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Behavior of Intermolecular Bond Formation in the Late Stage of Heat-Induced Gelation of Glycinin

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The gels were prepared by heating of 10% glycinin solution at 100 °C for 1 and 20 min. Gel hardness of the latter is about 4 times that of the former. The gels were examined for the nature of their network constitution. These two gels visualized by transmission electron microscopy did not differ significantly from each other in their network structures. However, destabilization of the network structures of the gels by reagents cleaving intermolecular bonds such as 2-mercaptoethanol and urea was quite different between the gels. Our results indicate that junction points of the constituent strands within the gel network structure consist of both disulfide bonding and noncovalent bonds such as hydrophobic interaction and/or hydrogen bonding formed on subsequent heating after the initial network structure is established (at 1 min of heating), thereby stabilizing the network structure and increasing gel hardness.

INTRODUCTION

Gelling ability of soybean proteins and physical properties of the gels formed are of significance with respect to their usage in food systems. Extensive studies have been done to understand the mechanism and the basic factors involved in the gelation phenomena, which has been adequately discussed by Hermansson (1978) and reviewed by Saio and Watanabe (1978), Kinsella (1979), and Peng et al. (1984). It has been suggested that the three-dimensional network of gel structure is formed by hydrophobic interactions, hydrogen bonds, ionic interactions, and disulfide bonding (Aoki, 1965; Catsimpoolas and Meyer, 1970, 1971; Circle et al., 1964; Furukawa et al., 1979; Ishino and Kudo, 1977; Shimada and Matsushita, 1980, 1981). Recently, Babajimopoulos et al. (1983) have investigated in detail the effects of specific anions on the gelation of soybean protein in thermally reversible systems. Their results led to the conclusion that the major forces involved in stabilizing the gel network are hydrogen bonding and van der Waals interactions. On the other hand, the 11S globulin (also referred to as glycinin), one of the major components of soybean proteins, has the ability to form heat-induced gels with thermal irreversibility (Mori et al., 1982b; Utsumi et al., 1982). In previous papers we demonstrated that glycinin gels as a result of aggregation to form strands followed by interaction of the strands to form the gel network, where hydrophobic interaction and disulfide bonding are suggested to be involved as the major molecular forces driven primarily by the reactivities of the subunits in glycinin molecules (Mori et al., 1982b; Nakamura et al., 1984). When heated at 100 °C, 10% glycinin solution forms a self-supporting gel in 1 min and hardness of the gel increases with subsequent time of heating and then reaches a plateau at 15 min; gel hardness after 20 min of heating is about 4 times that after 1 min of heating (Utsumi et al., 1982). Thus, thermal gelation of glycinin may be regarded as a three-stage process, i.e., aggregation of glycinin molecules to form strands, interaction of strands to form a gel network, and increase of gel hardness probably involving concurrent stabilization of the gel networks.

In this report, we have attempted to elucidate the molecular forces involved in the change of hardness of the gels by analyzing their network structures that are formed through polymerization of glycinin molecules in the late stage of the gelation process.

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MATERIALS AND METHODS

Materials. Soybean (*Glycine max*, var. Tsuru-no-ko) was purchased from Mizuno Seed Co., Ltd. DEAE-Sephadex A-50 was purchased from Pharmacia Co., Ltd. Urea and 2-mercaptoethanol, extrapure reagents, were obtained from Nakarai Chemicals. Sodium dodecyl sulfate ($NaDodSO_4$), extrapure reagent, was obtained from Wako Pure Chemicals. Other chemicals were guaranteed reagent grade.

Preparation of Glycinin. A crude glycinin fraction was prepared from soybeans according to the method of Thanh et al. (1975). Chromatographic fractionation of the crude glycinin fraction was performed on a column of DEAE-Sephadex A-50 as described previously (Mori et al., 1979), where the column was eluted with 35 mM potassium phosphate buffer (pH 7.6) containing 10 mM 2-mercaptoethanol, 0.02% NaN_3, and NaCl in a linear gradient of 0.25–0.5 M.

Method of Gelation. The 10% glycinin solution in 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl (heating buffer) was placed in a disposable micropipet and then heated at 100 °C to prepare gels according to the procedures described previously (Mori et al., 1982a). The gels formed in the micropipets were taken out, cut into 5-mm lengths, and subjected to further experiments.

