Relationships of Secondary Structure, Microstructure, and Mechanical Properties of Heat-induced Gel of Soy 11S Globulin

Yi-Chang Ker, Rong-Huei Chen, and Ching-Shyong Wu

Department of Marine Science, National Taiwan Ocean University, Keelung 20224, Taiwan, R.O.C.

Received July 8, 1992.

Secondary structure, microstructure, and mechanical properties of heat induced 11S globulin gel were studied to discover their relationships. Heat-induced 11S globulin gel at 80, 90, and 95°C were comprised of 7.2, 16.6, and 23.8% of z-helix; 19.4, 19.5, and 27.5% of $\beta$-sheet; and 73.5, 64.5, and 48.6% of random coil, respectively. This indicated the gel formed at higher temperatures contained more z-helix and less random coil structures. Micrographs of gels heated at 90 and 95°C had a more extended and integral matrix. Gel strength of heat-induced gels at 90 and 95°C were significantly greater than that of 80°C. These data indicated that the increase in $\alpha$-helix of heat-induced 11S globulin gel have facilitated the establishment of a good gel matrix.

Most food products consist of complex physico-chemical systems in different geometrical forms, fibrous, cellular, amorphous, crystalline etc., which are influenced by the thermodynamic instability of protein structures. A slight difference in enthalpy will change protein structure. It provides an enormous avenue to improve the physical properties in protein foods. Soy 11S globulin is a major component of soybean storage protein and has excellent heat-induced gel properties. However, the relationships among the three-dimensional structure of gel matrix, secondary structure, and mechanical properties are not clear.

The evaluation of the relationship between microstructure and physical properties have been studied by SEM microscopic observation and mechanical tests. However, SEM currently has a maximum resolution of 15 nm, it is limited to looking at the gross structure of large fibers or fibrous bundles. The technique of X-ray diffraction is a good tool to measure the molecular level of protein structure. It has been extensively used for structural analysis in single crystals, crystalline powders, oriented films, and fibers. However, the structure of processed protein system is not orderly enough to use X-ray diffraction.

The optical rotary dispersion (ORD) or circular dichroism (CD) method are two more molecular-level tools in measuring the secondary structure. However, the sample is limited to the liquid state. For gelled products, the sample was solid. The secondary structure of gels would be changed if the sample was dissolved with solvent. Therefore, the applications of ORD and CD are also limited. Also, the conformational changes in dilute aqueous solution are quite different to that of gel formation during or after the heat process. Therefore, it needs a method which could measure the molecular level structure in the solid state.

Fourier transform infrared (FTIR) spectroscopy has been widely applied to measure the secondary structures of proteins in aqueous and solid state. To compare with a known protein, the resolved components are used to measure the secondary structure. However, the procedure still has some problems and caveats. Alternatively, following the theoretical mode frequency of Amide I and Amide II of polypeptides and the mirror image of the deconvoluted and second derivative of deconvolute spectrum, the coupling peaks in Amide I and Amide II regions were used to measure the secondary structure. It was used to analyze the secondary structure of the solid state in an 11S globulin gel. It avoided empirical observation and curve fitting.

The objectives of this study are to use FTIR as a tool to measure the change of the secondary structure of 11S globulin in response to gel formation and to correlate the changes in secondary structure to the microstructure and the mechanical properties.

Materials and Methods

Preparation of soybean 11S globulin. The soybean 11S globulin fraction was purified by selective thermal denaturation and solubility in a high ionic strength envelope. The 11S globulin ratio was greater than 95%. It was lyophilized and stored in a desiccator until use.

Preparation of the gels. Five milliliters of 10% 11S globulin (lyophilized 11S globulin (weight)/ionic strength 0.5, pH 7.6 phosphate buffer (volume)) was piped into a 16 × 100 mm borosilicate glass disposable culture tube and heated at 80, 90, or 95°C for 30 min. Then, the heated 11S globulin was immediately cooled down with ice for 2 h. The heat-induced gel was lyophilized for FTIR spectroscopy measuring or for the SEM observation. On the other hand, for measuring the mechanical properties, gelation was done by placing the solution inside a 10-ml beaker with the same heating procedure. Then, the different heat-induced gels inside the beakers were used directly to measure the mechanical properties.

