run-off fraction at SP-Sepharose column chromatography	9,470	19.9	480	0.70	88
Activity-containing fraction at Q-Sepharose column chromatography	6,120	3.0	2,010	3.0	57
TSK-gel column chromatography					
P1[a]	330	0.63	520	0.75	3.1
P2	2,480	0.83	2,990	4.3	23
Repeated TSK-gel chromatography					
P1[a]	20	0.14	143	0.2	0.18
P1[b]	560	0.14	4,010	5.8	5.2
P2	2,380	0.87	2,740	4.0	22

[a]Activity of P1 in the elution buffer consisting of 143 mM NaCl, 5 mM 2-ME, 5 mM $CaCl_2$, and 10 mM Tris·HCl (pH 7.2) was estimated in the standard reaction mixture. [b]The activity was determined after dialysis against 5 mM 2-ME-5 mM $CaCl_2$-10 mM Tris·HCl (pH 7.2).

by TSK-gel filtration, respectively. We used P1 and P2 fractions for further analyses and characterization.

A typical purification of the two chorion TGases is summarized in Table I. The specific activity of the run-off fraction at step 2 was only 0.7 times that of the starting material. However, the SP-Sepharose column chromatography was essential for purification of the TGases in terms of the removal of unknown proteins and other substances. The final degree of purification was not high, suggesting that the starting material, crude extract of chorions, did not contain large amounts of proteins other than TGases. In fact, the 76-kDa TGase band was discernible in the SDS-PAGE pattern of the crude extract (Fig. 4). The specific activity of highly purified P1 (4,010 MIU/mg protein) was higher than that of P2 (2,740 MIU/mg protein).

Physicochemical Properties of Purified TGases—The molecular mass of the native form of P1 was estimated as 103 kDa by Toyopearl HW55S gel filtration and as 100 kDa by TSK-gel filtration. SDS-PAGE analysis showed that P1 consisted of two heterogeneous proteins of 82 and 76 kDa (Fig. 4). Electrophoresis of P1 not reduced by 2-ME also yielded two proteins with molecular masses of 82 and 76 kDa (data not shown). After separation by SDS-PAGE, the 82- and 76-kDa proteins were blotted onto PVDF membranes, and their amino acid compositions were analyzed. As represented by star diagrams (Fig. 5A), the 82-kDa protein was highly similar to the 76-kDa protein, and both were characterized by high content of Thr, Gly, and Pro residues as compared with P2. Therefore, it is reasonable to conclude that they are essentially similar to each other.

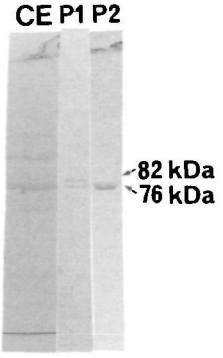

Fig. 4. **Sodium dodecylsulfate-polyacrylamide gel electrophoresis of chorion transglutaminases P1 and P2.** CE, P1, and P2 show SDS-PAGE patterns of crude extract of unfertilized egg chorion, purified P1 TGase, and purified P2 TGase, respectively. Numbers at right indicate molecular masses (kDa).

TABLE II. **Effect of various reagents on chorion transglutaminases P1 and P2.**

Reagents	Concentration (mM)	Remaining activity (%)[a] P1	P2
EDTA	0.1	0.4	1.7
Iodoacetamide	0.01	5.2	0.2
Hydroxylamine	10	93	95
	50	70	57
	100	52	39
Cadaverine	10	95	105
	50	84	71
	100	75	59
Spermidine	10	–	100
	50	–	60
	100	–	48
ATP	0.5	100	119
	1.0	104	114
GTP	0.5	102	106
	1.0	105	113

[a]The activity of P1 or P2 not treated with any reagent was taken as 100%. The effect of spermidine on P1 was not determined (–).

