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Effects of Microbial Transglutaminase for Gelation of Soy Protein Isolate during Cold Storage

Takahiko SOEDA

Ajinomoto Foundation For Dietary Culture, 1-16-7 Kyoubashi, Chuo-ku, Tokyo 104-8315, Japan

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On gelation of soy protein isolate during cold storage, strength, deformation, elasticity E_0 and Newtonian viscosity η_N of the gels greatly increased with addition of one unit/g protein of microbial transglutaminase when kept for 3 days at 5°C, while retardation time λ decreased. With addition of 3 units/g protein, the gels were too hard, but became brittle after being kept for 3 days at 5°C. The viscoelasticity of gel treated during cold storage was superior to that of gel heated after having been kept in cold storage. It was assumed that the texture of the gel treated for one day at 5°C was nearly equivalent to that of the gel treated at 40°C for 60 min, on enzyme reactivity of transglutaminase. ε-(γ-glutamyl)lysine bonds in the gels were not formed in the case of the gel without transglutaminase, but increased linearly in the gel with transglutaminase. Contents of the sulfhydryl group of the gel were found to decrease during cold storage, and the degree of decrease was smaller in the gel with transglutaminase than in the gel without transglutaminase. This indicated less contribution of disulfide bonds in the gels treated with transglutaminase during cold storage.

Keywords: soy protein, gel formation, cold storage, microbial transglutaminase, ε-(γ-glutamyl)lysine bonds, sulfhydryl groups

Transglutaminase (glutaminyl-peptide: amine γ-glutamyltransferase, E.C. 2.3.2.13; TGase) catalyzes an acyl transfer reaction between a γ-carboxyamide of peptide or protein-bound glutamine and a primary amine.

When TGase acts on protein molecules, ε-(γ-glutamyl)lysine (abbreviated ε-(γ-Glu)Lys) bonds are formed. Many studies have been carried out to use this unique enzyme reaction, crosslinking between protein molecules, to change rheological properties of food proteins (Whitaker, 1977; Ikura *et al*., 1980; Motoki & Nio, 1983). TGase, derived from a microorganism, has been found and mass produced (Ando *et al*., 1989). Enzymological properties and various basic effects on physical properties of food proteins have been reported (Soeda *et al*., 1992; Nonaka *et al*., 1992; Nonaka *et al*., 1994; Nonaka *et al*., 1996).

The principal mechanism of gelation of soy protein isolate (abbreviated SPI) is due to the formation of disulfide bonds, reported by Wolf and Smith (1961), Circle *et al*. (1964), Aoki and Sakurai (1969), Saio *et al*. (1971) on heat-induced gel, and by Watanabe *et al*. (1963), and Hashizume *et al*. (1974) on freeze-induced gel. On the other hand, as described in previous reports (Soeda, 1994a; 1994b; 1995a; Soeda & Baba, 1999), the gel (abbreviated Cold-gel) was obtained by keeping it in cold storage. The Cold-gel showed physical properties characterized by flexibility and springiness, which were excellent compared with the viscoelasticity of heat-induced or freeze-induced gel. We also reported that characteristics of gels required for application of SPI gel in food processing were flexibility and springiness related to masticability and swallowing in the mouth, and that Cold-gel provided an improved texture in processed foods (Soeda, 1995b). These superior textural properties are thought to be ascribable to the mechanism of gelation. In our studies, it was suggested that Cold-gel resulted in formation of a network structure of protein based on hydrophobic and hydrogen bonds (Soeda, 1994b), whereas heat-induced and freeze-induced gel resulted in formation of a network structure between proteins composed mainly of disulfide bonds.

As described above, gel formation by microbial transglutaminase (abbreviated MTG) could be obtained without heating, the same as the gelation in Cold-gel. However, in past studies on physicochemical properties of MTG-treated gels of SPI, the gels were heated at 90°C for one hour to inactivate enzymes after treatment with MTG, and were not studied without heating. In the present studies, the effects of MTG on the gelation of SPI during cold storage were investigated without heating.

Materials and Methods

SPI SPI was prepared by the same methods as described in the previous report (Soeda, 1994a). Briefly, soy protein solution was prepared from undenatured defatted soybean flakes by solubilizing into water and coagulating by sulfuric acid. After adding water to the coagulant, soy protein solution with about 7% solid matter (about 5% protein) was heated at 100°C for 2 min by steam, and then freeze-dried. The composition of SPI obtained was 84.9% protein, 4.5% carbohydrate, 4.1% ash and 3.3% moisture.

