Relationships of Molecular Forces to Rheological and Structural Properties of Legumin Gels from Broad Beans

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Rheological and structural properties of legumin gels, treated with various reagents, were studied. The gels were hardened by propylene glycol and ethanol, but softened by 2-mercaptoethanol, NaSCN, and formamide. The gel was completely dissolved by 8 M urea, but soluble aggregates, structural units of the gel network, remained. A combination of NaSCN with either 2-ME or formamide did not dissolve gels completely, but significantly decreased gel hardness. Treatments with 2-ME and urea decreased elastic parameters and viscous parameters, respectively. These results indicated that disulfide bonds play a role in stabilization and promote the elasticity of legumin networks, while hydrogen bonds and hydrophobic interactions predominate in maintaining the structure and increasing the viscosity.

The gelling ability of proteins and physical properties of the gels are of significance with respect to their use in food systems. Gels have been defined as three-dimensional networks of cross-linked polymeric molecules.\(^1\) The texture of many foods depends upon the gel network structures of proteins. Molecular forces are the main factors that affected the gel network structures. The effects of salts, reducing agents, and denaturants on the thermal gelation have been studied to explain the molecular forces involved in the gelation process of soybean proteins.\(^2\) These results indicated that the stabilization of gel network structures is related to the interactions between protein molecules such as the hydrophobic interactions and ionic, disulfide, and hydrogen bondings. In these studies almost all of the tests done on the physical characteristics of gels were measures of compression. Information on the relationship of viscoelastic and/or mechanical properties of gels to molecular interactions is lacking. Understanding the effects of molecular interactions on rheological properties would provide valuable information for the texture control of many proteins.

Gelation mechanism and gel microstructure of 11S globulin from broad beans were studied previously.\(^3\) The results showed that legumin formed a gel through formation of strands, their juncture forming network-like structures and self-supporting gel structures. Also, it was shown that molecular forces involved in polymerization of globulin molecules were mainly disulfide bonds and hydrophobic interactions.\(^4\) Mori et al.\(^5\) assumed that the disulfide and noncovalent bonding proceeds within the strands of networks on subsequent heating after the gel network has once been established. Then the junction points of constituent strands within the gel network are formed through further formation of both types of bonding. Therefore, it is important to explain the behavior of intermolecular bonding after a gel network has been established because it would provide valuable information for controlling the texture of protein gels in food systems.

In this study, we attempted to explain the molecular forces involved in the maintenance of gel network structures and their relationship to rheological properties of legumin gels by using compression tests and creep measurements.

Materials and Methods

Materials. Seeds of Vicia faba L. (var. Sanuki Nagasaya) were purchased from Takii Seed Co., Ltd. All chemicals used were reagent grade. Purified legumin was prepared from broad bean seeds as described previously.\(^6\)

Preparation of gel. A 12.5% legumin solution was injected into a silicone mold (4 mm both in diameter and length) filled up, degassed, and then the mold was sealed by covering with a glass plate on both sides and by clamping with clips. The mold containing the legumin solution was heated in a water bath of 100°C for different experimental times, followed by rapid cooling to room temperature by immersing in tap water. Then, the covering was removed, and the cylindrical gel in the mold was taken out for analysis.

Treatment of gels by reagents. The cylindrical gels were immersed in 5 ml of a 35 mM potassium phosphate buffer (pH 7.6) containing various concentrations of reagents or reagent combinations for 20 h at 20°C. After treatment, the gels were taken out from the buffer and gel hardness was measured with a Rheonener RE-3305 (Yamaden Co., Ltd.) as described previously.\(^7\)

Gel-permeation chromatography (GPC) measurement. GPC measurement was done using high performance liquid chromatography (HPLC) (Shimadzu LC-4A) with a TSK gel G4000SW column. Elution was done at a flow rate of 0.5 ml/min at room temperature. The eluent was monitored at a wavelength of 280 nm with a Shimadzu Spectrophotometric Detector SPD-2A.