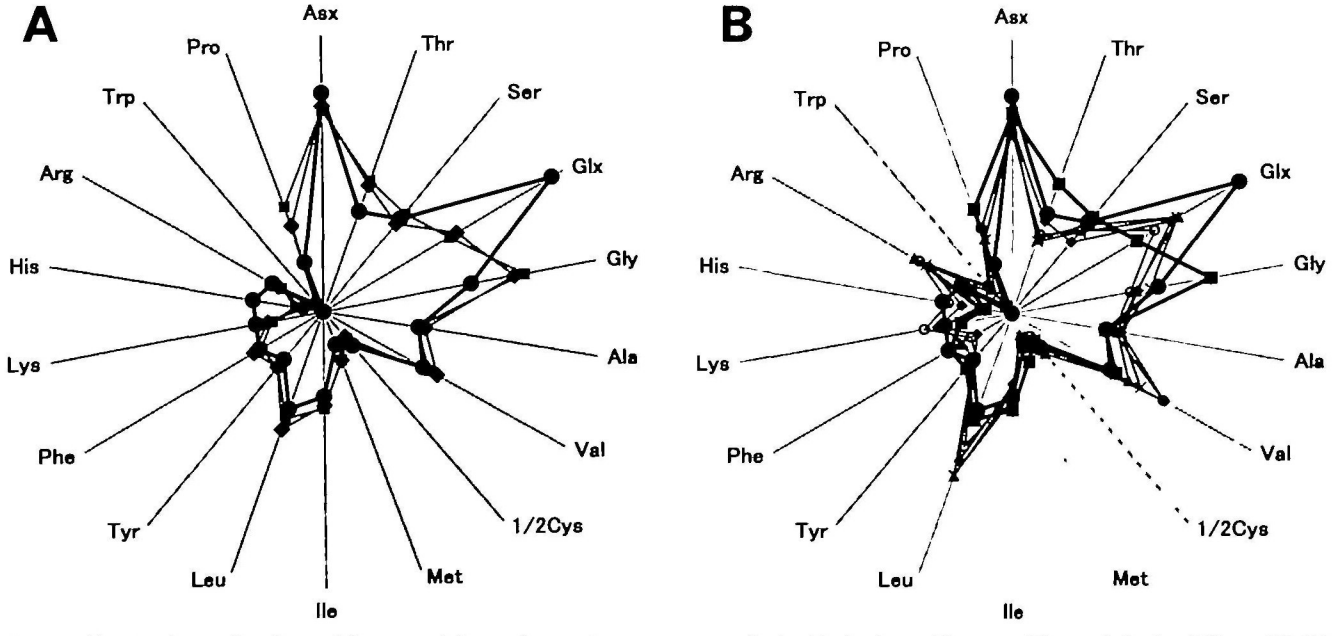

Fig. 5. **Comparison of amino acid compositions of some transglutaminases.** All the compositions are represented as star diagrams (Max. 16 mol%). A: Amino acid compositions of 82-kDa (■), 76-kDa (◆) P1 TGases, and P2 TGase (●) of egg chorion of rainbow trout, respectively. B: Amino acid compositions of chorion TGases P1-82-kDa (■) and P2 (●) of rainbow trout egg, tissue TGase of chum salmon (◆) (*51*), liver TGase of red sea bream (○) (*50*), aorta TGase of bovine (▲) (*53*), and liver TGase of guinea pig (×) (*52*).

Their heterogeneity will be discussed later.

The molecular mass of the native form of P2 was estimated as 83 kDa by the Toyopearl HW55S gel filtration and as 54 kDa by the TSK-gel filtration. SDS-PAGE analysis showed that P2 consisted of a 76-kDa protein (Fig. 4). In addition, the amino acid composition of P2 was different from that of P1 enzymes in its high content of Glx (Glu and Gln) and His residues (Fig. 5A).

Recently, the amino acid sequences of liver-type TGase of red sea bream (*50*) and tissue-type TGase of chum salmon (*51*) have been deduced from their cDNAs. As shown in Fig. 5B, the amino acid compositions of these fish TGases closely resemble those of mammalian TGases such as liver TGase of guinea pig (*52*) and tissue-type TGase of bovine aorta (*53*), but they differ from both the P1 and P2 enzymes in egg chorion of rainbow trout.

Enzymatic Properties—As summarized in Table II, TGase activities of P1 and P2 were completely inhibited by 0.1 mM EDTA or 0.01 mM iodoacetamide. In addition, Fig. 6 shows the relationship between the concentration of $CaCl_2$ and the enzymatic activities of the two TGases. Calcium chloride restored concentration-dependently the activity of EDTA-inactivated P1 and P2 enzyme. This result suggests that the two TGases are Ca^{2+}-dependent SH-enzymes like such well-characterized TGases as blood coagulation factor XIIIa (*32, 54*), guinea pig liver TGase (*38*), *Limulus* hemocyte TGase (*42*), and others (*33*). Ca^{2+} requirement for expression of the TGase activity in the fertilization/egg activation-associated chorion hardening will be discussed later.