Preparation of gel According to the method described in the previous report (Soeda, 1994a), SPI paste was adjusted to 19.5% protein concentration by adding water. After mixing, the paste was placed in a casing tube and kept for a maximum of 3 days at 5°C to prepare Cold-gel. Texture measurement of the control gel which had not been kept in cold storage was carried out directly after keeping it for one hour at 5°C in casing tubes filled with paste, to maintain a constant condition for measurement of the texture.

E-mail: takahiko_soeda_asb@ajinomoto.com

The heat-induced gels (abbreviated Heat-gel) were obtained by heating at 90°C for 50 min directly after having been kept in cold storage. Further, gel treated at 40°C for 60 min (abbreviated 40°C-gel) was prepared without cold storage treatment, for the purpose of investigating enzyme reactivity for protein of the substrate.

MTG MTG was prepared from the culture of a variant *Streptoverticillium mobaraense* as described (Ando *et al*., 1989). In all experiments the MTG product of 1.0 unit/mg of specific activity of enzyme was used. Enzyme activity was measured by the hydroxamate procedure with Carbobenzoxy-L-glutaminylglycine as the substrate (Folk & Cole, 1996). Specific activity was defined as follows: one unit was the amount of the enzyme which catalyzed the formation of one μmol of hydroxamic acid/min at 37°C.

Determination of ε-(γ-Glu)Lys bonds Analysis of ε-(γ-Glu)Lys bonds in the gel samples was performed as described by Kumazawa *et al*. (1993). Briefly, protein gel sample were lyophilized and an aliquot (2 ml) of 0.1 M sodium borate buffer (pH 8.0) was added to the lyophilized sample (equivalent 15–20 mg protein). This mixture was incubated with a combination of pronase, leucine aminopeptidase, prolidase and carboxypeptidase A. The digested samples were then lyophilized and subjected to a two-step fractionation of ε-(γ-Glu)Lys with high performance liquid chromatography (HPLC). Elution time and ε-(γ-Glu)Lys contents were estimated using a synthetic ε-(γ-Glu)Lys substance as a reference (Kumazawa *et al*., 1993). ε-(γ-Glu)Lys bonds reacted with *o*-phthalaldehyde (OPA) determined by measuring fluorescence.

The amount of ε-(γ-Glu)Lys bonds was expressed in terms of μmol per 100 g of dry matter of the sample.

Determination of sulfhydryl groups Sulfhydryl groups were determined according to Ellman's DTNB method (Beveridge *et al*., 1974). Seventy-five mg of sample was suspended in 1 ml of Tris-Gly(10.4 g Tris, 6.9 g glycine and 1.2 g EDTA per *l*, pH 8.0, denoted as Tris-Gly), 4.7 g of GuHCl was added, and the volume made up to 10 ml. For SH, to 1 ml of this solution was added 4 ml of urea-GuHCl (8 M urea containing 5 M GuHCl in Tris-Gly) and then 0.05 ml of Ellman's reagent (4 ml of DTNB in 1 ml of Tris-Gly) was added. For SS, to 1 ml of the solution was added 0.05 ml of 2-mercaptoethanol and 4 ml of Urea-GuHCl, and the mixture was incubated for 1 h at 25°C. After an additional 1 h incubation with 10 ml of 12% TCA in Tris-Gly, the tube was centrifuged at 5000×*g* for 10 min. The precipitate was dissolved in 10 ml of 8 M urea in Tris-Gly and 0.04 ml of Ellman's reagent was added. Absorbance was measured at 412 nm on a spectrophotometer.

Rupture test In accordance with the method described in the previous report (Soeda, 1994a), gel strength ($kg \cdot m^{-2}$) and deformation (%) were measured using a rheometer (Fudo Kogyo Inc., model NRM-2002 J).

Creep test Measurement of the creep test was carried out using a creep meter (YAMADEN Inc., model RE-3305) using the same method as described in the previous report (Soeda, 1995a), and elasticity E_0, Newtonian viscosity η_N and retardation time λ were calculated using a four factors kinetic model.