Creep measurement. Creep measurement under compression at 5 mm/s (cross-head speed) was done with a Rheonener RE-3305 interfaced with a computer (PC-9801NS/E, NEC, Japan). The elevation of the plate was continuously adjusted to produce a constant stress of 10 g force, and the displacement of the gel after the initial stress was recorded. For recovery measurements, the position of the holder was adjusted to produce zero stress. Creep and recovery were each measured for 5-minute intervals. The creep curves were analyzed by the procedure of Shama and Sherman,\(^8\) and Kamata et al.\(^9\) Linearity between stress and strain was maintained during these measurements. Creep measurements were repeated six times or more for each sample at 20°C.

Transmission electron microscopy. Negative staining was done as described previously.\(^10\)

Protein measurement. Protein was measured by the method of Lowry et al.\(^1\)

Results and Discussion

The 12.5% legumin solution formed a gel at 10 min, hardened on subsequent heating and the hardness reached
a plateau at 30 min. The gel microstructures of different heating times seen by SEM are similar, i.e., a cellular structure in which fibrous and sheet-like structures are interconnected (data not shown). This indicated that the increase of hardness with heating time may be due to changes in molecular bonding involved in network structures. To clarify this point, the effects of various reagents on the hardness of gels prepared by heating for 10 min (gel-10), 25 min (gel-25), and 60 min (gel-60) were investigated.

When the gels were treated with increasing concentrations of 2-mercaptoethanol (2-ME), the hardness decreased concomitantly and reached a plateau at concentration above 5 mM (Fig. 1). At the concentration of 10 mM, the hardness of gel-10, gel-25, and gel-60 decreased to 25, 26, and 31% of their initial hardness, respectively. A similar decrease in gel hardness was also observed when 2-ME was added before heating for gelation. The decrease in gel hardness might be due to a cleavage in the intermolecular disulfide bonding involved in the gel network by 2-ME. These results suggested that disulfide bonding is involved in the stabilization of gel network structures. In the case of soybean glycycin, a gel was not formed in the presence of 2-ME at concentrations over 10 mM. When a 10% glycycin gel (1 min heated) was treated with 2-ME, it was completely solubilized, and the 20 min-heated gel showed a solubilization of 33%. As described above, legumin gels heated for either 10 min or 60 min still kept their shape in the 2-ME treatment, although the gel hardness decreased. This fact indicated that the contribution of disulfide bonding in the maintenance of gel network structures was less in legumin than in glycycin. This presumption was consistent with the result that legumin has fewer cysteine residues than glycycin. Comparing the hardness of three gels at 10 mM 2-ME concentration, the longer the heating time, the harder the gel (Fig. 1). This may indicate that the noncovalent bonding involved in stabilizing network structures proceeds on subsequent heating after the initial gel network has been established.

The involvement of noncovalent bonding in stabilizing the network structure of gel was investigated by using three reagents, NaSCN, formamide, and propylene glycol (PG). The hardness decreased with increasing concentrations of NaSCN, and reached a plateau at a concentration over 0.2 M (Fig. 2). NaSCN weakened the hydrophobic interactions. Hence, hydrophobic interactions may be involved in the maintenance of legumin gel network structures. Formamide, which can form very strong hydrogen bonds with the imide group, can break apart intra/intermolecular hydrogen bonds. As shown in Fig. 3, the hardness of gels decreased dramatically with increasing concentrations of formamide. At a concentration over 40%, the gels became fragile ones, whose hardness was less than 15% of that of the control. It is worth noting that the effects of formamide on the hardness of gel were more dramatic than that of NaSCN. This showed that hydrogen bonding is more important than hydrophobic interaction for stabilizing networks. On the other hand, in the presence of PG, the hardness of the gels increased markedly (Fig. 4). Ethanol also had a similar hardening effect with PG on legumin gels (data not shown). Alcohols weaken electrostatic interaction by lowering the dielectric constant and promote hydrogen bonding. Treatment with NaCl had little effect on the hardness of gels (data not shown), suggesting that contributions of electrostatic and
ionic interactions were limited. These results regarding the effect of several reagents on gel hardness indicated that hydrogen bonding was the main force involved in the maintenance of gel network structures of all noncovalent bonding.

