Crosslinking of Casein Components by Transglutaminase*

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Transglutaminase catalyzes the formation of ϵ -(γ -glutamyl)lysyl crosslinks and the substitution of a variety of primary amines for the γ -carboxamide groups of protein-bound glutamyl residues. As a first step in the use of transglutaminase for enzymic modification of food proteins, the reactivity of bovine casein components (α_{s1} -, β -, and κ -caseins) in the transglutaminase reaction was compared, and the properties of casein modified by this enzyme were examined.

The reactivity of κ -casein was lower than that of α_{s1} - or β -caseins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis indicated that each casein component was polymerized through formation of intermolecular crosslinks by transglutaminase.

Ultracentrifugal experiments in the absence of calcium ions revealed that complex formation between α_{s1} -casein (or β -casein) and κ -casein was not impaired by this modification. The functional properties of α_{s1} - and β -caseins in the presence of calcium ions (*e.g.* precipitation and micelle formation) were essentially retained after the modification. These results suggest that transglutaminase would be a useful tool to polymerize casein without damaging its special properties.

Transglutaminase catalyzes a calcium-dependent acyl-transfer reaction in which the γ -carboxamide groups of peptide-bound glutamyl residues are the acyl donors. Primary amino groups in a variety of compounds may act as acyl acceptors with the subsequent formation of monosubstituted γ -amides of peptide-bound glutamic acid.¹⁾

If R-NH₂ is a suitable exogeneously added amine, appropriate R groups can be introduced to the protein at the glutamyl residue. ϵ -Amino groups of appropriate lysyl residues in proteins can also serve as substrates, generating intra- or intermolecular ϵ -(γ -glutamyl)lysyl crosslinks (isopeptide bonds).

Transglutaminases are widely distributed in nature^{1,2)} and several of them have been

shown to perform biologically important functions by catalyzing the formation of ϵ -(γ -glutamyl)lysyl crosslinks with protein substrates. These functions include the stabilization of the fibrin structure during blood coagulation,³⁾ the clotting of seminal fluid,⁴⁾ the stiffening of the erythrocyte membrane,⁵⁾ and the formation of the cornifying envelope in keratinocytes.⁶⁾ The occurrence of the ϵ -(γ -glutamyl)lysyl crosslink and its physiological role have been the subjects of a recent review.²⁾

The capacity to mediate the attachment of specific labels to proteins, and to introduce crosslinks between them, should make transglutaminase very useful for effecting specific modifications of proteins and for the analysis of protein structure and organization. Some reports have indicated that transglutaminase is generally useful in crosslinking membrane proteins and in attaching to them a variety of chemical probes and labels. These studies involve the membranes of mouse⁷⁾ and human^{8,9)} erythrocytes, of sarcoplasmic reticulum from rabbit skeletal muscle,^{7,9,10)} of human plate-

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lets¹¹⁾ and of bovine retinal rod.^{9,12)} Transglutaminase has also been used to label viral proteins^{9,13)} and skeletal muscle myofibril proteins.¹⁴⁾

The demand for high quality food protein is increasing. Modification of proteins with enzymes or chemical reagents helps improve the functional properties and nutritive values of currently available food proteins.¹⁶⁾ Enzymic modification has a great advantage in that we need not have much concern about the production of unknown components which may be toxic, because enzyme-catalyzed reactions proceed specifically under mild conditions. Although a number of enzymes which modify proteins have been found, few papers have appeared describing the use of enzymes other than hydrolases to produce food protein of high quality.

It has been shown that bovine casein is a good substrate for various transglutaminases in the presence or the absence of added amines. The properties of modified casein, however, have not been reported. In this study, the reactivities of purified bovine casein components (α_{s1} -, β -, and κ -caseins) in the transglutaminase reaction catalyzed by guinea pig liver enzyme were compared, and the properties of the casein components modified by the enzyme were examined.