Results and Discussion

Behavior of rupture test on gels The effects of MTG on gelation of SPI during cold storage were studied by carrying out a rupture test. As shown in Fig. 1, the strength of Cold-gel without MTG gradually increased with prolongation of the period of cold storage. On the other hand, the strength of Cold-gel containing one unit/g protein of MTG greatly increased after one day of cold storage, and was constant after 3 days. The strength of Cold-gel containing 3 units/g protein attained maximum after one day of cold storage, and decreased to the same level as Cold-gel containing one unit/g protein after 3 days. This decrease of gel strength might have been due to the brittleness of the gel which was induced by the excess formation of ε-(γ-Glu)Lys bonds via MTG reaction during long periods of cold storage. When treated in cold storage for one day, strength of the gels containing one and 3 units/g protein of MTG was 3.5 times and 5.5 times, respectively for that of the Cold-gel without MTG.

The strength of Heat-gel heated for 50 min at 90°C after being kept in cold storage increased in the cases without MTG and with one unit/g protein when the period of cold storage was prolonged, especially in the latter case. The Heat-gels containing 3 units/g protein did not show a big change of gel strength after being kept in cold storage.

The deformation of Cold-gel without MTG increased with prolongation of the period of cold storage. In the case of gels with MTG, the deformation of Cold-gels containing one unit/g protein increased after one day of cold storage, and decreased after 3 days. Deformation of Cold-gels containing 3 units/g protein decreased with prolongation of the period of cold storage, especially after 3 days. The deformation of the Heat-gels increased in a case without MTG and with one unit/g protein, and decreased in a case with 3 units/g protein and prolongation of the period of cold storage.

In Fig. 1, the reason that gel strength and deformation of H-gel with MTG were lower than C-gel with MTG added with units/g protein could be attributed to the more brittle characteristics of the former gel based on the excess formation of ε-(γ-Glu)Lys bonds by heat.

Behavior of creep test on gels Regarding the creep test, as shown in Fig. 2, elasticity E_0, and Newtonian viscosity η_N of the Cold-gels without MTG slightly increased as the period of cold storage was prolonged, while retardation time λ decreased.

When MTG was added to gels, elasticity E_0, and Newtonian viscosity η_N increased linearly in proportion to the increasing

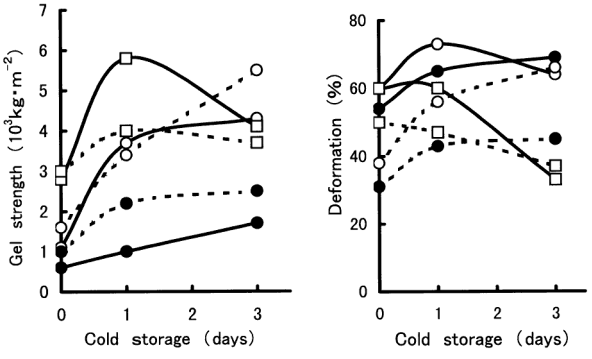

Fig. 1. Changes of texture on rupture test in gels prepared from soy protein isolate during cold storage. ●: MTG 0 unit/g protein, ○: MTG 1 unit/g protein, □: MTG 3 units/g protein. —: Cold-gel, ------: Heat-gel.

dose of MTG, with prolonged periods of cold storage. The retardation time λ of Cold-gels containing one and 3 units/g protein of MTG decreased as did Cold-gels with no MTG. On the other hand, on creep behavior of Heat-gels, elasticity E_0 and Newtonian viscosity η_N of the gels without and with MTG increased, and the retardation time λ decreased with a prolonged period of cold storage.

Optimum temperature of MTG is about 40–50°C. Thus, the creep test of the 40°C-gel was carried out by adding one and 3 units/g protein of MTG. The results are given in Fig. 3. In this figure, 40°C-gels were compared with Cold-gels kept at 5°C for one and 3 days. The creep parameters of the 40°C-gel showed the same behavior as those of Cold-gel with one unit/g protein of MTG during cold storage, and were lower than those of Cold-gel added with 3 units/g protein. It was suggested that treatment for minutes at 40°C was equivalent to that for one day of cold stor-

