Gelation Mechanism of Protein Solution by Transglutaminase

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Received August 9, 1985

Transglutaminase catalyzes the formation of \( \varepsilon-(\gamma\text{-glutamyl})\text{lysyl} \) cross-links within and between protein molecules. Therefore, transglutaminase polymerizes proteins through the formation of isopeptide bonds. We have reported that several high-concentration protein solutions formed firm gels when they were incubated with transglutaminase. This paper presents unambiguous evidence that \( \alpha_{s1}\)-casein solution is gelled by the formation of \( \varepsilon-(\gamma\text{-glutamyl})\text{lysyl} \) cross-links. Gelation of \( \alpha_{s1}\)-casein was effected by the amount of transglutaminase and pH value, and \( \alpha_{s1}\)-casein gel was not dissolved by various denaturants. Succinylated \( \alpha_{s1}\)-casein, in which \( \varepsilon \)-amino groups of lysine residues are blocked, was not gelled by transglutaminase. Therefore, it is confirmed that \( \alpha_{s1}\)-casein is formed by the action of transglutaminase.

It has been recognized that intramolecular disulfide bonds play an important role in the protein structure.\(^1\) In addition, much attention has been paid from the physiological aspect to other covalent cross-links within and between protein molecules. There are two types that comprise these cross-links. One type, which occurs in collagen\(^2\) and elastin,\(^3\) is the Shiff bases formed by the condensation between lysine- and hydrolysine-derived aldehyde and \( \varepsilon \)-amino groups of protein-bound Lys residues. The other type is \( \varepsilon-(\gamma\text{-glutamyl})\text{lysyl} \) cross-links which occur in fibrin clot\(^4\) and epidermal keratinocytes.\(^5\) It is known that cross-links of this type are formed by transglutaminase. Transglutaminase (R-glutaminyl-peptide: amine \( \gamma\text{-glutamyl} \)transferase, EC 2.3.2.13) is a \( \text{Ca}^{2+} \)-dependent enzyme which catalyzes the intra- and intermolecular cross-linking of certain proteins by \( \gamma\text{-glutamyl}\varepsilon\text{-lysine} \) side chain bridges.\(^6\)\(^7\)

\[
\begin{align*}
\text{Glu-CO} & \text{N} & \text{H}_2 & \text{N} & \text{Lys} & \xrightarrow{\text{Transglutaminase}} & \text{Glu-CO} & \text{N} & \text{Lys} & + & \text{NH}_3 \\
\quad & & \quad & & \quad & & \quad & & \quad & & \quad \quad \text{Ca}^{2+}
\end{align*}
\]

It has been also suggested that this enzyme is a useful tool to produce new food protein materials with new functional properties\(^8\)\(^9\)\(^10\) and better nutritive values.\(^11\) Furthermore, we have found that high-concentration protein solutions were firmly gelatinized by transglutaminase.\(^12\) It was suggested that transglutaminase and a higher concentration of a substrate protein are indispensable for firm gel formation.

This report presents evidence that the gel of \( \alpha_{s1}\)-casein formed by transglutaminase is stabilized by \( \varepsilon-(\gamma\text{-glutamyl})\text{lysine} \) cross-links between the \( \alpha_{s1}\)-casein molecules.

MATERIALS AND METHODS

Materials. Transglutaminase was prepared from fresh guinea pig livers by the method of Connellan \textit{et al.}\(^13\) The characteristics of the isolated transglutaminase were reported previously.\(^10\) \( \alpha_{s1}\)-Casein was prepared from fresh cows' milk by the method of Zittle and Custer.\(^14\) Other chemicals were extra-pure reagents from Wako Pure Chemicals Co.

Succinylation of \( \alpha_{s1}\)-casein. Succinylation of \( \alpha_{s1}\)-casein was performed by modifying the method of Franzen and Kinsella.\(^15\) \( \alpha_{s1}\)-Casein (1 g) was dissolved in 6.6 M urea
aqueous solution (40 ml) and added to 2 M Tris–HCl buffer (pH 8.0, 40 ml). Fifty mg increments of succinic anhydride were added up to a total of 500 mg, and the mixture was stirred for over 2.5 hr at 0°C. During this succinylation, the pH was maintained at 8.0 with 2 N NaOH. After pH stabilization, the solution was dialyzed against distilled water at 3°C for 24 hr to remove the impurities and excess reagent. Succinylated $\alpha_s$-casein (910 mg) was recovered by lyophilization. It was found that the amino groups had been completely succinylated by this procedure, the degree of succinylation being determined by the method using trinitrobenzenesulfonic acid. 16