It is known that urea destabilizes both hydrophobic interactions and hydrogen bonds. The hardness of gels was decreased greatly by treatment with increasing concentrations of urea, and finally the gels were completely dissolved in 8 M urea buffer (Fig. 5). This indicated that hydrophobic interactions and hydrogen bonds behave cooperatively to maintain legumin gel network structures against the solubilization. In the case of glycmin, a gel that was formed by heating for 20 min still kept its shape in the 8 M urea buffer. This difference may be due to the degree of covalent bonding, such as disulfide bonding within gel network structures. In other words, more disulfide bonds were formed in glycmin gel structures than in the legumin. Again, this result was consistent with the fact that glycmin has more sulphydryl and disulfide residues than legumin.

Gel filtration patterns with HPLC showed that the dissolvable materials of the gel-10 by 8 M urea are in an aggregate form as shown in the inset of Fig. 5. Same results were also observed in the case of gel-25 and gel-60. Figure 6 shows micrographs at a magnification of 5000 × of negative staining of these soluble materials. In the dissolved materials of gel-10, straight strands with rough surfaces were visible (Fig. 6A). In that of gel-25, entanglement and elongation of strands were apparent; straight strands were also observed (Fig. 6B). In the gel-60, network-like structures were visible; straight and branched strands were also observed (Fig. 6C). The results shown in Fig. 6 demonstrated that the longer the heating time, the larger the soluble aggregates. The molecular forces involved in these aggregates would be disulfide bonds, because these aggregates disappeared and dissociated into acidic and basic subunits in the presence of 2-ME (data not shown). This indicated that the intermolecular cross-linking through disulfide bonding continued to proceed between the constituent strands within gel network structures, and that this cross-linking may be one of the reasons for the hardening of legumin gel during heating. A complete breakdown of gels was also caused by the treatment of formamide plus 2-ME (Table I). This means that breaking hydrogen bonds and disulfide bonds is necessary to disrupt the networks. Complete solubility of gels by either urea or

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Hardness (g·w)</th>
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<tbody>
<tr>
<td></td>
<td>Gel-10</td>
</tr>
<tr>
<td>Control</td>
<td>18.6</td>
</tr>
<tr>
<td>0.5 M NaSCN + 50% formamide</td>
<td>2.6</td>
</tr>
<tr>
<td>0.5 M NaSCN + 20% PG</td>
<td>30.4</td>
</tr>
<tr>
<td>0.5 M NaSCN + 10 mM 2-ME</td>
<td>4.5</td>
</tr>
<tr>
<td>50% formamide + 10 mM 2-ME</td>
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</table>

Fig. 5. Effects of Urea Treatment on Hardness of 12.5% Legumin Gels as a Function of Concentration. Symbols are the same as in Fig. 1. The insertion shows the HPLC pattern of solubilized material.

Fig. 6. Transmission Electron Micrographs of Negatively Stained Materials. A, B, and C correspond to the soluble materials of gel-10, gel-25, and gel-60, respectively.
formamide plus 2-ME implied that the main role of hydrogen bonding is in maintenance of gel network structures. Disulfide bonding and hydrophobic interaction may also play a complementary role.

A combination of other reagents did not dissolve gels completely, but did affect gel hardness. A combination of PG with NaSCN resulted in an increase of gel hardness as compared with that of the control but it was lower than that of gel treated by PG alone (Table I) (compare with Fig. 4). This result also suggests that hydrogen bonding is important, and that hydrophobic interactions complement hydrogen bonding partly in the maintenance of gel network structures. A combination of NaSCN with 2-ME significantly decreased gel hardness (Table I) compared with gel that was treated by these reagents separately (Figs. 2 and 3). This result indicates that the breaking of hydrophobic interaction and SS bonding severely decreases the hardness of gels. From the results of these treatments using several reagents, it is clear that disulfide bonding, hydrophobic interactions, and hydrogen bonds cooperatively contribute to the stabilization of gel network structures of legumin. In particular, the hydrogen bonds are important.