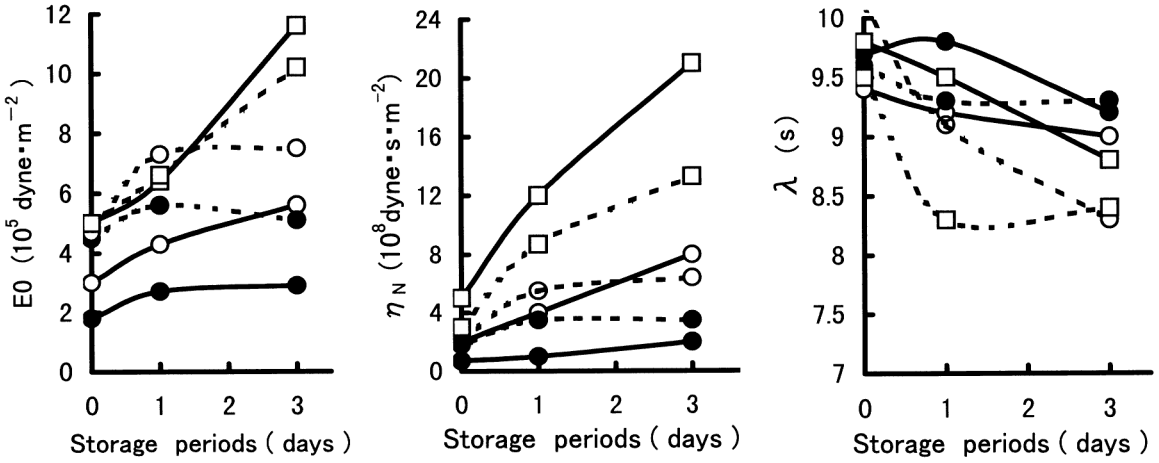

Fig. 2. Changes of texture on creep test in gels prepared from soy protein isolate during cold storage. ●: MTG 0 unit/g protein, ○: MTG 1 unit/g protein, □: MTG 3 units/g protein. —: Cold-gel, ------: Heat-gel.

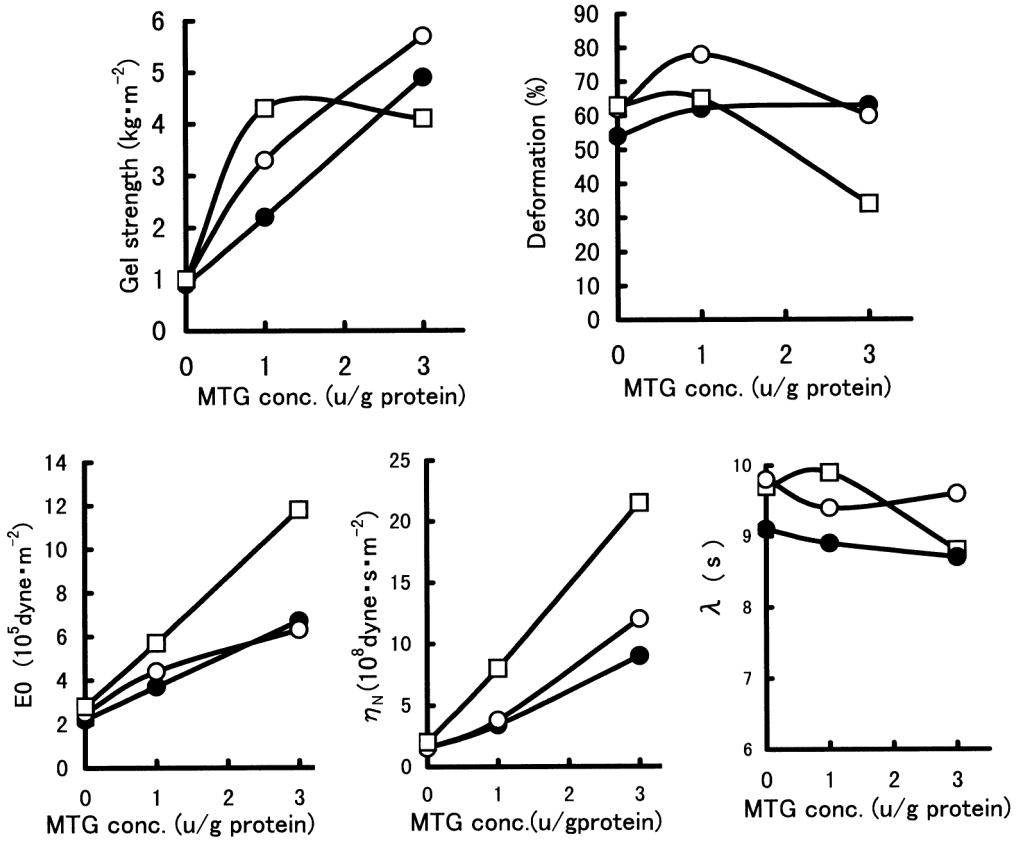

Fig. 3. Relationships of texture between 40°C-gel and Cold-gel prepared from soy protein isolate. ●: 40°C-gel treated at 40°C for 60 min, ○: Cold-gel treated at 5°C for one day, □: Cold-gel treated at 5°C for 3 days.