Preparation of the gel

Gelation with transglutaminase. The protein solutions were prepared with 0.1 M Tris–HCl buffer (pH 8.0) containing 5 mg CaCl$_2$ and 10 mM dithiothreitol (DTT) with the concentration and pH indicated in the legends to the figures. Transglutaminase was added to them at the ratio of 0.03 units/mg of $\alpha_s$-casein, and followed by incubation at 37°C for 1 hr.

Gelatin gel. The concentration of gelatin in the gel sample was 10% (w/v). To the gelatin was added distilled water, the mixture being incubated at 37°C for 30 min and allowed to stand at 3°C for 1 hr.

Polyacrylamide gel. 14% Polyacrylamide gel was prepared by the method described in the previous paper. 10

Viscosity. Gel formation was followed by observing the changes of viscosity in a low-shear viscometer (Contraves LS-30), which allowed accurate measurements under low shear rates. The 5% $\alpha_s$-casein solution already described was used to fill the cuvette cylinder (800 $\mu$l). Transglutaminase was then added at the ratio of 0.03 units/mg of $\alpha_s$-casein. After 10 sec, the reaction mixture was subjected to a shear rate of 50 s$^{-1}$ at 30°C. Changes in the shear stress were recorded with a personal computer (Hewlett Packard HP-85) and the data were used to illustrate the time course of the viscosity change.

Solubility of the gel in several denaturants. The gels were formed in test tubes of 12 mm diameter by the method just described. Several denaturants (10% SDS, 10% 2-mercaptoethanol, 6.6 M urea and 6 M guanidium hydrochloride solution) were then added to them. After the mixtures had been shaken at 25°C for 10 min, the appearance of the gels in each denaturant was observed. Heat denaturation was attained by heating in boiling water for 10 min.

SDS-polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was performed on slab gel in the presence of sodium dodecyl sulfate (SDS) as described in the previous paper. 10

Scanning electron microscopic observation. Protein gels in small test tubes were frozen at $-78$°C and then freeze dried. The dried samples were coated with gold sputtering, and microphotographs were taken under a Hitachi electron microscope (type S-430) at a voltage of 20 kV.

RESULTS

Effect of transglutaminase

The change in viscosity of a reaction mixture was measured as an indication of the $\alpha_s$-casein gel formation. Figure 1 shows the relationship between the time course of the viscosity and amount of transglutaminase. When an $\alpha_s$-casein solution was not treated with transglutaminase (in the case of 0 units/mg of $\alpha_s$-casein), the reaction mixture did not show any remarkable change in viscosity. However, an increase of viscosity was observed to accompany an increase in the amount of transglutaminase. When transglutaminase was added by more than 0.015 units per milligram of $\alpha_s$-casein, the viscosity increased rapidly and the $\alpha_s$-casein solution formed a gel.

Effect of pH

Figure 2 shows the time course of the viscosity of a protein solution treated by transglutaminase at pH 7.0–10.0. It is suggested that

![Fig. 1. Effect of the Amount of Transglutaminase.](image-url)
Mechanism for Gelation by Transglutaminase

The concentration of αs1-casein was 5%. Transglutaminase was added to the reaction mixture at the ratio of 0.02 units/mg of αs1-casein.

**Table 1. Solubility of Gels in Denaturants**

<table>
<thead>
<tr>
<th>αs1-Casein Gel* (TG)$^a$</th>
<th>Gelatin$^b$</th>
<th>PAGE$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% 2-Mercaptoethanol</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6 M Guanidium HCl</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>6.6 M Urea</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>10% SDS</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Heating at 100°C</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

+, dissolved; −, not dissolved.

* αs1-Casein gel (5%) formed with transglutaminase.

$^a$ Gelatin gel (10%) formed by cooling.

$^c$ Polyacrylamide gel (14%).

the gel formation by transglutaminase was pH dependent. In the case of a 5% αs1-casein solution, the change of viscosity at pH 9.0 was most significant, compared with the other pH values.

