Purification and Substrate Specificity of Transglutaminases from Blood and *Streptoverticillium mobaraense*

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A procedure for a fast and simple purification of bovine plasma transglutaminase was developed, which resulted in a homogeneous enzyme preparation. Two different procedures were developed for the purification of pig erythrocyte transglutaminase, both of which resulted in partial purification. Both enzymes were used in cross-linking reactions of α-lactalbumin, β-lactoglobulin, bovine serum albumin, casein, hemoglobin, glycinin, and myosin. The substrate specificity was compared to that of bacterial transglutaminase isolated from *Streptoverticillium mobaraense*. The bacterial transglutaminase caused cross-linking of a wider range of proteins and, thus, exhibited a lower substrate specificity than the blood transglutaminases. In addition, differences exist in the necessity of the addition of reducing agents. These differences allow specific applications of blood and bacterial transglutaminases at protein cross-linking in single or complex protein systems.

Keywords: *Transglutaminase; substrate specificity; purification; blood*

INTRODUCTION

Transglutaminase (protein-glutamine γ-glutamyltransferase, EC 2.3.2.13; TG) catalyzes acyl-transfer reactions introducing covalent cross-linkages between proteins, creating high molecular weight polymers. In the early stages of transglutaminase research, attention was focused on the cross-linking of proteins involved in blood coagulation (*1*, *2*). In a later stage cross-linking of other proteins received more attention. Guinea pig liver TG was the most used enzyme for these cross-linking studies (*3*−*8*). In addition, partially purified transglutaminase was used from bovine blood (factor XIII) (*9*) or human placenta (*10*). Guinea pig liver TG and factor XIII are calcium dependent, which plays a very important role in the conditions necessary for the cross-linking reactions. The discovery of a Ca-independent TG isolated from *Streptoverticillium mobaraense* (*11*) enhanced transglutaminase research and utilization in food products, because of the better availability of this enzyme.

In addition to plasma transglutaminase, mammal blood contains two other types of transglutaminases. One is the transglutaminase from blood platelets, which is very closely related to plasma TG (*12*). The other one is a transglutaminase found in erythrocytes and because of that location is called erythrocyte TG (*13*, *14*). Purification of this transglutaminase has been performed from human erythrocytes (*13*, *14*). However, the relatively small quantities of human blood and its origin make this enzyme uninteresting for large scale applications in food. On the contrary, the large availability of bovine and pig blood from slaughterhouses would make it more practical to isolate transglutaminases from these sources.

Our objective was to isolate pig erythrocyte TG and bovine plasma TG and to compare their cross-linking activities with the transglutaminase from *S. mobaraense*.

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MATERIALS AND METHODS

Materials. All chromatographic materials were purchased from Pharmacia (Uppsala, Sweden). *N*,*N*-Dimethylcasein, monodansylcadaverine, dithiotreitol (DTT), α-lactalbumin, β-lactoglobulin, bovine serum albumin (BSA), casein, and hemoglobin were obtained from Sigma. Glycinin was prepared according to the method given in ref *15*, with an additional ammonium sulfate precipitation. Myosin was prepared according to a modified procedure (*16*). All other reagents were of analytical grade.

Purification of Erythrocyte TG from Pig Blood (Procedure 1). Fresh pig blood was obtained from a slaughterhouse. During collection, 10% (v/v) of an 8% trisodium citrate solution was added to the blood. The citrated blood was centrifuged at 4000*g* for 30 min at 10 °C, and the erythrocyte fraction was collected by careful removal of the plasma. The erythrocyte fraction (200 mL) was diluted with cold demineralized water (800 mL) in order to obtain a hemolysate. After 10 min of incubation at 4 °C, 25 mL of 1 M Tris-HCl, pH 7.5, and 4 mL of 250 mM EDTA were added, followed by centrifugation at 25000*g* for 20 min. The supernatant was applied to DEAE-Sepharose fast flow in a sintered glass funnel (15 × 5 cm) equilibrated in 25 mM Tris-HCl, pH 7.5, containing 1 mM EDTA (buffer A). The ion exchanger was washed with, respectively, buffer A and buffer A containing 0.15 M NaCl. Subsequently, the transglutaminase activity was eluted with 0.3 M NaCl in buffer A. This fraction was diluted with buffer A to a concentration of 0.2 M NaCl and applied to a Blue Sepharose column (Pharmacia, 5 mL) previously equilibrated with the same buffer. The column was washed with 0.2 M NaCl in buffer A, and the transglutaminase activity was eluted with buffer A without salt addition. The active fractions were applied to a Source Q column (Pharmacia, HR 10/10). For elution a gradient of 0−0.5 M NaCl in buffer A was applied. The pooled active fractions were stored at −80 °C.