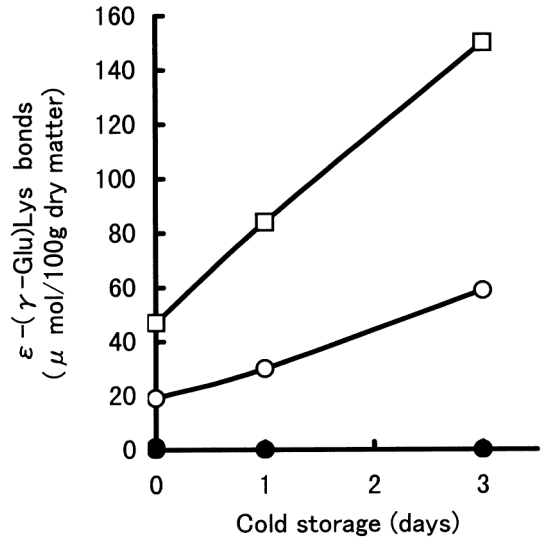

Fig. 4. Changes of ε-(γ-Glu)Lys bonds on gels stored at 5°C. ●: MTG 0 unit/g protein, ○: MTG 1 unit/g protein, □: MTG 3 units/g protein.

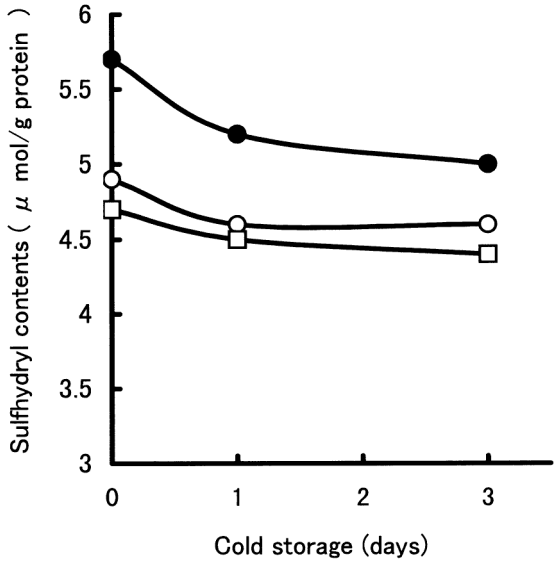

Fig. 6. Changes of sulfhydryl contents in gels stored at 5°C. ●: MTG 0 unit/g protein, ○: MTG 1 unit/g protein, □: MTG 3 units/g protein.

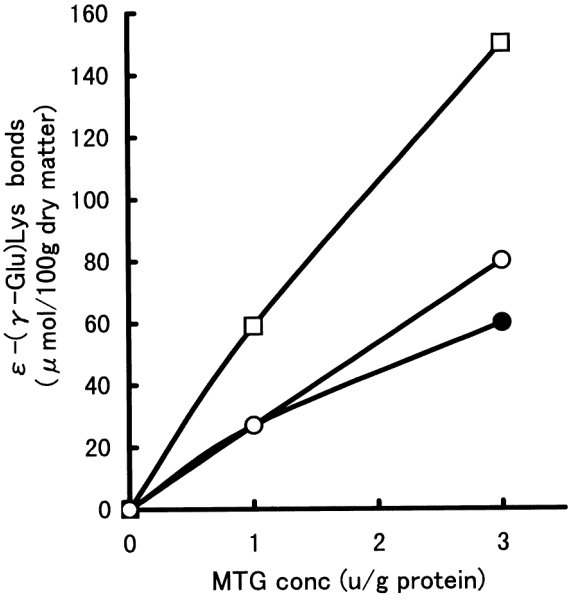

Fig. 5. Changes of ε-(γ-Glu)Lys bonds in gels stored at 5°C. ●: 40°C-gel treated at 40°C for 60 min, ○: Cold-gel treated at 5°C for one day, □: Cold-gel treated at 5°C for 3 days.

age at 5°C, on enzyme reactivity for protein substrate.