MATERIALS AND METHODS

Transglutaminase. Transglutaminase was purified from fresh guinea pig livers by the method of Connellan *et al.*¹⁶⁾ During the purification, enzyme activity was determined by a colorimetric hydroxamate procedure using an ethanol extract of a tryptic digest of casein as substrate.¹⁷⁾ The prepared enzyme exhibited 95% of the reported specific activity upon assay by hydroxamate formation with *N*-carbobenzoxy-L-glutaminylglycine,¹⁸⁾ and gave a single band on electrophoresis in a polyacrylamide gel containing SDS.* This sample exhibited no proteolytic activity. The enzyme was stored at -80°C in 0.4 ml aliquots. An extinction coefficient of $E_{280}^{1\%} = 15.8^{16)}$ was used to determine the enzyme concentration.

Casein components. α_{s1} -, β -, and κ -Caseins were

prepared by the methods described in a previous paper.¹⁸⁾ The number of lysyl residues was 14 for α_{s1} -casein,^{20,21)} 11 for β -casein,^{21,22)} and 9 for κ -casein.²³⁾ Values of 23,500, 24,000, and 19,000 were used for the monomer molecular weights of α_{s1} -, β -, and κ -caseins, respectively.

Chemicals. A tryptic digest of casein (*N*-Z-Amine Type E) was obtained from Humko Sheffield. *N*-Carbobenzoxy-L-glutaminylglycine was from Vega Biochemicals. L-Glutamate dehydrogenase (Type II) was from Sigma. The molecular weight calibration kit (HMW) was from Pharmacia Fine Chemicals. Other reagents (of guaranteed reagent grade) were purchased from Wako Pure Chemical Industries, Ltd. and from Nakarai Chemicals, Ltd.

Crosslinking. The standard reaction mixture contained, in a total volume of 100 ml, 0.1 M Tris-HCl buffer (pH 7.5), 5 mM CaCl_2 , 10 mM dithiothreitol, 5 mg/ml casein component, and 30 $\mu\text{g/ml}$ transglutaminase. Incubations were performed at 25°C , and 15 ml aliquots were taken at intervals. The reaction was terminated by adding 1.5 ml of 0.4 M EDTA (pH 8.0) on ice. The control non-crosslinked samples (the zero-time product) were prepared by mixing the reaction mixture with EDTA before addition of the enzyme. The formation of crosslinked caseins was followed by measuring the ammonia released and by analyzing the products with SDS-polyacrylamide gel electrophoresis. For other experiments using crosslinked proteins, the terminated reaction mixtures were heated for 3 min in boiling water and then dialyzed extensively against distilled water. Dialysates were lyophilized and stored at room temperature.

Determination of ammonia. Ammonia released during the crosslinking reaction was determined enzymically by the use of bovine liver glutamate dehydrogenase. Aliquots of 0.1 to 1.0 ml of the terminated reaction mixtures were brought to a final volume of 2 ml in assay mixtures containing 0.1 M Tris-HCl buffer (pH 8.0), 10 mM α -ketoglutarate, 0.25 mM NADH, and 19.2 units of glutamate dehydrogenase. After 1.5 hr at 25°C , the decrease in absorbance was measured at 340 nm. Ammonia was estimated using a molar extinction coefficient for NADH of 6.22×10^3 .

Determination of amino groups. The number of free amino groups (ϵ -amino groups of lysyl residues and *N*-terminal amino groups) of the protein was determined by the trinitrobenzenesulfonate method²⁴⁾ with minor modifications. One ml of sample solution to be analyzed, 1 ml of 4% NaHCO_3 , and 1 ml of 0.05% trinitrobenzenesulfonate were mixed and kept in a dark place at 37°C for 5 hr. Absorbance at 340 nm was measured after adding 1 ml of 1 N HCl containing 5%

* SDS, sodium dodecyl sulfate.

SDS to the reaction mixture.

SDS-polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in SDS was carried out on column gels (3% stacking gel and 10% separating gel) or on slab gels (2% stacking gel and 3~25% gradient separating gel) using the SDS-Tris-glycine discontinuous buffer system described by Laemmli.²⁵⁾ Prior to electrophoresis, the sample proteins were incubated at 90°C for 3 min in the presence of 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.002% bromophenol blue. The column gel electrophoresis was done at a constant current (4 mA per gel for 100 min) and the slab gel electrophoresis at a constant voltage (40 V for 1 hr and 120 V for the next 8 hr). The column gel was stained with 0.5% amido black 10B in 20% acetic acid, and the slab gel with 0.1% Coomassie brilliant blue in 50% trichloroacetic acid. Both gels were destained in 7% acetic acid.