Fourier transform infrared analysis. A Boman DA3002 FTIR spectrometer with a Hg-Cd-Tc detector was used for the experiment. A piece of lyophilized heat-induced 11S globulin gel without severe grinding (about 2 mg) was homogeneously mixed with KBr (about 10 mg), and then pressed to form a pellet. All the pellet was transparent except the sample area; it was installed in the sample cell. Before the scanning, the spectrometer was purged with liquid nitrogen and then scanned 200 times. Spectral resolution was set in 2 cm⁻¹. Difference spectra were generated by digital subtraction of a spectrum of blank KBr recorded under the same conditions as the sample spectra.

Fourier self-deconvolution was done with the technique of Kauppinen et al. a method for resolving intrinsically overlapped bands. In the primary experiment, the parameters of self-deconvoluted spectra and second derivative of deconvoluted spectra procedure conducted in k of 2.4, a resolution enhancement, and s of 6.5 cm⁻¹, the half width at half height of the chosen line shape function were appropriately used to separate the overlapped peaks. Where the peaks displayed in the deconvoluted spectra (since function), was identical with the spectra shown in the...
downward spectra of the second derivative of deconvolution (cosine function). After the peak deconvolution and coupling, the deconvoluted peaks position and half-width of the peak were used directly to measure the secondary structure.

Scanning electron microscopy. Heat-induced gels were fixed, dehydrated, and observed by the method previously described in Chen et al. On the other hand, a sample without 3% glutaraldehyde fixing was also used to observe the microstructure as a comparison. The gelled materials were directly frozen at −40°C following by freeze drying. A clump from each dried sample was mounted on an aluminum stub using epoxy glue, and coated with a gold-platinum alloy using a sputter coating device. Scanning electron micrographs were obtained using a Hitachi 550 microscopy.

Mechanical properties. Gel strength was measured from the force required to penetrate the gel (deformation at break) with a 0.5 cm disc diameter probe installed in an Sun Rheometer (CR-2000). All experiments were done in duplicate. Measurements on samples from each experiment were done in triplicate. Values were averaged and standard deviations were calculated for those presented in a graphical format.

Results and Discussions

1. Changes in secondary structure analysis by FTIR

Figure 1 illustrated the Amide I and Amide II of undeconvoluted spectra and deconvoluted spectra of heat-induced 11S globulin gel at 80, 90, and 95°C. The frequencies of deconvoluted spectra contributed to the order structure (α-helix and β-sheet) are listed in Table. The peaks presented (Fig. 1) showed that some features were different from those seen in the aqueous system, where the model proteins used were low molecular weight and simple-structured proteins, such as bovine serum albumin, ribonuclease A or B, lysozyme, carbonic anhydrase, or α-chymotrypsinogen. However, the structure of 11S globulin contains 12 subunits and has a high molecular mass of 350 kDa. It was a complex and compact protein.

Fig. 1. The Amide I and Amide II Spectra of Heat-induced 11S Globulin Gel at Various Temperatures.

A and B, the spectra of second derivative of deconvolution and deconvolution of 80°C one, respectively; C, the spectra of deconvolution of 90°C; D and E, the spectra of deconvolution and undeconvolution of 95°C, respectively.

Fig. 2. Changes in Secondary Structure of Heat-induced 11S Globulin Gel.