Creep curves of the gels were measured and their viscoelastic parameters were calculated using a six-element mechanical model to examine the difference in viscoelastic properties of gels treated by several reagents. The model consisted of one Hookean body (one spring, $E_0$), two Voigt bodies (two spring, $E_1$, $E_2$ and two dashpots, $\eta_1$, $\eta_2$) and one Newtonian body (one dashpot, $\eta_N$). The viscoelastic constants for gel-10 showed the values of $2.90 \times 10^8$, $1.62 \times 10^6$, $2.68 \times 10^8$ dyn/cm², $7.12 \times 10^7$, $1.47 \times 10^9$, and $6.62 \times 10^8$ poise for $E_0$, $E_1$, $E_2$, $\eta_1$, $\eta_2$, and $\eta_N$, respectively. On subsequent heating, up to 60 min, the parameters increased to the values of the control (Table II). The results indicated that the elastic property was strengthened, and the difficulty of perpetual fluidity (caused by sliding between strands) was increased because of heating. The time-dependent increase of the parameters may be related to intermolecular bonding involved in the stabilization of gel networks. The treatment of the gel-60 with 2-ME decreased its viscoelastic constants (Table II). Similar decreases were also observed with treatment with either NaSCN or urea (Table II). Upon treatment with formamide, the viscoelastic constants except $\eta_N$ also decreased (Table II). The increase of $\eta_N$ by formamide treatment means that the breaking of hydrogen bonds damaged the elastic and viscoelastic parts but not the viscous parts. Change in viscoelastic parameters by 2-ME treatment was different from those with treatments with the other reagents. In spite of the smaller effect of 2-ME on gel hardness than formamide, the 2-ME treatment was more effective in decreasing elastic parameters ($E_0$, $E_1$, and $E_2$) than formamide. Elastic parameters of gels treated by NaSCN or urea were also higher than those of gels treated by 2-ME. This indicates that disulfide bonding seems to contribute significantly to the elastic properties of gels. The $\eta_1$, $\eta_2$, and $\eta_N$ of gels treated by urea were lower than those treated by 2-ME. The $\eta_1$ and $\eta_2$ of gels treated by NaSCN or formamide were also lower than those treated by 2-ME. This suggested that noncovalent forces contribute to the viscous properties of gel networks.

In conclusion, covalent cross-linking (disulfide bonds) plays a role in stabilization and promotes the elasticity of legumin gel networks, while noncovalent forces (hydrogen bonds and hydrophobic interactions) predominate in maintaining the structure and increasing the viscosity. These covalent and noncovalent bonds are probably formed by subsequent heating after a self-supporting gel has been established. This might relate to textural properties of the gel.

### References


### Table II. Effects of Various Reagents on Viscoelastic Parameters of Gels

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>2-ME (mm)</th>
<th>NaSCN (m)</th>
<th>Formamide (%)</th>
<th>Urea (m)</th>
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<tr>
<td>$E_0$ ($\times 10^3$ dyn/cm²)</td>
<td>3.32</td>
<td>3.03</td>
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<td>2.21</td>
<td>2.79</td>
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<td>$E_1$ ($\times 10^3$ dyn/cm²)</td>
<td>2.95</td>
<td>2.79</td>
<td>2.28</td>
<td>2.06</td>
<td>2.34</td>
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<tr>
<td>$E_2$ ($\times 10^3$ dyn/cm²)</td>
<td>4.25</td>
<td>3.38</td>
<td>3.14</td>
<td>2.12</td>
<td>3.28</td>
</tr>
<tr>
<td>$\eta_1$ ($\times 10^3$ poise)</td>
<td>9.12</td>
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<td>8.61</td>
<td>7.94</td>
<td>8.51</td>
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<tr>
<td>$\eta_2$ ($\times 10^3$ poise)</td>
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<td>1.39</td>
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<tr>
<td>$\eta_N$ ($\times 10^9$ poise)</td>
<td>7.64</td>
<td>7.41</td>
<td>7.00</td>
<td>6.75</td>
<td>7.46</td>
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Hardness (g·w) 59.4 50.2 41.3 33.0 50.0 44.3 36.9 56.0 48.1 30.0 34.7 29.8 25.7