Relationship between the Thermal Denaturation and Gelling Properties of Legumin from Broad Beans

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The effects of NaCl and heating temperature on the gel-forming ability of legumin were studied. The addition of NaCl progressively increased the denaturation temperature of legumin. Heating to around the denaturation point, i.e., below the onset temperature (zone 1), between the onset and maximal temperatures (zone 2), between the maximal and final temperatures (zone 3), and above the final temperature (zone 4), affected both the gel-forming ability and gel properties. No gel was formed in zone 1, while the gel was harder in zone 3 than in zones 2 and 4. The gel hardness gradually decreased with increasing temperature in zone 4. Differences in the viscoelastic and microstructural properties between gels heated at various temperatures around the denaturation point were observed.

The gelation process in proteins by heating involves denaturation of the proteins followed and subsequent aggregation of denatured proteins, resulting in the formation of a gel network structure. Calorimetric and optical techniques have been used for studying the denaturation step, and these studies provide basic information about the thermal stability of each protein in different environments. Such information on thermal stability is very useful for determining the proper heat conditions (heating temperature, ionic strength, etc.) and for predicting or explaining the gelation behavior of proteins. For example, the effect of heating temperature on the aggregation or network formation of proteins has been investigated for glycine,5 sesame x-globulin,6 bovine serum albumin,7 and ovalbumin8,9 on the basis of their DSC data. From these results, it is generally accepted that a gel will be formed by heating protein to roughly above its denaturation temperature. No more attention was paid to the effect on gelation of a small difference in heating temperature around the denaturation point. However, it is possible that an alteration to the heating temperature around the denaturation point would affect the network structure and physical properties of a gel. If this is true, changing the temperature could be a useful means by which to control the textural characteristics of protein gels. The denaturation temperature and gelation behavior of proteins are both affected by several factors (the concentration of neutral salts, type of salts, added detergents, etc.). The effect of NaCl concentration on the thermal gelling properties of proteins has been extensively investigated,10 because NaCl can be used as an ingredient in food manufacturing, unlike some neutral salts and detergents. It was found that the addition of NaCl radically affected the denaturation temperature.11 The NaCl concentration also influenced protein–protein interactions, the formation of network structures and the physical properties of gels.12 Several reports have demonstrated an optimum NaCl concentration to produce the maximum gel hardness for whey protein,13 egg protein,9 and oat globulin.14

In the previous study,15 the process for the denaturation-aggregation of legumin from broad beans was examined. It was expected that this process would also be affected by NaCl concentration, as soybean glycine and oat globulin are. In this study, the effect of NaCl concentration on the thermal denaturation of legumin was investigated. The physical properties and microscopic structure of legumin gels formed at different temperatures around denaturation point were also examined in order to learn the relationship between the heating temperature and gel properties.

Materials and Methods

Materials. Seeds of Vicia faba L. (var. Sanuki Nagasaya) were purchased from Takii Seed Co. (Japan). All chemicals used were of reagent grade.

Preparation of legumin. A partially purified legumin fraction was prepared according to the method reported by Suchkov et al.16 with some modifications. The partially purified legumin fraction was further purified in a column of DEAE-TOYOPEARL as described previously.17

Differential scanning calorimetry (DSC). The purified legumin in the eluting buffer was thoroughly dialyzed against a 35mM potassium phosphate buffer (pH 7.6) containing various concentrations of NaCl. DSC was conducted with SEIKO DSC2000 equipment. Fifty microliters of the legumin solution were sealed in silver pans, and an empty pan was used as a reference. The heating rate was 1°C/min over a temperature range of 20 to 120°C. The extrapolated temperature for the onset of denaturation (T onset), peak temperature (T p), final temperature (T f) and enthalpy were systematically computed from the thermograms.

Preparation of the gels. A 12.5% legumin solution was injected into an aluminum cup and heated at 100°C for various lengths of time in a water bath. Details have been described previously.17 The gel hardness was measured with a Rheoner RE-3305 (Yamaden Co.) as described previously.18

Creep measurement. Creep measurements under compression at a 5mm/s cross-head speed were performed with the Rheoner RE-3305 (Yamaden Co., Japan) linked to a personal computer (PC-9801NS/E, NEC, Japan). The creep curves were analyzed according to the procedure described by Shama and Sherman,18 and Kamata et al.19 Linearity between stress and strain was maintained during these creep measurements, which were repeated at least six times for each sample at 20°C.

Scanning electron microscopy (SEM). A small piece of gel was fixed in 2% glutaraldehyde at pH 7.2 and 4°C for 2h, and then in 1% osmium tetroxide at pH 7.2 and 4°C for 1h. After fixation, the gel was dehydrated by immersing in ethanol with a series of increasing concentrations, and at the critical point, it was dried with CO2. Each dried sample was mounted
on an aluminum stub and coated with gold, the specimens being observed with a Hitachi S-450 scanning electron microscope.