Treatment of the Gels. The gels thus prepared were placed in 12 × 90 mm test tubes to which was added 0.5 mL of heating buffer containing one of the following: no additive (buffer-1·none), 0.2 M 2-mercaptoethanol (buffer-2·MeSH), 8 M urea (buffer-3·urea), or 0.2 M 2-mercaptoethanol plus 8 M urea (buffer-4·Mix). The test tubes were shaken gently at ambient temperature for the times indicated in the appropriate figures. The supernatant liquids (solubilized material) were removed carefully by means of a pipet. The gels were then taken out and their surfaces dried with tissue. The solubilized materials and residual gels were subjected to further experiments.

Transmission Electron Microscopy. Negative Staining. A small drop of a solution of the solubilized materials was applied to a carbon-coated electron microscope grid. The grid was floated on a top of a 2% solution of potassium phosphotungstate (pH 7.0). Excess liquid was drained off with a filter paper and examined in a Hitachi H-700H electron microscope.

Thin Sectioning. Small pieces of the gels, without or with subsequent treatment, were fixed in 2% glutar-

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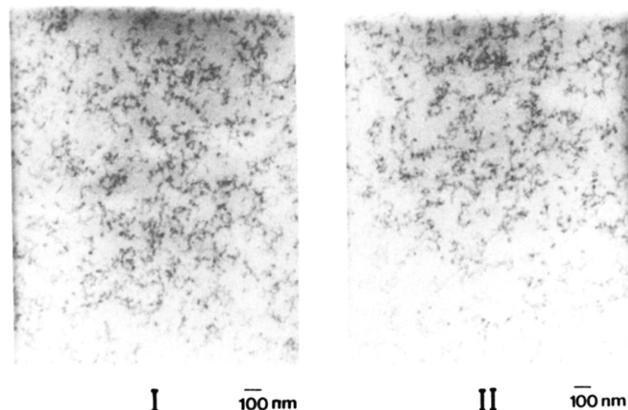

Figure 1. Transmission electron micrographs of glycinin gels: I, heated for 1 min (gel-1); II, heated for 20 min (gel-20).

aldehyde at pH 7.2 (50 mM sodium phosphate buffer) and 4 °C for 2 h and then in 1% osmium tetraoxide at pH 7.2 (0.1 M sodium phosphate and 0.1 M sucrose) and 4 °C for 1.5 h. Ethanol dehydration was then performed with a series of increasing ethanol concentrations, and the gel was embedded by using a mixture of propylene oxide and epon resin. The gel block was sectioned, and thin sections were stained with uranyl acetate and lead citrate and finally viewed under a Hitachi H-700H electron microscope.

Electrophoresis. The solubilized materials obtained as described above were analyzed by polyacrylamide gel electrophoresis (PAGE) and $NaDodSO_4$–PAGE following the methods of Davis (1964) and Laemmli (1970), respectively. Electrophoreses were performed in 6.5% and 10% polyacrylamide gels, and the gels were stained with amido-black 10B as described previously (Mori and Utsumi, 1979). Fifty micrograms of protein was used for electrophoresis.

Determination of Gel Hardness. Hardness of the gels, without or with subsequent treatment, was measured with a texturometer (General Food Corp., GXT-2) and expressed as texturometer units (kgw). Details of the procedure have been described previously (Mori et al., 1982a; Utsumi et al., 1982).

Protein Determination. Protein was determined by the method of Lowry et al. (1951). When the samples contained 2-mercaptoethanol, protein was determined by Lowry's method as modified by Ross and Schatz (1973).

RESULTS AND DISCUSSION

Gels were prepared by heating 10% glycinin solutions for either 1 min (gel-1) or 20 min (gel-20). Figure 1 shows a micrograph at a magnification of 30 000× of ultrathin sections of glycinin gels. The network structures of gel-1 and gel-20 consisted mainly of similar thin strands. No significant difference of the pattern of networks was observed between the two gels, whereas the gels differ from one another in hardness (Utsumi et al., 1982). This result suggests that the molecular forces involved in stabilizing network strands change with time of heating both qualitatively and quantitatively and thereby cause the increase in gel hardness. Therefore, effects of denaturants such as urea in combination with 2-mercaptoethanol on destabilization of gel network and its constituent strands and on the hardness of gels were investigated.

Figure 2 shows the solubilization of the gels in buffers 1–4 (see Materials and Methods). In the case of treatment with buffer-1·none, both gel-1 and gel-20 gave a low degree of solubilization (around 10%) and retained their shapes. When gel-1 and gel-20 were treated with either buffer-2·MeSH or buffer-3·urea, solubilization differed greatly.