We examined inhibitory effect of various amines on P1 and P2 TGases (Table II). Hydroxylamine was a relatively effective inhibitor of both the P1 and P2 enzymes, while cadaverine and spermidine were less effective, confirming previous reports that a relatively high concentration of cadaverine (50-100 mM) was required to inhibit both chorion hardening and chorion protein polymerization in rainbow trout egg (*15, 16*). There was no essential difference between P1 and P2 TGases in their susceptibility to the amines.

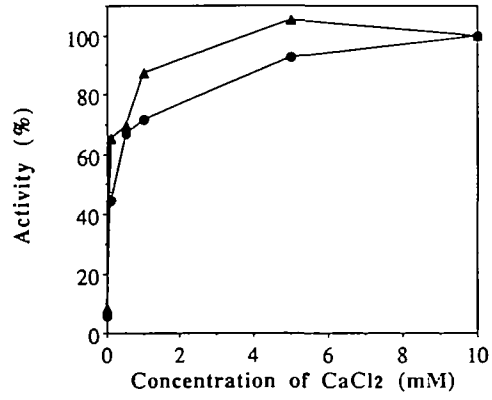

Fig. 6. **Relationship between the activity of transglutaminases P1 or P2 and the concentration of $CaCl_2$.** As mentioned in "MATERIALS AND METHODS," calcium chloride was added to EDTA-inactivated enzyme. Activity of P1 (3.7 MIU, ●) or P2 (1.8 MIU, ▲) was measured in the reaction mixture consisting of 0.5 mM MDC, 0.2% dimethylcasein, 5 mM 2-ME, 0.01 mM EDTA, 50 mM TrisHCl (pH 7.2), and various amounts of $CaCl_2$. Each activity at 10 mM $CaCl_2$ was taken as 100%.

Liver TGase of guinea pig (*55*) and human erythrocyte TGase (*56*), and blood coagulation factor XIII (*57*) are known to be inhibited by GTP and ATP. Neither the P1 nor P2 enzyme was inhibited by ATP or GTP (Table II).

As shown in Fig. 7, the highest peak of activity was observed at pH 6.0 and a minor peak was at the pH 9-10 for both P1 and P2. Although the TGase activity was assayed using unnatural substrates, its highest activity at pH 6 was consistent with pH dependency of chorion protein polymerization occurring during chorion hardening (*15*). The pH dependency profiles of the two enzymes seemed to differ somewhat, especially at pH 8 to 11.

Incorporation of MDC into Chorion and Polymerization of Chorion Subunits Catalized by Purified Enzymes—Throughout the present study, we used unnatural substrates, MDC and dimethylcasein, to assay TGase activity. Therefore, we examined whether or not the purified enzymes actually catalyzed incorporation of MDC into natural substrate, chorion, and polymerization of chorion subunit proteins.

Figure 8A shows that both P1 and P2 catalyzed the MDC-incorporation into chorions, and that the reactions followed similar courses. In addition, the chorion protein polymerization was catalyzed by both the purified P1 and P2 enzyme as shown in Fig. 8, B and C. The major subunits of 49, 56, and 65 kDa of unfertilized egg chorions gradually disappeared, while 123-kDa protein appeared. Finally, all protein components in the SDS-PAGE gel were considerably diminished, probably having become too highly poly-

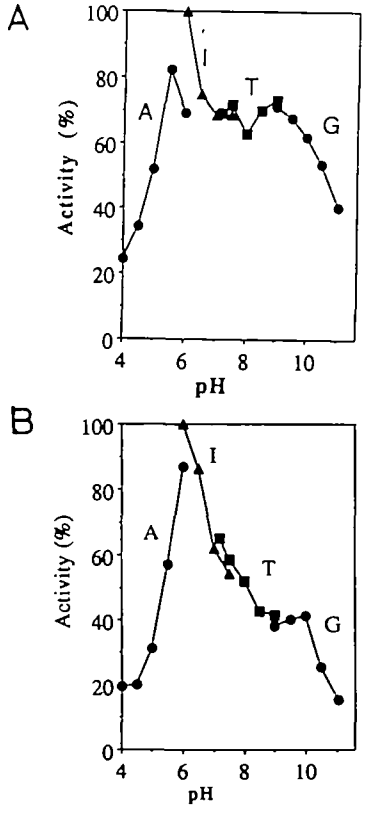

Fig. 7. **pH-dependency of transglutaminase activity of P1 or P2.** A, I, T, and G indicate 50 mM acetate, 50 mM imidazole, 50 mM Tris·HCl, and 50 mM glycine buffer, respectively. The highest peak of the activity was taken as 100%. (A) P1, 2.4 MIU; (B) P2, 4.0 MIU.

