Polymerization of Several Proteins by Ca^{2+} -Independent Transglutaminase Derived from Microorganisms

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Received March 24, 1989

α_{s1} -Casein and soybean globulins were polymerized and gelatinized by Ca^{2+} -independent transglutaminase that was isolated from the culture filtrate of a microorganism thought to belong to *Streptovercillium* sp. of actinomycetes. This enzyme polymerized such albumins as bovine serum albumin, human serum albumin and conalbumin in the presence of dithiothreitol. Rabbit myosin was polymerized by the present enzyme but actin was not. An RP-HPLC analysis after enzymic digestion of the polymerized α_{s1} -casein showed existence of the ϵ -(γ -Glu)Lys bond. Thus, it was confirmed that the polymerization was formed by a catalytic reaction of the transglutaminase.

The incorporation of inter- or intramolecular covalent crosslinks into food proteins seemed to be available for improving the physical and textural properties of many food products such as tofu, boiled fish paste and sausage. There are chemical and enzymic procedures to introduce covalent crosslinks into proteins. Because of its natural origin and high specificity for substrates, the enzymic procedure is more acceptable for regulatory authorities and the consumer.

Transglutaminase (*R*-glutaminyl-peptide: amine γ -glutamyltransferase; EC 2. 3. 2. 13) is one of the enzymes which is capable of introducing covalent crosslinks between proteins. The enzyme catalyses a Ca^{2+} dependent acyl-transfer reaction, in which the γ -carboxyamido groups of peptide-bound glutaminyl residues are the acyl donors. When amino groups of peptide-bound lysine residues are the acyl acceptor, ϵ -(γ -Glu)Lys crosslinks are generated.

Ikura *et al.* found that guinea pig liver transglutaminase polymerized bovine casein components¹⁾ and soybean proteins.²⁾ It was

further shown that guinea pig liver transglutaminase catalysed the formation of heterologous polymers through ϵ -(γ -Glu)Lys crosslinks between acetylated α_{s1} -casein and various native proteins.³⁾ Kurth and Rogers reported crosslinking of myosin to soybean protein, casein and gluten by using bovine plasma transglutaminase.⁴⁾

In spite of those reports, it was thought to be difficult to use such mammalian transglutaminase for food production because of its high supply cost.

To open the way to the mass production of transglutaminase by fermentation, we have been screening for microorganisms. Consequently, as described in the previous paper,⁵⁾ in the culture filtrate of the microorganism thought to belong to *Streptovercillium* sp., the enzyme which catalyzed transacylation between the γ -carboxyamido of the glutamine residue in *N*-carbobenzoxy-L-glutaminylglycine and hydroxylamine was found. This enzyme was purified to a uniform state from the culture filtrate by a chromatographic procedure and studied for its enzymatic properties.

The present enzyme differed from known mammalian transglutaminases, required no calcium ions in the hydroxamate-forming activity, and thus, appeared to be a new transglutaminase.

In this report, we found polymerization of various proteins by using present enzyme, and the existence of an ϵ -(γ -Glu)Lys bond in those polymerized proteins.

Materials and Methods

Enzymes. Microbial transglutaminase (11.3 units/mg) was prepared from a culture filtrate of *Streptovorticillium* sp. by the method in the former report.⁵⁾ Pronase, leucine aminopeptidase and carboxypeptidase A were purchased from Boeringer Mannheim. Prolidase was purchased from Sigma.

Protein substrates. Crude α_{s1} -casein was prepared from fresh defatted milk by the method of Zittle and Custer.⁶⁾ Crude soy 7S and 11S globulin were prepared from defatted soybean flour (Ajinomoto Co., Inc.) by the method of Thanh and Shibasaki.⁷⁾ Rabbit myosin and actin were prepared from back muscle by the methods of Connell,⁸⁾ and Squidich and Watt,⁹⁾ respectively. Bovine serum albumin, human serum albumin and conalbumin were purchased from Sigma.

Chemicals. ϵ -N- γ -Glutamyllysine and *o*-phthalaldehyde reagent solution were purchased from Sigma. *N*-Carbobenzoxy-L-glutamylglycine was purchased from Kokusan Chemical Works, all other chemicals being purchased from Wako Pure Chemical Co.