Protein determination. Protein was determined by the method of Lowry et al.\textsuperscript{20}

Results and Discussion

By using various NaCl concentrations, DSC was used to investigate the denaturation behavior of legumin. As shown in Fig. 1, $T_o$, $T_a$, $T_f$, and enthalpy increased progressively with increasing NaCl concentration. At an NaCl concentration higher than 0.8 M, legumin had a denaturation temperature higher than 100°C. These results clearly indicate that NaCl stabilized the quaternary and tertiary structure of legumin, and thereby probably protected the protein against denaturation. A similar result has also been observed for glycinein,\textsuperscript{11} although the thermal stability of glycinein is lower. As shown in Fig. 2, the NaCl concentration had a profound effect on the gel-forming ability of 12.5% legumin at 100°C. The gel hardness increased gradually with increasing NaCl concentration up to a maximum of 0.5 M, and then decreased significantly with further addition of NaCl. No gels could be obtained at an NaCl concentration above 1.5 M. Gel formation is governed by the equilibrium between attractive (hydrogen bonding, hydrophobic interaction, and disulfide bonding) and repulsive (electrostatic) forces among thermally altered molecules.\textsuperscript{21} NaCl has a charge-shielding effect,\textsuperscript{12} and the suppression of ionic repulsion at higher NaCl concentrations would enhance protein-protein interaction and the formation of a stable gel network. However, an NaCl concentration above 0.5 M may cause extensive and intensive protein-protein interaction, which would result in the formation of an irregular aggregate structure. This may lead to the collapse of protein matrices with syneresis.\textsuperscript{14}

In addition to the effect of NaCl, the relationship between the heating temperature and denaturation temperature may also have a crucial effect on gel-forming ability. The addition of 1.5 M NaCl increased $T_o$ and $T_a$ of legumin to 101.5°C and 108°C, respectively. This means that the experimental heating temperature at 100°C was below the denaturation point and, naturally, no gel was formed. Therefore, the relationship between the heating temperature and gel-forming ability was examined more precisely. Legumin solutions containing 0.2, 0.4, and 0.8 M NaCl were heated for 60 min at various temperatures (Fig. 3). From the DSC curve, four temperature zones were designated: below $T_o$ (zone 1), between $T_o$ and $T_a$ (zone 2), between $T_a$ and $T_f$ (zone 3), and above $T_f$ (zone 4), respectively. At a 0.2 M NaCl concentration, legumin gave values of 87.8, 93.7, and 98.8°C for $T_o$, $T_a$, and $T_f$, respectively (Fig. 3A). When the legumin was heated in zone 1, no gel was obtained (Fig. 3A). In zone 2, a gel was formed, and its hardness increased with increasing temperature toward $T_o$. The hardest gel was obtained in zone 3. In zone 4, the gel hardness progressively decreased with increasing temperature. Similar results were also observed in the case of legumin containing 0.4 and 0.8 M NaCl (Figs. 3B and C). These results clearly indicate that the gel formation and its properties were very dependent on

Fig. 1. Effect of NaCl Concentration on the Thermal Denaturation Temperature.
- $T_o$, onset temperature; $T_p$, peak temperature; $T_f$, final temperature; $\Delta$, enthalpy.

Fig. 2. Effect of NaCl Concentration on Gel Hardness.
The 12.5% legumin solution was heated at 100°C.

Fig. 3. Effect of Temperature on Gel Hardness Related to the Denaturation Temperature of Legumin.
NaCl concentration: (A) 0.2 M, (B) 0.4 M, and (C) 0.8 M. ---, DSC curves; $\cdots$, gel hardness.
small differences in heating temperature around the de-
naturation point. Figure 2 shows that the 12.5% legumin
containing 0.1 M NaCl formed a softer gel when heated at
100°C, the reason for this being that the heating tempera-
ture (100°C) in zone 4 (Fig. 1). On the other hand, legumin
containing 0.5 M NaCl at 100°C was in zone 3, where the
hardest gel was obtained. When legumin was heated with
1.0 M NaCl at 100°C (zone 2), a soft gel was formed. In
the case of 1.5 M NaCl at 100°C, in temperature zone 1, no
gels could be produced from the legumin. The maximum
gel hardness of legumin containing 0.2, 0.4, and 0.8 M
NaCl was with 0.4 M NaCl concentration. This result
indicates that the gel-forming ability was governed by the
NaCl concentration as well as by the heating temperature
already mentioned.

In order to confirm the effect of heating temperature on
the physical properties of legumin gels, typical creep and
creep recovery curves for gels containing 0.2 M NaCl heated
in different temperature zones, 90°C (zone 2), 95°C (zone
3), and 105°C (zone 4), were investigated as shown in Fig.
4. The 90°C gel had the highest creep compliance, in-
dicating that the gel became soft and more deformable.
Differences in the properties of these three gels were also
clearly observed by the residual strain after removing the
stress. The 95°C gel yielded the lowest value of residual
strain, while the 90°C gel had the highest value. This
suggests that the gel formed at the temperature in zone 3
(95°C) was more rubber-like and elastic, while that in zone
2 (90°C) was likely to have been more like plastic and less
elastic.