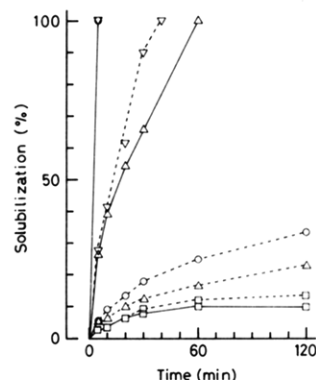

Figure 2. Effects of urea and 2-mercaptoethanol on solubilization of glycinin gels. For each treatment the amount of protein of the solubilized materials was determined as described in the text and expressed as the solubilization in percent of total protein of the gel used: (□) buffer-1·none; (○) buffer-2·MeSH; (△) buffer-3·urea; (▽) buffer-4·Mix. Key: —, gel-1; - - -, gel-20.

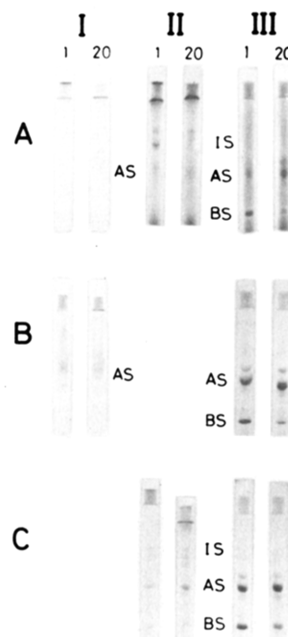

Figure 3. PAGE and $NaDodSO_4$–PAGE of the solubilized materials. Treatment of glycinin gel (vertical): (A) buffer-1·none; (B) buffer-2·MeSH; (C) buffer-3·urea. Condition of electrophoresis and glycinin gel samples (horizontal): (I) PAGE; (II) and (III) $NaDodSO_4$-PAGE in the absence and presence of 2-mercaptoethanol, respectively. Key (1) and (20), gel-1 and gel-20, respectively; IS, intermediary subunits in which the acidic and basic subunits are linked by disulfide bridges in a 1:1 ratio; AS and BS, acidic and basic subunits of glycinin, respectively. Migration is from top to bottom.

Gel-1 was completely solubilized by treatment with either buffer-2·MeSH for 5 min or buffer-3·urea for 60 min, while in the case of gel-20 the solubilization was 33% and 22% by treatment for 2 h with buffer-2·MeSH and buffer-3·urea, respectively, where the gel still kept its shape. On treatment with buffer-4·Mix for 40 min gel-20 was completely solubilized.

Figure 3 shows analyses by PAGE and $NaDodSO_4$–PAGE of the solubilized materials obtained as described in Figure 2 by treating the glycinin gels for 2 h. In the case of treatment with buffer-1·none, the solubilized material of gel-1 consisted of an aggregate that did not migrate into the stacking gel plus traces of free acidic subunits that were barely detected, while that of gel-20 was composed of an aggregate that migrated into the stacking gel but not into

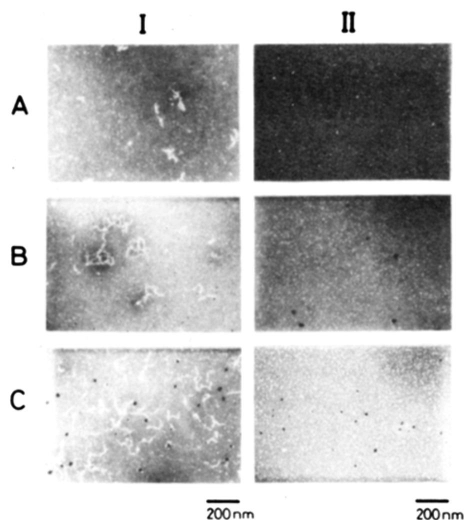

Figure 4. Transmission electron micrographs of negative staining of solubilized materials. Treatment of glycinin gel (vertical): (A) buffer-1·none; (B) buffer-2·MeSH; (C) buffer-3·urea. Glycinin gel samples (horizontal): (I) gel-1; (II) gel-20.