Ultracentrifugation. Sedimentation analyses were performed at 35°C with a Beckman model E analytical ultracentrifuge. Lyophilized caseins were dissolved in 10 mM imidazol buffer (pH 7.1) containing 70 mM KCl and 2 mM dithiothreitol.

Precipitation of α_{s1} - and β -caseins. The precipitation of α_{s1} - and β -caseins caused by CaCl_2 was tested as follows. The reaction mixture contained, in a total volume of 1.0 ml, 2.5 mg/ml lyophilized casein component, 10 mM imidazol buffer (pH 7.1), 70 mM KCl, and 0~25 mM CaCl_2 . After preincubation of the protein solution for 10 min at 37°C, 200 mM CaCl_2 was added to the desired concentration. The reaction mixture was further incubated for 1 hr and centrifuged at 2000 rpm for 1 min at the same temperature. The amount of protein in the supernatant was determined from the absorbance at 280 nm after dilution with 50 mM potassium citrate.

Stabilization of α_{s1} - and β -caseins by κ -casein. Stabilization against precipitation in the presence of CaCl_2 of α_{s1} - and β -caseins by κ -casein was tested under the same conditions as in the precipitation experiments, except that the reaction mixture contained 25 mM CaCl_2 and 0.125 to 0.625 mg/ml κ -casein. The amount of α_{s1} - or β -casein in the supernatant was calculated by subtracting the amount of added κ -casein from the total protein in the supernatant.

RESULTS AND DISCUSSION

Crosslinking of casein components

The transglutaminase reaction with each casein component was followed by estimating the ammonia released by the protein (Fig. 1).

FIG. 1. Transglutaminase Reaction with Casein Components.

The determination of ammonia and the conditions of the reaction were as given in MATERIALS AND METHODS (●—●) α_{s1} -casein; (○—○) β -casein; (▲—▲) κ -casein.

Both α_{s1} - and β -caseins had the same high reactivity, but a much lower reactivity was found for κ -casein. Since amine was not added to the transglutaminase reaction system, the amount of ammonia released during the reaction corresponds to the number of inter- or intramolecular ϵ -(γ -glutamyl)lysyl crosslinks formed by the action of the enzyme on casein.²⁶⁾ Figure 1 shows that, after 120 min under the experimental conditions mentioned above, 3.5 ϵ -(γ -glutamyl)lysyl crosslinks per molecule were formed with α_{s1} -casein, 3.8 with β -casein, and 1.0 with κ -casein. With each casein component, the amount of ammonia released during the reaction corresponded to the decrease of free amino groups (Table I).

At present, the reasons for the lesser reactivity of κ -casein are not clear. The numbers

TABLE I. AMOUNT OF AMMONIA RELEASED AND THE DECREASE IN FREE AMINO GROUPS DURING THE TRANSGLUTAMINASE REACTION

Casein	Number (mol/mol of casein) of:	
	Ammonia released	Decrease of free amino group
α_{s1}	3.5	3.7
β	3.8	3.5
κ	1.0	0.6

of glutaminyl and lysyl residues in the κ -casein molecule (Gln, 14; Lys, 9) are comparable to those of α_{s1} -casein (Gln, 14; Lys, 14) and β -casein (Gln, 21; Lys, 11), so the concentrations of these residues in the reaction of each casein component are at the same level under experimental conditions. The data obtained from experiments using several native or denatured proteins and a labeled low molecular weight amine ($[^{14}\text{C}]$ glycine ethyl ester) as substrates have suggested that the substrate effectiveness

of the peptide-bound glutaminyl residue depends not on the primary structure of the polypeptide containing the glutaminyl residue but on its conformation.²⁷⁾ Bovine casein components have been thought to have a disordered structure.²⁸⁾ Recently it was postulated that κ -casein has a fairly ordered structure.²⁹⁾ It is possible that most of the glutaminyl and lysyl residues of κ -casein are buried inside the molecule and thus inaccessible to transglutaminase.