Preparation of proteins polymerized by transglutaminase. Polymerization of the proteins was carried out without the calcium ion. α_{s1} -Casein and soybean globulin solutions (10 mg/ml) were prepared in a 0.1 M Tris-HCl buffer (pH 7.6). When the albumins were protein substrates, protein solutions were prepared in a 0.1 M Tris-HCl buffer (pH 7.6) containing 10 mM dithiothreitol. Rabbit myosin and actin solutions were diluted to 10 mg/ml with a buffer of 0.5 M KCl and 20 mM Tris-maleate (pH 7.5). Except for myosin and actin, each protein solution (1.0 ml) was mixed with 0.2 unit of microbial transglutaminase and incubated for 60 min at 55°C. Following the addition of the present enzyme (1.0 unit), the myosin and actin solutions (1.0 ml) were incubated for 60 min at 10°C. After incubation, the reaction mixtures were immediately analyzed by HPLC (gel filtration) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). On the other hand, the enzyme was inactivated by heat-treatment at 100°C for 3 min to carry out a control experiment.

Gel formation by microbial transglutaminase. Protein solutions (50 mg/ml) were prepared in a 0.1 M Tris-HCl buffer (pH 7.6). Aliquots of 2.0 ml from the protein solutions and 2 units of microbial transglutaminase were mixed and put into a small test tube, with subsequent incubation at 55°C for 60 min. Protein gel formation was confirmed by standing the test tubes on their heads as shown in Fig. 5.

Conditions for HPLC and SDS-PAGE. Gel filtration analyses were performed on a Waters HPLC system (M600 + M490 + M470). The reaction mixture (20 μ l) was injected in a TSK-GEL 3000SW column (600 mm $\times$ 7.5 mm i.d., TOSOH). Solvent flow rate was 1.0 ml/min and the mobile phase was 0.1 M sodium phosphate buffer (pH 7.0). The detector monitored absorbance at 280 nm. SDS-PAGE was performed on a Phastgel gradient 10 ~ 15 (Pharmacia) with the Phastsystem (Pharmacia). Sample solutions for SDS-PAGE were prepared by mixing 0.2 ml of the reaction mixture with 0.8 ml of an SDS reagent solution (0.01 M Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, 2.5% SDS and 5% 2-mercaptoethanol) and heating for 5 min in boiling water. The samples were separated under the programmed conditions and stained with Coomassie brilliant blue.

Analysis of the ϵ -(γ -Glu)Lys bond in polymerized α_{s1} -casein. An α_{s1} -casein solution (10 mg/ml) was prepared in distilled water. Microbial transglutaminase (0.2 unit) was added to 1.0 ml of this solution, and after incubating for 60 min at 55°C, the reaction mixture was boiled for 3 min. An aliquot of 1.0 ml from 0.2 M ammonium bicarbonate buffer (pH 7.8) and 1 crystal of thymol were added to the cooled reaction mixture. Proteolytic digestion of this mixture was carried out by the sequential addition of proteolytic enzymes. Pronase was applied in two additions (to total 0.8 mg), each for a duration of 24 hr at 37°C. After inactivation of pronase by heating at 100°C for 15 min, digestion was continued at 37°C for 24 hr by adding 14 μ l of leucine aminopeptidase and 13 μ l of prolidase. This was followed by a second addition of leucine aminopeptidase for the same duration of time. Finally, 10 μ l of carboxypeptidase A was added for a further period of 24 hr at 37°C. This proteolytic digests were lyophilized, and the dried digests were taken up in 5 ml of water. Aliquots of 50 μ l from these were mixed with 450 μ l of an *o*-phthalaldehyde reagent solution and kept for 3 min at room temperature. Aliquots of 100 μ l from this reaction mixture were applied to an RP-HPLC system and analyzed by the method of Griffin *et al.*¹⁰⁾ Synthetic ϵ -N- γ -glutamyllysine (0.1 mM solution) was used as the internal standard.

Results and Discussion

Enzymatic polymerization of proteins

When the α_{s1} -casein solution was treated

with microbial transglutaminase, a polymer fraction was detected at the void volume (MW 500,000) on the gel filtration chromatogram (Fig. 1).