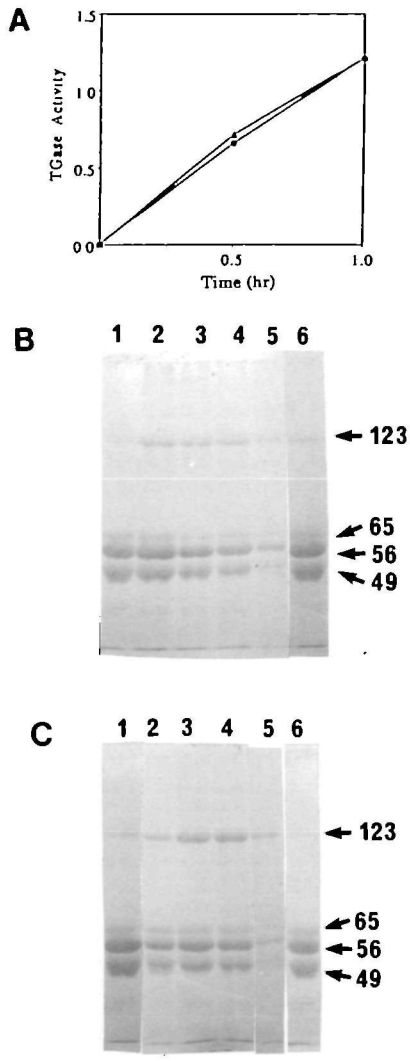

Fig. 8. **Incorporation of monodansylcadaverine (MDC) into chorion and polymerization of chorion proteins catalyzed by chorion transglutaminases P1 and P2.** A: Incorporation of MDC (nmol) into chorion catalyzed by purified P1 (▲) or P2 TGase (●) was measured as a function of reaction time (h) at 10°C. P1 or P2 (4.6 MIU) and 1.9 mg (dry weight) of isolated chorions were used. B and C: Time course of polymerization of chorion subunit proteins catalyzed by purified P1 (B) or purified P2 TGase (C) was examined by SDS-PAGE as described in the text. P1 or P2 (3.0 MIU) and 1.9 mg (dry weight) of isolated chorions were used in the present study. Lanes, 1, 2, 3, 4, and 5 show SDS-PAGE patterns of chorion proteins after 0, 1, 3, 6, and 24 h of incubation, respectively. Lane 6 indicates the pattern of chorion proteins after incubation for 24 h without the enzyme. Numbers at right indicate molecular masses (kDa).

merized to enter the gel. In addition, chorions treated with the enzyme for 24 h and over could not be solubilized even after boiling in 12 M urea and 100 mM sodium phosphate (pH 6.8) prior to SDS-PAGE. The polymerizations of chorion proteins catalyzed by P1 and P2 enzyme essentially resembled each other.

DISCUSSION

We found two TGases in chorions isolated from unfertilized eggs of the rainbow trout, *Oncorhynchus mykiss*, and purified them: One is P1 TGase consisting of 76- and 82-kDa proteins, the other is P2 TGase consisting of a homogeneous 76 kDa protein. The P2 enzyme was comparable to an enzyme purified in the previous study (*31*).

Both the P1 and P2 TGases required calcium ions to exhibit their enzymatic activities. In the dog salmon, *Oncorhynchus keta*, 1.44% of total egg Ca^{2+} (0.58 μg) was released from an egg within 2 h after the egg activation (*58*). Because of the extremely narrow perivitelline space, such a Ca^{2+} efflux may be sufficient for expression of the activity of chorion TGases and may be one of the triggers for chorion hardening *in situ*.