Changes in the molecular size of casein components during the transglutaminase reactions shown in Fig. 1 were analyzed by SDS-polyacrylamide gel electrophoresis. In all cases, as the reaction proceeded, the monomer content decreased and the polymer content increased (Figs. 2, 3). In the cases of α_{s1} - and β -caseins, the formation of oligomer was observed during the first 30 min, and by 120 min all monomers and oligomers were turned to polymers. With κ -casein, only about 60% of the monomers had changed into oligomers and polymers after 120 min. When the enzyme concentration is elevated, all of the κ -casein is converted to polymer (data not shown). These results indicate the formation by transglutaminase of intermolecular crosslinks for each of the casein components.

In the early reaction steps of α_{s1} - and β -caseins (during the first 30 min with α_{s1} -casein, and

FIG. 2. SDS-Polyacrylamide Gel Electrophoretic Patterns of Casein Components during the Transglutaminase Reaction.

Samples were obtained from the transglutaminase reactions shown in Fig. 1. 40 μg of protein was applied to each column. Migration is from top to bottom. M, O, and P indicate the positions of bands of monomers, oligomers, and polymers.

(A) α_{s1} -casein; samples on gels a~f, incubated with transglutaminase for 0, 10, 20, 30, 60, and 120 min, respectively. (B) β -casein; a~f, for 0, 4, 10, 20, 60, and 120 min. (C) κ -casein; a~f, for 0, 10, 30, 50, 90, and 120 min.

FIG. 3. Monomer Content of Casein Components during the Transglutaminase Reaction.

The disappearance of the monomer bands shown in Fig. 2 was analyzed quantitatively with densitometer tracings. The monomer content (in %) is based on the amount obtained from each gel loaded with zero-time product.

(●—●) α_{s1} -casein; (○—○) β -casein; (▲—▲) κ -casein.

Fig. 4. SDS-Polyacrylamide Gradient Gel Electrophoresis of Polymer Products.

Samples were the 120 min products obtained from the transglutaminase reactions shown in Fig. 1. 40 μ g of protein was applied to each lane. Migration is from top to bottom. P, O, and M indicate the position of bands of polymer, oligomer, and monomer. (A) Standard proteins; (1) thyroglobulin (669,000), (2) ferritin (440,000), (3) 1/2 thyroglobulin (330,000), (4) lactate dehydrogenase (140,000), (5) bovine serum albumin (67,000), (6) 1/4 catalase (60,000), (7) 1/4 lactate dehydrogenase (36,000). (B) α_{s1} -casein. (C) β -casein. (D) κ -casein.

during the first 60 min with β -casein), bands with higher mobility than that of the monomer band were detected, and, with each of the caseins, the mobility of the oligomer bands did not indicate a molecular weight corresponding to an integral multiple of the monomer molecular weight by calibration with standard proteins. The formation of intramolecular crosslinks might bring about an abnormal mobility in SDS-polyacrylamide gel of the protein molecules.

To determine the molecular weight of the polymer products, each of the 120 min products was subjected to SDS-polyacrylamide gradient gel electrophoresis (Fig. 4). From a standard curve (not shown) drawn from the electrophoretic mobilities of proteins of known molecular weight, the molecular weights of α_{s1} -, β -, and κ -casein polymer products were calculated to be $5.0 \sim 10 \times 10^5$. α_{s1} -Casein had heterogeneous bands, while β - and κ -caseins had homogeneous ones indicating molecular weights of 8.8×10^5 and 10×10^5 , respectively.

Properties of crosslinked casein components

Each of the casein components forms soluble

polymers by self-association, and α_{s1} - and β -caseins form soluble complexes with κ -casein. In the presence of calcium ions, α_{s1} - and β -caseins precipitate as the calcium caseinates, and κ -casein stabilizes these calcium caseinates against precipitation as casein micelles. Therefore, the properties of casein components modified by transglutaminase were examined in the presence and in the absence of calcium ions. The samples used in these experiments were products of the transglutaminase reactions shown in Fig. 1.

1) *Properties in the absence of CaCl_2 .* In ultracentrifugal experiments, effects of the modification by transglutaminase on the self-association of casein components were studied in the absence of calcium ions. Figure 5 shows sedimentation patterns of casein components in the presence of 70 mM KCl and 2 mM dithiothreitol at 35°C. After modification, sedimentation coefficients (S_{85}) of α_{s1} -caseins increased from 4.9 S to 8.3 S and of β -caseins from 14.9 S to 20.4 S. These results indicate that, under our experimental conditions, each of the modified components exists as a larger

Fig. 5. Sedimentation Patterns of Casein Components.