SDS-PAGE of the α_{s1} -casein and soybean globulins treated with the present enzyme shows that the monomeric fraction of the intact protein diminished or disappeared, and

Fig. 1. Gel Filtration Analysis for the Polymerization of α_{s1} -Casein by Microbial Transglutaminase (BTGase).

α_{s1} -Casein solution incubated (55°C for 60 min) with inactivated BTGase (—), and with BTGase (---).

Fig. 2. SDS-PAGE of Proteins Polymerized by Microbial Transglutaminase (BTGase).

α_{s1} -, 7S and 11S represent α_{s1} -casein, soybean 7S and 11S globulins, respectively. Each protein solution was incubated with inactivated BTGase at 55°C for 60 min (-Cont.), and with BTGase (+BTGase).

BSA: bovine serum albumin
HSA: human serum albumin
CA: conalbumin

Fig. 3. Effect of Dithiothreitol on the Polymerization of Albumins by Microbial Transglutaminase.

BSA, HSA, CA and DTT represent bovine serum albumin, human serum albumin, conalbumin and dithiothreitol, respectively. Each sample was prepared under the same conditions as those in Fig. 2.

Fig. 4. SDS-PAGE of Rabbit Myosin and Actin Treated with Microbial Transglutaminase (BTGase).

Each protein solution was incubated with inactivated BTGase at 10°C for 60 min (-Cont.), and with BTGase (+BTGase).

Fig. 5. Gelation of a Protein Solution by Microbial Transglutaminase.

The lefthand test tube of each pair was incubated with inactivated BTGase, and the righthand test tube of each pair was incubated with BTGase. The concentration in each protein solution was 50 mg/ml.

a novel polymer fraction, which could not enter into the gradient gels, was formed (Fig. 2). Since enough 2-mercaptoethanol was contained in the sample solution for SDS-PAGE, the polymer detected could not have been formed by disulfide bonds.

Bovine serum albumin, human serum albumin and conalbumin were polymerized by the present enzyme when dithiothreitol was added to the reaction mixture (Fig. 3). For these albumins, a conformational change caused by reduction of disulfide bonds was needed to be the substrate for polymerization.

On the SDS-PAGE of rabbit myosin treated with microbial transglutaminase, the monomeric myosin heavy chain was decreased and a polymer fraction was detected in the gradient gel. Although rabbit actin was incubated with the same enzyme under the same condition, it revealed no change (Fig. 4). Myosin is essential for the textural properties of meat products, and it is useful that microbial transglutaminase catalyzes the crosslinking between myosin heavy chains at low temperatures.

Gelation of the protein solution by microbial transglutaminase

We have reported the gelation of a protein

Fig. 6. HPLC analysis of the ϵ -(γ -Glu)Lys (EGGL) bond in the proteolytic digest of polymerized α_{s1} -casein.

(A) mixture of 20 amino acids and EGGL; (B) proteolytic digest of α_{s1} -casein incubated with inactivated microbial transglutaminase; (C) proteolytic digest of α_{s1} -casein polymerized by microbial transglutaminase.

solution by guinea pig transglutaminase.¹¹⁾ The protein solutions with higher concentrations turned into self-supporting gels with the addition of microbial transglutaminase as well as was shown in the case for guinea pig transglutaminase (Fig. 5). The protein solution did not gel unless transglutaminase was added.

ϵ -(γ -Glu)Lys bond in the polymerized α_{s1} -casein

The peak of synthetic ϵ -N- γ -glutamyllysine on HPLC (RP-HPLC) was detected at 10.8 min. The peak of the ϵ -(γ -Glu)Lys bond was recognized on an HPLC chromatogram of the sample solution derived from polymerized α_{s1} -casein, but not of control. Thus, the ϵ -(γ -Glu)Lys bond in the polymer was formed by

the transacylation between γ -carboxyamides of glutamine residues and ϵ -amino groups of lysine residues in α_{s1} -casein catalyzed by the microbial transglutaminase. It was calculated that the quantity of the ϵ -(γ -Glu)Lys bond in the polymerized α_{s1} -casein was *ca.* 1.3 mol/mol of α_{s1} -casein. This fact confirmed that microbial transglutaminase is one of the transglutaminases.

Microbial transglutaminase can be produced by the industrial fermentation method, so studies of this enzyme for food processing or applications in medical treatment will be accelerated.

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