On the sensory profile, the physicochemical properties of the Cold-gels with MTG during cold storage tended to increase in hardness and tightness. It was understood that the gels hardened due to an increase in elasticity E_0 and Newtonian viscosity η_N, and became brittle due to the decrease in retardation time λ. As described in previous reports (Soeda *et al.*, 1992; Nonaka *et al.*, 1994), the changes in physicochemical properties of the gel are assumed to be due the formation of a network structure between protein molecules as a result of cross-linking of polymerization between glutamine and lysine by the action of MTG.

Content changes of ε-(γ-Glu) Lys bonds in gels As shown in Fig. 4, ε-(γ-Glu)Lys bonds were not formed in the gel with no MTG. In contrast, when MTG was added to the gel system, ε-(γ-Glu)Lys bonds linearly formed according to prolongation of the period of cold storage and in proportion to the dose of MTG. Comparision of Fig. 4 with Fig. 2 shows that the contents of ε-(γ-Glu)Lys bonds formed are in good agreement with the values of elasticity E_0 and Newtonian viscosity η_N obtained by the creep test.

For the purpose of investigating the enzyme reactivity of MTG, the contents of ε-(γ-Glu)Lys bonds were measured using gels treated for 60 min at 40°C which was the optimum temperature of MTG. As shown in Fig. 5, the contents of ε-(γ-Glu)Lys bonds in the gels obtained by treatment at 40°C for 60 min showed 31.4 μmol with the addition of one unit/g protein, and 57.4 μmol with the addition of 3 units/g protein of MTG. On the other hand, the contents of ε-(γ-Glu)Lys bonds in the gels kept in cold storage for one day at 5°C were 25.2 μmol in one unit/g protein of MTG, and 78.0 μmol in 3 units/g protein. Consequently, the enzyme reactivity of MTG for one day at 5°C was nearly equal to that in 40°C for 60 min.

Content changes of sulfhydryl groups in gels As described in the previous report (Soeda, 1995a), the decrease in contents of sulfhydryl groups in Cold-gel during cold storage was understood to be due to the formation of disulfide bonds between protein molecules. Thus, the contents of sulfhydryl groups in gels obtained with and without MTG were determined during cold storage. As shown in Fig. 6, contents of these groups were recognized to decrease during cold storage, however, the degree of decrease was smaller in gels with MTG than in those without MTG. It was suggested that MTG had less contribution to the formation of a network structure between protein molecules by disulfide bonds.

Conclusions

The effects of MTG on gelation of SPI during cold storage were studied. Gelation during cold storage was markedly promoted by the addition of MTG. Gel strength, deformation, elas-

ticity E_0, and Newtonian viscosity η_N of Cold-gels clearly increased by adding MTG during cold storage, while retardation time λ decreased. However, after 3 days of cold storage, the strength of gels containing 3 units/g protein of MTG was lower than that of gel containing one unit/g protein, and decrease in the deformation was also greater. Addition of MTG caused an increase in hardness and toughness in the mouth, as evidenced by the increased gel strength, elasticity E_0 and Newtonian viscosity η_N, and the decreased retardation time λ.

The Cold-gels with MTG were markedly greater in gel strength, elasticity E_0 and Newtonian viscosity η_N than H-gels heated after cold storage. It was suggested that gel characteristics were changed to be more rigid but brittle by the excess formation of cross-linkage between protein molecules by heat, in the case of a large dosage of MTG and/or long periods of cold storage. In the gels containing MTG, the contents of ε-(γ-Glu)Lys bonds linearly increased according to prolongation of the period of cold storage. When the dose of MTG was increased, the contents of ε-(γ-Glu)Lys bonds that had formed also increased.

With regard to physicochemical properties of the gel, the changes of elasticity E_0 and Newtonian viscosity η_N of Cold-gels as observed in the creep test were in good agreement with changes of the contents of ε-(γ-Glu)Lys bonds formed. The contents of sulfhydryl groups in the gels were found to decrease during cold storage, and the degree of decrease was smaller in the gels treated with MTG than in those treated with no MTG. This indicated less contribution of disulfide bonds in the gels treated with MTG during cold storage.

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