Samples were from the transglutaminase reactions shown in Fig. 1. "n" represents native protein (zero-time product) and "m" the modified protein (120 min product). Centrifugation was performed at 35°C in 10 mM imidazol buffer (pH 7.1) containing 70 mM KCl and 2 mM dithiothreitol. The concentration of each casein component was 5 mg/ml. The pictures were traced from photographs taken 18 ± 1 min after reaching maximum speed (60,000 rpm). Values in this figure are S_{85} .

molecular species than a self-associate of the corresponding native component would be. The degree of self-association of the modified component is not known at present. With κ -casein, the sedimentation coefficient did not change significantly, probably because modification was slight. Figure 5 also shows that each of the modified α_{s1} - and β -caseins can form complexes with κ -casein as well as the native ones could. Sedimentation coefficients of the complexes of modified components were

larger than that of the complex from native ones. These results seem to reflect the increase in molecular size caused by the modification.

2) Properties in the presence of CaCl_2 .

Properties of casein components modified by transglutaminase were tested in the presence of calcium ions in two areas; the precipitation of calcium-sensitive caseins (α_{s1} - and β -caseins) in the presence of 0~25 mM CaCl_2 , and the stabilization by κ -casein of α_{s1} - and β -caseins

FIG. 6. Effect of Modification by Transglutaminase on the Precipitation of α_{s1} - and β -Caseins in the Presence of CaCl_2 .

Precipitation was tested as described in MATERIALS AND METHODS. The percentage content of the casein in the supernatant was based on the amount obtained in the absence of CaCl_2 . Samples were from the transglutaminase reactions shown in Fig. 1.

(A) α_{s1} -casein; (●—●) not modified (zero-time product), (○—○) modified (120 min product). (B) β -casein; (●—●) not modified (zero-time product), (○—○) modified (120 min product). (C) Relationship between precipitation in the presence of 10 mM CaCl_2 and the extent of modification; (●—●) α_{s1} -casein, (○—○) β -casein.

FIG. 7. Effect of Modification by Transglutaminase on the Stabilization of α_{s1} - and β -Caseins by κ -Casein.

Stabilization by κ -casein was tested as described in MATERIALS AND METHODS. The percentage content of the casein in the supernatant was based on the amount obtained in the absence of κ -casein. Samples were from the transglutaminase reactions shown in Fig. 1.

(A) α_{s1} -casein; (●—●) not modified (zero-time product), (○—○) modified (120 min product). (B) β -casein; (●—●) not modified (zero-time product), (○—○) modified (120 min product). (C) Relationship between stabilization by κ -casein and the extent of modification. The ratio of the amount of κ -casein to the amount of α_{s1} - or β -casein was 0.1; (●—●) α_{s1} -casein, (○—○) β -casein.

against precipitation in the presence of 25 mM CaCl_2 . Figure 6 shows that precipitation of α_{s1} - and β -caseins at lower concentrations of CaCl_2 was reduced by the modification but, at higher concentrations of CaCl_2 (25 mM for α_{s1} -casein, 15~25 mM for β -casein), both native and modified caseins precipitated to the same extent. The decrease in precipitation at lower CaCl_2 concentrations might be caused by an increase in the negative net charge or by the restriction of Ca^{2+} -induced conformational changes in the modified casein components. Figure 7 shows that both native and modified α_{s1} -caseins were stabilized by κ -casein in the same manner, and β -casein turned out to be stabilized more easily. The modification of κ -casein, the extent of which is very low (Fig. 1), did not affect its stabilizing ability on both α_{s1} - and β -caseins (data not shown).

The results above indicate that transglutaminase polymerizes each of the casein components by the formation of intermolecular cross-links, but that it does not impair their functional properties exhibited in both the presence and the absence of calcium ions. This suggests that transglutaminase is a useful tool to change the rheological properties of caseins without damaging their special functional properties, and that it can also be used to localize the casein components in the casein micelle structure.

Transglutaminase will be useful for producing food protein materials with new functional properties and high nutritive values through the formation of complexes between proteins from different sources and through the introduction of primary amines, including amino acid esters, to proteins. These areas are under investigation.

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