Unfertilized eggs of rainbow trout are usually inseminated in isotonic saline and activated by addition of a large volume of fresh water. This activation is termed "water activation." After the activation, chorion hardening proceeds rapidly at low ionic strength such as that of fresh water. In the previous study (*17*), we extracted TGase activity from isolated chorions by homogenization with isotonic saline (143 mM NaCl-10 mM Tris·HCl, pH 7.2). When dialyzed against 10 mM Tris·HCl (pH 7.2), the activity of the crude extract was increased by 3- to 4-fold. Such augmentation of chorion TGase activity is probably due to the P1 enzyme in the extract. P1 TGase activity, which becomes apparent in medium of low ionic strength, is responsible for the acceleration of chorion hardening after the water activation. In addition, the specific activity of highly purified P1 TGase was about 1.5-fold that of P2 TGase.

The activity of P1 TGase is latent in isotonic saline, while that of P2 is apparent in this solution. The coexistence of the TGases with their substrate, chorion, suggests that chorion proteins are automatically polymerized in oocytes or unfertilized eggs. In fact, we demonstrated that the 123-kDa chorion protein formed by the action of the TGase was found little in ovarian oocytes, while it was found to some extent in eggs ovulated into the ovarian or coelomic cavity (*16*). On the other hand, an inhibitor activity against TGases was found in the coelomic fluid (unpublished data). Some properties of the inhibitor are under investigation.

As mentioned in "RESULTS," the molecular mass of the native form of P1 was estimated as 103 or 100 kDa, while SDS-PAGE analysis showed that it consisted of two heterogeneous proteins with molecular masses of 82 and 76 kDa. From the present results, we cannot yet confirm whether P1 TGase is a monomeric or a dimeric protein.

Comparison of amino acid composition showed that the 82- and 76-kDa P1 proteins were highly similar to each other and quite different from P2 TGase (76 kDa). We tentatively examined the partial amino acid sequence of the two P1 proteins from their N-termini. The N-terminal amino acid of the 82-kDa protein could not be determined by Edman degradation, while that of the 76-kDa protein was determined as HPYSD-. These findings suggest that the heterogeneity in P1 is ascribable to a modification of its N-terminal region: linkage of an unknown atomic group to the terminal amino group and/or proteolytic processing of the N-terminal region. The N-terminal amino acid of P2 enzyme could also not be determined by Edman degradation.

As described above, amino acid compositions of the P1 and P2 TGases of rainbow trout chorion were different from those of the previously-sequenced fish TGases (*50, 51*). Moreover, we searched amino acid sequences of blood

coagulation factor XIIIa of human (*59, 60*), keratinocyte TGase of human (*61*), rat (*61*), and rabbit (*62*), prostate TGase of human (*63*) and rat (*64*), and erythrocyte TGase of human (*65*), mouse (*66*), and chicken (*67*). None was similar to the P1 or P2 TGases in amino acid composition. Although further confirmatory studies are required, the present results suggest that the chorion P1 and P2 TGases of rainbow trout egg are unique and new types of TGases. At least, it seems reasonable to conclude that they are not members of the fish liver or tissue TGases.

With reference to their role in chorion hardening, it is interesting to examine whether the two TGases differ in enzymatic properties. However, when TGase activity was assayed using unnatural substrates, no essential difference was found between the two TGases in the effect of amines, ATP, or GTP, Ca^{2+} requirement, and pH dependency. In addition, the polymerizations of unfertilized egg chorion proteins by P1 and P2 were essentially similar.

Chorion proteins, substrates of the TGases, are known to be produced as precursors (choriogenins) in the spawning female liver of the medaka, *Oryzias latipes* (*68-70*), rainbow trout (*71, 72*), and other fishes (*73*), transported into the ovary by the blood, and accumulated as an extracellular matrix between oocytes and follicle cells. The hepatic production of choriogenins occurs in response to oestradiol-17β (*68, 69, 71*). Because TGases are present in unfertilized egg chorion, they must be incorporated and integrated into chorion simultaneously with the formation of chorion structure. However, we do not have any information of the origin of chorion TGases or the mechanism of the TGase-integration into chorion. Identification of the origin of chorion TGases using antibodies against the TGases and cloning of cDNAs for the TGases are under investigation. In addition, we plan to examine the fertilization/egg activation-associated changes of chorion TGases in the near future.

In the spring of 1998, Dr. Kenjiro Yamagami retired as Professor of the Laboratory of Developmental Biology, Life Science Institute, Sophia University, Tokyo. We dedicate the present study to him as a token of gratitude for his support for our research activities including the present study, for his valuable advice, and for reading our manuscripts. Thanks are also due to Irikawa Trout Hatchery (Tokyo) and Fuji Trout Farm (Shizuoka) for supplying rainbow trout eggs.

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