**Solubility of gel in several denaturants**

The solubilities of gels in several denaturants are summarized in Table I. Gelatin gel, which was formed by hydrogen bonding, was easily dissolved by a 6.6 M urea solution and 6 M guanidium hydrochloride, or by heating. However, the αs1-casein gel formed with transglutaminase was not dissolved by treating with all denaturants tested as well as with polyacrylamide gel, which was formed by covalent bonding.

![Fig. 2. Effect of pH on Gelation.](image)

The concentration of αs1-casein was 5%. Transglutaminase was added to the reaction mixture at the ratio of 0.02 units/mg of αs1-casein.

![Fig. 3. Viscosity Change in Reaction Mixtures of αs1-Casein (A) and Succinylated αs1-Casein (B).](image)

transglutaminase was added to the reaction mixture at the ratio of 0.03 units/mg of substrate protein; ---, transglutaminase was not added to it. The concentration of proteins was 5%, and the pH value of the reaction mixture was 8.0.

![Fig. 4. SDS-Polyacrylamide Gel Electrophoretic Pattern of the Reactions with αs1-Casein (A) and Succinylated αs1-Casein (B).](image)

Lane a, transglutaminase was not added to the protein solution; lane b, transglutaminase was added to it. The conditions of the reaction mixtures were the same as shown in Fig. 3. αs1, Sucαs1 and P indicate the positions of bands of αs1-casein, succinylated αs1-casein and polymers, respectively.
Reaction with succinylated \( \alpha_{\text{s1}} \)-casein

When \( \alpha_{\text{s1}} \)-casein was incubated with transglutaminase, the viscosity of the reaction mixture increased rapidly, the polymer formation being detected by SDS-PAGE (Figs. 3 and 4). However, in the absence of transglutaminase, the viscosity did not change and a polymer was not observed. On the other hand, the succinylated \( \alpha_{\text{s1}} \)-casein solution did not show any remarkable change in viscosity regardless of the transglutaminase treatment (Fig. 3). Further, the monomer fraction did not decrease and no polymer formation was found as shown by SDS-PAGE (Fig. 4). 5\% Succinylated \( \alpha_{\text{s1}} \)-casein was not gelled in spite of 5 hours' incubation.

Structure

The microstructures of protein gels made by transglutaminase were visualized by scanning electron microscopy. As shown in Fig. 5, the gel had a three-dimensional network structure.

DISCUSSION

In the previous paper\(^{12}\), we reported that several protein solutions of high concentration formed a firm gel with transglutaminase, it being supposed that the gel formation was caused by a transglutaminase reaction. This postulation is supported by the findings that gelation of native \( \alpha_{\text{s1}} \)-casein was dependent on the amount of transglutaminase and pH value (Figs. 1 and 2). When succinylated \( \alpha_{\text{s1}} \)-casein, whose \( \varepsilon \)-amino groups of lysine residues were blocked, was incubated with transglutaminase, it did not form a gel. The molecular weight by SDS-PAGE and viscosity of succinylated \( \alpha_{\text{s1}} \)-casein also did not change (Figs. 3 and 4). These facts indicate that the gelation of native \( \alpha_{\text{s1}} \)-casein was due to the formation of \( \varepsilon-(\gamma \text{-glutamyl}) \)-lysyl cross-links. Since the gelation of native \( \alpha_{\text{s1}} \)-casein was performed in the presence of a reducing reagent (10\% DTT) and the electrophoresis procedure included 2-mercaptoethanol, the polymers detected in the gel could not have been formed by disulfide bonds. These protein gels were not dissolved by a 6.6\% urea solution, 10\% SDS solution, 6\% guanidium hydrochloride, or by heating (Table I). Therefore, it is concluded that these gels were formed not by the hydrogen bond and hydrophobic interaction, but by covalent bonds with \( \varepsilon-(\gamma \text{-glutamyl}) \)-lysyl cross-linking. The gels formed with transglutaminase had the three-dimensional network structure shown in Fig. 5. Consequently, it is suggested that this network structure was formed through the formation of intermolecular \( \varepsilon-(\gamma \text{-glutamyl}) \)-lysyl cross-links with transglutaminase. \( \varepsilon-(\gamma \text{-Glutamyl}) \)-lysine cross-links would contribute to maintaining a gel network structure as well as disulfide and hydrogen bonds.

REFERENCES

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