Gelation of Protein Emulsion by Transglutaminase

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Transglutaminase is a Ca\(^{2+}\) dependent enzyme that covalently polymerizes proteins through the formation of \(\varepsilon-(\gamma\text{-glutamyl})\text{lysyl cross-links}\). We investigated the transglutaminase reaction in emulsions stabilized by protein. When emulsions prepared with proteins (\(\alpha_s\text{-casein}, \text{and soybean 11S and 7S globulins}\)) and soybean oil were incubated with transglutaminase, the emulsions turned into gels. Gel formation depended on the amount of transglutaminase and the pH. The results on SDS–polyacrylamide gel electrophoresis indicated that protein in the emulsions was polymerized by the action of transglutaminase. Gelation of the emulsions was attributed to the formation of \(\varepsilon-(\gamma\text{-glutamyl})\text{lysyl cross-links}\).

MATERIALS AND METHODS

Materials. Transglutaminase was prepared from fresh guinea pig livers by the method of Connellan et al.\(^{10}\) The characteristics of the isolated transglutaminase were reported previously.\(^{5}\) \(\alpha_s\text{-casein}\) was prepared from fresh cows’ milk by the method of Zittle and Custer.\(^{11}\) Soybean 11S and 7S globulins were prepared from defatted soybean flour by the method of Thanh et al.\(^{12}\) Other chemicals were extra pure reagents obtained from Wako Pure Chemicals Co.

Acetylation of \(\alpha_s\text{-casein}\). \(\alpha_s\text{-casein}\) was acetylated by a modification of the method of Franzen and Kinsella.\(^{13}\) \(\alpha_s\text{-casein}\) (500 mg) was dissolved in 6.6 \(\text{m}\) urea (20 ml) and mixed with 2 \(\text{m}\) Tris–HCl buffer (pH 7.6, 20 ml). Acetic anhydride was added in 80 \(\mu\text{l}\) increments up to a total of 1.5 ml over a 2 hr period while keeping the mixture stirred at 0°C. During acetylation the pH was maintained at 8.0 with 2 \(\text{m}\) NaOH. After the acetylation, the solution was dialyzed against water (3°C, 24 hr) to remove impurities and excess reagents. Acetylated \(\alpha_s\text{-casein}\) (480 mg) was recovered by lyophilization. It was found that the amino groups had been completely acetylated by this procedure. The degree of acetylation was measured by the method using trinitrobenzenesulfonic acid.\(^{14}\)

Preparation of emulsion. Protein solution was prepared with 0.1 \(\text{m}\) Tris–HCl buffer containing 5 \(\text{mM}\) CaCl\(_2\) and 10 \(\text{mM}\) dithiothreitol at the concentration and pH indicated in the legends to figures. Soybean oils (0.6 ml, Ajinomoto Co., Inc.) was added to the protein solution (1.4 ml). The mixture was emulsified by a sonicator (Ohtake Seisaku-Sho, Model 5202, 20 kHz, 60 W) in an
ice-bath for 3 min. Duplicate preparations were made for each experiment.

**Gel preparation with transglutaminase.** To 500-μl sample of the emulsion was added transglutaminase at the ratio of 0.03 units/mg substrate protein. The reaction mixture was incubated at 37°C for 1 hr.

**Measurement of viscosity.** Gel formation was detected by following the change of viscosity. The viscosity was measured on a Contraves LS-30 low shear viscometer, which allows accurate measurements under low shear rates. Samples (800 μl) of the emulsion were placed in the cuvette cylinder. Transglutaminase was then added at the ratio of 0.03 units/mg substrate protein. After 10 sec, the mixtures were sheared at a shear rate of 50 s⁻¹ at 30°C for 300 sec. Changes in the stress were registered on a personal computer (Hewlett Packard, HP-85) and the data were used to illustrate the course of the viscosity change.

**SDS-polyacrylamide gel electrophoresis.** Polyacrylamide gel electrophoresis was done on a slab gel in the presence of sodium dodecylsulfate (SDS) as reported in our previous paper. To analyze the formation of the covalent cross-linkages of protein in the emulsions, emulsion was defatted by n-hexane and mixed with an equal volume of 50% glycerol, 2% SDS, and 5% 2-mercaptoethanol in 0.02 M phosphate buffer (pH 7.2). After 2 min of incubation at 100°C, it was put on to the SDS-polyacrylamide gel.

**Solubility in denaturants.** The gels (0.5 ml) were formed in test tubes of 8 mm diameter by the method described above. Several denaturants (10% SDS, 10% 2-mercaptoethanol, 6.6 M urea, or 6 M guanidine hydrochloride solutions) (5 ml) were added to them. After the mixtures were shaken at 25°C for 10 min, the gel was observed. To observe the heat stability, distilled water (5 ml) was added to the gels (0.5 ml) and then they were heated at 100°C for 20 min.

**RESULTS AND DISCUSSION**

When emulsions prepared with several proteins (αsl-casein, and soybean 11S and 7S globulins) and soybean oil were incubated with transglutaminase, these emulsions turned into gels. The gels retained the initial shapes even on inversion as shown in Fig. 1. However, the emulsions did not gel, even after 4 hours' incubation, unless transglutaminase was added. The proteins extracted from gels formed by transglutaminase were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). The monomer fractions of the intact protein diminished or disappeared. Polymer fractions which did not enter the running gels were formed. Since the reaction mixture included a reducing reagent (10 mM dithiothreitol) and the electrophoresis system included 2-mercaptoethanol, the polymers detected could not have been formed by disulfide binds. These results indicated that
Gelation of Emulsion by Transglutaminase

The polymers were formed by intermolecular cross-links, which were produced by transglutaminase. The increase of viscosity was affected also by the pH, as shown in Fig. 4. When transglutaminase was added to 7S globulin emulsions of pH 6 and 7, they turned very quickly into gels. On the other hand, when the emulsion gels formed by transglutaminase were diluted 10 times by several denaturants (10% SDS, 10% 2-mercaptoethanol, 6.6M urea, or 6M guanidine hydrochloride solutions) and shaken at 25°C for 10 min, they did not dissolve. Even if they were diluted 10 times by distilled water and heated at 100°C for 10 min, they remained the initial shape. In addition, an increase in viscosity of the 7S globulin emulsion was not observed in the presence of 0.4M EDTA (pH 8.0), which is an inhibitor of transglutaminase, regardless of the transglutaminase treatment. When an emulsion prepared with acetylated αs1-casein was incubated with transglutaminase, the viscosity of the reaction mixture did not increase and no polymer fraction on SDS-PAGE was formed. This shows that acetylated αs1-casein in an emulsion is not a substrate for transglutaminase, because the reaction sites, the ε-amino groups of lysine residues, have been blocked by acetylation. Therefore, the gels are not formed by hydrogen bonds and/or hydrophobic interaction, but by covalent bonds, ε-(γ-glutamyl)lysyl cross-linking.

REFERENCES

4) K. Ikura, T. Kometani, M. Yoshikawa, R. Sasaki