Purification of Erythrocyte TG from Pig Blood (Procedure 2). Purification of erythrocyte TG was performed on DEAE-Sepharose fast flow as described above. After DEAE-Sepharose, the eluted active fraction was diluted twice with buffer A (pH 9) and applied to a Source Q column (Pharmacia, 300 mL) previously equilibrated with buffer A. The column was eluted with a gradient of 0−0.5 M NaCl in buffer A. The active fractions were pooled and diluted twice with buffer A (pH 9) and applied to a Source Q column (Pharmacia, 300 mL)

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previously equilibrated with 25 mM MES, pH 6.0, and 1 mM EDTA. Subsequently, a gradient of 0−0.5 M NaCl in 25 mM MES, pH 6.0, and 1 mM EDTA was applied.

The pooled active fractions were diluted twice with buffer A (pH 9) and concentrated with a Source Q column (Pharmacia, HR 16/10) by using a small gradient of 0− 0.5 M NaCl in buffer A. The concentrated active fractions were stored at −80 °C.

Purification of Plasma TG from Bovine Blood. Fresh bovine blood was obtained from a slaughterhouse. During collection, 10% (v/v) of an 8% trisodium citrate solution was added to the blood. The citrated blood was centrifuged at 4000*g* at 10 °C for 30 min to remove the erythrocytes. Ten milliliters of cold ethanol (0 °C) was added to the stirred bovine plasma (90 mL). After 30 min of stirring at 0 °C, the mixture was centrifuged for 10 min at 25000*g* and the pellet suspended in buffer A. After suspension, the preparation was incubated at 56 °C for 4 min. The denatured protein, predominantly fibrinogen, was removed by centrifugation, and the supernatant containing the transglutaminase activity was applied to a DEAE-Sepharose fast flow column (15 × 5 cm) equilibrated in buffer A. The column was washed with buffer A and eluted with a gradient of 0−0.5 M NaCl in buffer A.

Active fractions were pooled and bound to Source Q (Pharmacia, HR 10/10) after 4 times dilution with buffer A. The transglutaminase was eluted by applying a gradient from 0 to 1.0 M NaCl in buffer A. Pooled active fractions were further purified by gel filtration on a Pharmacia Superdex 200 column equilibrated with 0.2 M NaCl in buffer A and the eluted active fractions stored at −80 °C.

Purification of Bacterial TG from *S. mobaraense*. *S. mobaraense* was grown as described (*17*), and purification of transglutaminase was performed according to the method given in ref *11*.

Measurement of Transglutaminase Activity. Transglutaminase activity was determined by the incorporation of monodansylcadaverine into methylated casein (*18*). The activity of the transglutaminase-containing fractions was expressed as the increase in fluorescence at 480 nm. Specific activities are expressed as the relative increase in fluorescence per milligram of protein per minute. The quantitative hydroxamate assay using methylamine incorporation into benzyloxycarbonyl (Cbz)-glutaminylglycine (*19*) gave negative results in both blood transglutaminases and could be used only for bacterial TG.

Cross-Linking Experiments. The standard reaction mixture (total volume of 2 mL) contained 100 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 5 mg/mL protein substrate, 0 or 10 mM dithiotreitol, and variable concentrations of the transglutaminases. Incubations were performed at 37 °C, and 250 μL samples were taken at time intervals. The reaction was terminated by adding 80 μL of 0.25 M EDTA and incubation at 80 °C for 5 min, before the mixture was put on ice. Analyses of the cross-linking reactions were performed by gel electrophoresis of the cross-linked protein or by measuring the ammonia released (*20*).

Polyacrylamide Gel Electrophoresis (SDS—PAGE). Subunit molecular masses were determined under denaturing conditions by SDS-PAGE (*21*) using ready gels and Mini Protean equipment (Bio-Rad). Enzyme or reaction mixture samples were denatured by incubation for 5 min at 100 °C in 2% SDS and 1% DTT. Gels were stained for protein with Coomassie Brilliant Blue G250. A high molecular weight calibration kit (Pharmacia) was used to derive the molecular masses.