The creep curves obtained were analyzed by the six-
element mechanical model shown in the inset to Fig. 4,
the parameters \(E_0, E_1, E_2, \eta_1, \eta_2, \) and \(\eta_n\) being calculated
as listed in Table. The results shows that the viscoelastic
properties of gels are related to the heating temperature.
The 95°C gel had higher values for the viscoelastic pa-
rameters than those of the 90°C and 105°C gels. This means
that the gel formed in zone 3 (95°C) was the most visco-
aelastic. By comparing the viscoelastic parameters of the
90°C gel with those of the 105°C gel, it was noted that the
90°C gel had higher values for viscous parameters \(\eta_1, \eta_2,\) and \(\eta_n\) while the 105°C gel had higher elastic modulus
values \(E_0, E_1,\) and \(E_2\) (Table). These results suggest that
the gel formed in zone 4 (105°C) was more elastic, while
that formed in zone 2 (90°C) was more viscous. Similar
results were also found in the case of legumin gels con-
taining 0.4 and 0.8 M NaCl. These results demonstrate
that the viscoelastic properties of legumin gels changed
with heating temperatures around \(T_d\). Therefore, a
small change in temperature around the denaturation
point of legumin could be a useful means for controlling
the textural characteristics of legumin gels.

The effect of heating temperature on the microstructure
of legumin gels is shown in Fig. 5. The 90°C gel had a
granular structure (Fig. 5A), clumps of aggregated legumin

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Fig. 4. Relationships between the Heating Temperature and Creep
Compliance in Terms of the Six-element Model.
Heating temperature: ·····, 90°C; ——, 95°C; ——, 105°C.

Fig. 5. Scanning Electron Micrographs of Legumin Gels at Various Heating Temperatures.
(A) 90°C; (B) 95°C; (C) 105°C. The NaCl concentration was 0.2 M.
molecules being observed, but no fibrous or sheet-like structures existing. The 95 and 105°C gels had quite different microstructures when compared to the 90°C gel, showing a cellular structure in which fibrous and sheet-like formations were interconnected (Figs. 5B and C). However, the structure of the 95°C gel was more regular than that of the 105°C gel. This indicates that a heating temperature above \( T_c \) caused disorder in the legumin gel structure, and demonstrates that differences in the gel microstructure are related to the rheological properties. The 95°C gel, which had a regular network structure, had the highest viscoelastic properties (Table) and gel hardness (Fig. 3A). The 90°C gel had no fibrous structures and its data gave with the lowest values for the elastic parameters (Table) and gel hardness (Fig. 3A). Although somewhat irregular, the fibrous structure of the 105°C gel contributed to the higher values of elastic parameters when compared to the 90°C gel. The differences in structural regularity between the 95 and 105°C gels appears to have significantly influenced the values for their viscoelastic parameters. These results show that the fibrous and sheet-like structures characteristic of the 95°C gel (Fig. 5B) were responsible for viscoelasticity, and particularly for the elastic properties of gels. On the other hand, an aggregated or granular structure (Fig. 5A) contributed to a decrease in gel elasticity. The results show that fibrous and granular structures of ovalbumin gels have high and low elasticity, respectively, as has already been reported.\(^{22}\)

The foregoing results show that the addition of NaCl increased the denaturation temperature of legumin, and that the rheological and structural properties of a legumin gel closely depended on the heating temperature. In the gelation process of legumin, molecules associate to form strands and then a network structure by intermolecular forces, \( i.e., \) hydrophobic interaction and disulfide bonding.\(^{20}\) However, the main forces involved in the gelation process would have been from hydrophobic interaction, because legumin contains a single free SH residue,\(^{23}\) so that the formation of intermolecular SS bonding was limited. Conformational change and exposure of the hydrophobic regions by heating was essential for the subsequent association of legumin molecules.\(^{15}\) Heating in zone 3 (between \( T_d \) and \( T_c \)) could cause a conformational change in the legumin molecules, which would lead to the formation of a regular fibrous structure to result in higher elasticity and a harder gel. On the other hand, heating in zone 2 (between \( T_c \) and \( T_d \)) might not be enough for a complete conformational change of the legumin molecules and subsequent strand formation. This is probably why the gel formed in zone 2 exhibited less elasticity. The irregular fibrous structure of the gel formed in zone 4 would be one reason for it being less viscoelastic than the one formed in zone 3. In order to explain the effect of heating at a temperature around the denaturation point on the network structure and physical properties of gels more clearly, other proteins should also be studied.

In conclusion, it was demonstrated that a change in heating temperature around the denaturation point (zones 1 to 4) clearly affected the network structure and physical or viscoelastic properties of a legumin gel. These findings might be useful when selecting appropriate conditions for controlling a particular texture in protein gels.

References