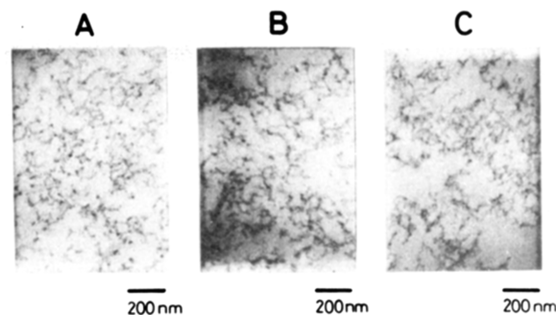

Figure 5. Transmission electron micrographs of thin sections of gel-20 after leaching with buffer-1·none (A), buffer-2·MeSH (B), and buffer-3·urea (C).

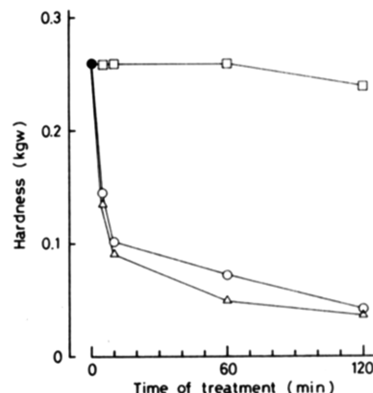

Figure 6. Effects of buffer treatments on hardness of glycinin gels. Treatment of the gel: (●) none; (□) buffer-1·none; (○) buffer-2·MeSH; (△) buffer-3·urea. Gel hardness was measured immediately after the gels were treated for indicated times.

the separating gel and traces of free acidic subunits (Figure 3, A-I). The aggregate in gel-1 consisted of the acidic and basic subunits, while the aggregate in gel-20 was composed mainly of acidic subunits (Figure 3, A-III). On treatment with either buffer-2·MeSH or buffer-3·urea gel-1 was completely solubilized as described above; however, the resultant solubilized materials consisted primarily of aggregates staying at the top of stacking gel (Figure 3, B-I and C-II). The aggregates consisted of acidic and basic subunits (Figure 3, B-III and C-III) as expected. The solubilized materials of gel-20, in buffer-2·MeSH (Figure 3, B-I and B-III), gave an electrophoretic pattern similar to that in buffer-1·none (Figure 3, A-I and A-III), while those in buffer-3·urea also contained the basic subunits to some extent (Figure 3, C-II and C-III). All the aggregates staying at the tops of the stacking and separating gels were found to contain disulfide and/or hydrophobic bondings (compare the electrophoretic patterns horizontally). From the results of gel electrophoretic analyses it is inferred that, in gel-1, the networks are formed through association by either disulfide or hydrophobic bondings of some of the aggregates to such a size that stays on top of the stacking gel and a part of the aggregates exists in the free form; the forces involved in the association of aggregates appear to be comprised of both types of bondings during the course of subsequent heating for 20 min resulting in stabilization of the network structure. It is also suggested that the aggregates staying on the top of separating gel consisted of acidic subunits preferentially formed and liberated from the network strands during the course of heating. Gel-1 was completely solubilized by treatment with buffer-2·MeSH (Figure 2), and the solubilized materials disaggregated in the presence of $NaDodSO_4$ (Figure 3, B-I-1 and B-III-1). This indicates that the contribution of hydrogen bondings in stabilization of the network structure of gel-1 may be negligible, since $NaDodSO_4$ is known to destabilize hydrophobic interaction but not hydrogen bonding.

Figure 4 shows micrographs at a magnification of 60 000× of negative staining of the solubilized materials similar to those described in Figure 3. In the case of treatment with buffer-1·none, strands with varying sizes were visible in the solubilized material of gel-1, while strands were not seen but small dots alone in that of gel-20 (Figure 4, A-I and A-II). On treatment with either buffer-2·MeSH or buffer-3·urea, both the strands and the fragments of network structure were visible in the solubilized material of gel-1 (Figure 4, B-I and C-I), while no such structures was seen in that of gel-20 (Figure 4, B-II and C-II). The strands seem to correspond to the entities with a molecular weight of 8×10^6, which are constitutive units of the network structure (Nakamura et al., 1984). The results of electron microscopic analysis are consistent with those of gel electrophoretic analysis and substantiate the inferences described there. It is likely from the results shown in Figure 4 (B-I and C-I) that the complete solubilization of gel-1 by either of the buffers is due to severance of the bondings by which the strands associate to form the network structure; the forces involved in the formation of the strands and partly in the association of strands within the network fragments seem to be comprised of both disulfide and hydrophobic bondings. However, in the case of gel-20, the results shown in Figure 4 did not reveal whether such severance of bondings did not occur or did so only to a limited degree. In order to clarify this point, the gels remaining after leaching of gel-20 with the buffers 1–3 for 2 h were examined for a network structure by microscopy at a magnification of 60 000×. As shown in Figure 5, the patterns of networks of the unsolubilized gels revealed no significant differences from the untreated one shown in Figure 1. This may indicate that the forces involved in the formation of the strands and their associations within the networks of gel-20 consist of both types of bondings, which could prevent destruction of the networks upon severance of one of the bondings by treatment with either 2-mercaptoethanol or urea. Apparently both bonds need to be broken simultaneously to disrupt the networks.