RESULTS

Purification of Erythrocyte TG from pig blood (See Table 1). The purification procedure for pig erythrocyte TG (procedure 1) resulted in an active fraction containing 50% transglutaminase (Figure 1A). The total yield of this purification, however, was very low. The Blue Sepharose column was especially responsible for a great loss of transglutaminase activity. To obtain sufficient enzyme for cross-linking experiments, an alternative purification procedure had to be developed. Procedure 2 had a higher total yield (Table 2); the purity was only 10%, but this was adequate for cross-linking experiments.

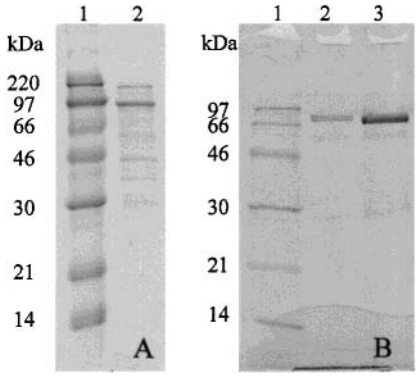

Figure 1. SDS-PAGE analysis of the purified transglutaminases: (A) pig erythrocyte TG (lane 1, molecular weight marker; lane 2, pig erythrocyte TG purified according to procedure 1); (B) bovine plasma TG [lane 1, molecular weight marker; lane 2, bovine plasma TG (2 μg) purified according to the procedure under Materials and M; lane 3, similar to lane 2 (20 μg)].

Table 1. Purification of Erythrocyte TG According to Procedure 1

purifn step	total protein (mg)	specific activity (AU/mg)	yield (%)	purifn factor
hemolysate	150000	0.475	100	
DEAE-Sepharose	564	40	32	84
Blue Sepharose	13.2	212	3.9	446
Source Q pH 7.5	0.96	1583	2.1	3332

Table 2. Purification of Erythrocyte TG According to Procedure 2

purifn step	total protein (mg)	specific activity (AU/mg)	yield (%)	purifn factor
hemolysate	150000	0.475	100	
DEAE-Sepharose	564	40	32	84
Source Q pH 7.5	62	286	25	600
Source Q pH 6	30	472	20	990

Difficulties in the purification and especially in the total recovery of the activity were caused by self-cross-linking of transglutaminase prior to and during the purification procedure, as could be determined by gel filtration (data not shown). This phenomenon occurred even in the presence of citrate or EDTA, known binders of the necessary calcium for this type of transglutaminase.

Purification of Plasma TG from Bovine Blood (See Table 3). The purification procedure for bovine plasma TG resulted in a homogeneous fraction (Figure 1B). However, the total recovery of transglutaminase activity could not be calculated because of turbidity in the activity assay of the initial plasma. Further purification showed that thrombin treatment in combination with the presence of DTT caused precipitation of a protein of molecular weight <100 kDa. Recovery of the transglutaminase was calculated after ethanol precipitation.

Cross-Linking Experiments. The appearance of cross-linking of protein substrates with transglutaminases was analyzed with SDS-PAGE and the determi-