On the other hand, as shown in Figure 6, gel hardness decreased remarkably by treatment with either 2-

mercaptoethanol or urea, while treatment with buffer-1-none did not cause any change of gel hardness. The shapes of the gels were not changed significantly by the treatments. The decrease of gel hardness may be due to severance of the bonds involved in the structure of the gel networks by 2-mercaptoethanol or urea. The partial solubilization of gels may not be a major factor contributing to the change of gel hardness, since the change in gel hardness with time of the treatment did not parallel that found in solubilization of the gels shown in Figure 2. It is not clarified here whether either or both of noncovalent bonds, hydrophobic interactions and hydrogen bondings, contributes in stabilization of the network structure of gel-20, since urea destabilizes both types of bondings.

From the results obtained here, it is deduced that intermolecular bondings, mainly disulfide bondings and noncovalent bonds, probably hydrophobic interactions, and/or hydrogen bondings proceed within the strands of networks on subsequent heating after the gel network has once been established, and then the junction points of constituent strands within the gel network are formed through further formation of both types of bondings. Thus, stabilization of the gel network structure and concomitant increase of the gel hardness may occur in the late stage of the thermal gelation process of glycinin.

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High-Performance Liquid Chromatographic Method for the Quantification of Cholesterol Epoxides in Spray-Dried Egg

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A simple and sensitive method for the quantification of cholesterol epoxides in egg products was developed by using reversed-phase high-performance liquid chromatography (HPLC). This method involved the addition of total lipids to a disposable column containing silica to obtain the cholesterol fraction and the subsequent derivatization with *p*-nitrobenzoyl chloride prior to separation for HPLC. Cholesterol α- and β-epoxides were found in commercial spray-dried egg at levels about 0.1% and 0.2% of cholesterol, respectively.

Cholesterol is known to be susceptible to air oxidation by a free-radical mechanism similar to unsaturated fatty acid. There have been extensive studies on the autoxidation products of cholesterol (Smith, 1980). Among such products, 5α-cholestane-5,6α-epoxy-3β-ol (α-epoxide) has been suggested to be involved in carcinogenesis (Bischoff, 1969; Black and Douglas, 1973; Reddy and Wynder, 1977; Parsons and Goss, 1978; Kelsey and Pienta, 1979, 1981). Sevanian and Peterson (1984) have recently found that α-epoxide is a direct-acting mutagen in mammalian cells. This oxygenated cholesterol seems to be formed in vivo or incorporated as a food contaminant. Bowden et al. (1979) reported that a small percentage of α-epoxide and its metabolites was found in a wide variety of organs when administered to mice by gastric intubation, although most of the epoxide was excreted in the feces. Therefore, attention should be paid to the content of α-epoxide in foods (Shepard and Shen, 1980).

Spray-dried egg is a popular foodstuff containing a high concentration of cholesterol. Chicoye et al. (1968) detected 5β-cholestan-5,6β-epoxy-3β-ol (β-epoxide) in photooxidized spray-dried egg. Tsai and Hudson (1984) isolated α- and β-epoxides from egg products after the spray-drying process. However, no quantitative analysis of each epoxide in egg products has yet been carried out, although the contents of total epoxides in cheese and butter oil were recently estimated by thin-layer chromatographic analysis (Finocchiaro et al., 1984). Tsai et al. (1980) proposed the use of high-performance liquid chromatography (HPLC) with a differential refractometer for the resolution of α- and β-isomers in egg products, but the sensitivity was not high. In this experiment, we developed a reversed-phase HPLC analysis for the quantification of α- and β-epoxides through their *p*-nitrobenzoyl derivatives (Watabe et al.,

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