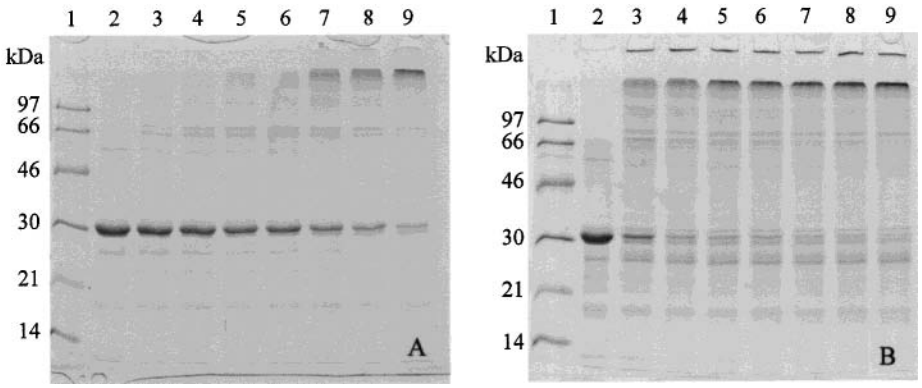

Figure 2. SDS-PAGE analysis of the cross-linking of β-casein with transglutaminases: (A) cross-linking of β-casein with bovine plasma TG (5 μg) (lane 1, molecular weight marker; lane 2, β-casein; lane 3, β-casein after 0.5 h of incubation with plasma TG at 37 °C; lane 4, 1 h; lane 5, 1.5 h; lane 6, 2 h; lane 7, 4 h; lane 8, 6 h; lane 9, 24 h); (B) cross-linking of β-casein with erythrocyte TG (10 μg) in the presence of 10 mM DTT (lane 1, molecular weight marker; lane 2, β-casein; lane 3, β-casein after 0.5 h of incubation with erythrocyte TG at 37 °C; lane 4, 1 h; lane 5, 1.5 h; lane 6, 2 h; lane 7, 4 h; lane 8, 6 h; lane 9, 24 h).

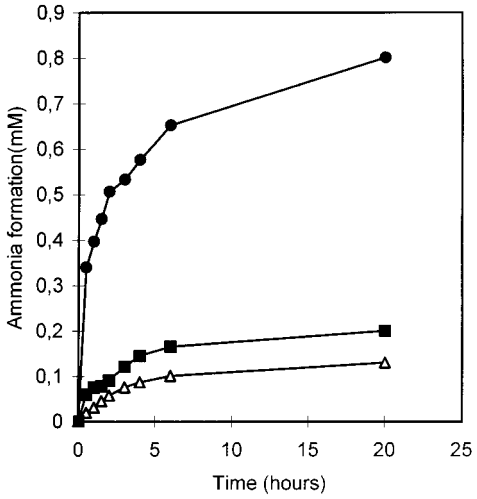

Figure 3. Analysis of the ammonia formation after cross-linking of β-casein with three transglutaminases: (●) 12.5 μg/mL bacterial TG; (■) 25 μg/mL plasma TG; (△) 100 μg/mL erythrocyte TG (20% purity).

Table 3. Purification of Plasma TG from Bovine Blood

purifn step	total protein (mg)	specific activity (AU/mg)	yield (%)	purifn factor
plasma	15200	21		
ethanol precipitate	460	392	100	
DEAE-Sepharose	86	297	14.2	14
Source Q	33	593	12.4	28
gel filtration	5	3535	9.8	168

nation of released ammonia. Figure 2 shows the cross-linking of β-casein with the two types of blood TGs. In the cross-linking of β-casein with erythrocyte TG, DTT had to be added; otherwise, no cross-linking was observed. Both plasma and bacterial TGs cross-link β-casein without the addition of reducing agents. Analysis of the released ammonia at the cross-linking of β-casein is shown in Figure 3. This figure shows in analogy with Figure 2 that β-casein is cross-linked much more quickly by bacterial TG than by plasma TG. In Table 4 the results are given of the cross-linking of seven protein substrates with the transglutaminases as judged by SDS-PAGE.

DISCUSSION

A striking observation was made at the purification of pig erythrocyte TG. It was observed that self-cross-

Table 4. Cross-Linking of Different Substrates with Transglutaminases[a]

	pig erythrocyte TG		bovine plasma TG		bacterial TG	
substrate	− DTT	+ DTT	− DTT	+ DTT	− DTT	+ DTT
α-lactalbumin	−	±	−	±	+	++
β-lactoglobulin	−	−	−	±	−	++
BSA	−	+	−	+	−	++
casein	−	++	++	++	++	++
hemoglobin	−	−	±	±	±	±
myosin	−	−	++	++	++	++
glycinin	−	++	−	−	++	++

[a] Substrates were cross-linked with the isolated TGs and a comparison of the rates of cross-linking was based on the formation of higher weight protein polymers and the disappearance of the monomer on SDS-PAGE. Definition of the amount of cross-linking observed: −, no cross-linking; ±, slow cross-linking; +, moderate cross-linking; ++, fast cross-linking.

linking of this enzyme took place prior to and during the purification procedure, as could be determined by gel filtration (data not shown). This phenomenon occurred even in the presence of citrate or EDTA, known binders of the necessary calcium for this type of transglutaminase. This self-cross-linking will also have an impact on the activity of the enzyme after calcium addition during cross-linking reactions with other substrates. Although erythrocyte TG is considered to be closely related to guinea pig liver TG, the former did not exhibit hydroxylamine incorporation into Cbz-glutaminylglycine, whereas guinea pig liver TG performs well in the hydroxamate assay (*3*). This shows that a difference exists in the substrate specificities of related transglutaminases. The purification of the erythrocyte TG as described here is performed to give an indication of the industrial applicability. This is in contrast to the purification procedures described for human blood. One paper describes a purification procedure using two size exclusion steps (*14*); a second article describes the use of a size exclusion step and two additional preparative gel electrophoresis steps (*13*). Gel filtration is not easily implemented in an industrial large scale purification process; preparative electrophoresis can be used on a laboratory scale only for very low amounts of proteins.

Purification of bovine plasma TG was easily performed as described here and resulted in a homogeneous preparation. The activity of plasma TG also could not be detected in the hydroxamate assay. Bacterial TG was purified with a specific activity of 23 units/mg/min

measured in the hydroxamate assay. This activity is similar to that found previously (*11*).

Using the two types of animal blood TGs and bacterial TG, cross-linking experiments with seven proteins showed large differences in substrate recognition and in cross-linking rates.

Bacterial TG showed the lowest substrate specificity, as it was able to cross-link all seven proteins tested. However, cross-linking of BSA and β-lactoglobulin is observed only after reduction of the disulfide bridges by DTT, which will promote the unfolding of the protein. The unfolding of BSA and β-lactoglobulin will increase the accessibility of glutamine and lysine residues for the cross-linking reaction.

Erythrocyte TG was able to cross-link BSA, casein, and glycinin, indicating a higher substrate specificity for this enzyme. The presence of DTT was necessary for these cross-linking reactions, which was confirmed in the fluorescence assay. However, the latter assay uses monodansylcadaverine and dimethylcasein as substrates, so no disulfide bridges are present. Consequently, not only should the role of DTT be ascribed to that of an agent reducing disulfide bridges, but DTT plays a role in the reduction state of cysteine in the active site of erythrocyte TG.

Plasma TG does not need DTT for its activity, as can be deduced from the observed cross-linking of casein, hemoglobin, and myosin in the absence of DTT.

In some cases such as α-lactalbumin and glycinin, the presence of calcium in the cross-linking buffer caused a solubility problem. α-Lactalbumin and glycinin were much more quickly cross-linked by bacterial TG without calcium than in the presence of calcium. This means that cross-linking of these proteins by the two blood TGs can be inhibited by the decreased solubility in the presence of calcium.

This study shows that the erythrocyte TG has the highest substrate specificity, whereas the bacterial enzyme has the lowest specificity. An intermediate specificity is exhibited by the plasma TG. These differences should be ascribed to the roles of these enzymes in their concomitant natural processes. The fact that these transglutaminases are able to cross-link proteins different from their natural substrates means that they can be used in several applications for which enzymatic protein cross-linking is desired instead of chemical cross-linking. Applications may be aimed toward the development of protein polymers with modified functional properties but also to direct applications in complex systems, such as foods. Depending on the number and types of proteins in an application and the need for specific cross-linking of particular proteins in such an application, one can select the most suitable transglutaminase.

With respect to applications related to food products or protein ingredients, the cross-linking of the described substrates looks promising, especially as it is known that cross-linking of protein can have substantial effects on functional properties, for example, gelling capacity, emulsifying capacity, and solubility. The possibility of using different types of transglutaminases for the desired effect can be interesting, especially because the rates and numbers of cross-links produced will differ depending on the type of transglutaminase used. The problems concerning the erythrocyte TG regarding self-cross-linking, the necessity of using a reducing agent, and the difficulties in the purification process will narrow the possibilities of this enzyme. Plasma TG offers better possibilities, although purification to a homogeneous enzyme preparation may not be necessary. A good example of the use of a partially purified plasma TG is in the area of meat processing, for which plasma TG is used in combination with fibrinogen to form a system that enables cross-linking of meat parts. Bacterial TG shows the lowest substrate specificity and offers the greatest possibilities in cross-linking of protein ingredients. Cross-linking of proteins with this enzyme is favored because of its independence from calcium, which can be beneficial when proteins are to be cross-linked because the solubility is negatively influenced by the addition of calcium.

ABBREVIATIONS USED

BSA, bovine serum albumin; Cbz, benzyloxycarbonyl; DTT, dithiothreitol, kDa, kilodalton; TG, transglutaminase.

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