Table

<table>
<thead>
<tr>
<th>Conformation</th>
<th>Mode</th>
<th>80</th>
<th>90</th>
<th>95</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amide I</td>
<td>Amide II</td>
<td>Amide I</td>
</tr>
<tr>
<td>α-Helix</td>
<td>(V_1(0))</td>
<td>1651.5</td>
<td>1515.0</td>
<td>1651.8</td>
</tr>
<tr>
<td></td>
<td>(V_2(2\pi/n))</td>
<td>1647.8</td>
<td>1540.1</td>
<td>1648.5</td>
</tr>
<tr>
<td>Antiparallel</td>
<td>(V_1(0, \pi))</td>
<td>1530.2</td>
<td>1683.3</td>
<td>1530.1</td>
</tr>
<tr>
<td>β-sheet</td>
<td>(r(\pi, \pi))</td>
<td>1669.2</td>
<td>1668.7</td>
<td>1666.5</td>
</tr>
<tr>
<td></td>
<td>(r(\pi, 0))</td>
<td>1631.2</td>
<td>1628.7</td>
<td>1630.8</td>
</tr>
</tbody>
</table>

* The positions of wavenumber are verified by self-deconvolution spectra and second derivative of deconvolution spectra, which are printed out directly from the computer program.

* The selecting of the coupling wavenumber combination are referred to by Miyazawa and Blout (1961) in ±2 wavenumber.
Therefore, the different features, especially frequencies that were not found in model protein, might be due to intra- or inter-chain interaction. The results also indicated that the molecular vibrations of protein in solid are different from aqueous systems.

Theoretically calculated frequency of Amide I (from 1630 to 1687 cm⁻¹) and Amide II (from 1514 to 1552 cm⁻¹) of polypeptides has been used by Chen et al. to study and measure the relative ratio of secondary structure in the solid protein. They reported the native 11S globulin contained 7.9, 28.3, and 63.8% of α-helix, β-sheet, and random coil respectively, reaffirming the 11S globulin is a relatively low order structure and a relatively high random coil structure, which indicated the method used was feasible. The relative ratio of secondary structure of constituent molecules of gels was calculated and plotted in Figure 2. The percentages were 7.2, 16.6, and 23.8% of α-helix; 19.4, 19.5, and 27.5% of β-sheet and 73.5, 64.5, and 48.6% of random coil for gel prepared at 80, 90, and 95°C, respectively. It showed that gel prepared at higher temperatures of 90 or 95°C, contained more ordered structure (α-helix and β-sheet) and less random coil. Where the increase in α-helix was mainly in the increase of vibration mode \( V_{ii}(0) \), which appeared at 1650, 1516 cm⁻¹ coupling wavenumber. The result was consistent with the band frequency around 1650 cm⁻¹ assigned to discover the characteristics of a high helix protein, e.g., hemoglobin.

The secondary structure of native 11S globulin was

Fig. 3. The SEM Micrograph of Heat-induced 11S Globulin Gel with Fixation by Glutaraldehyde (A, 95°C; B, 90°C; C, 80°C) and without Fixation (D, 95°C; E, 90°C; F, 80°C)
reported to be 7.9, 28.3, and 63.8% \(^{19}\) or 5.2, 34.8, and 60% \(^{30}\) or 6, 40, and 54% \(^{31}\) of \(\alpha\)-helix, \(\beta\)-sheet, and random coil, respectively. Basically, the native 11S globulin without any treatment, is a low order and highly unordered structured protein (Fig. 2). After the heating treatment at 80°C, the \(\alpha\)-helix kept constant, the \(\beta\)-sheet decreased by roughly the same amount as the increase of random coil (Fig. 2). It implied that the ordered structure of \(\beta\)-sheet was destroyed and converted to random coil at 80°C. As the temperature increased to 90°C, the \(\beta\)-sheet was still lower than that of native, but was almost equal to that of 80°C. The \(\alpha\)-helix increased to about 10% more than the native, or 9% more than that of 80°C. But, the amount of random coil decreased to that of the native one, where the decrease was at about the same magnitude of the increase of \(\alpha\)-helix. Therefore, this implied that the increase of \(\alpha\)-helix was at the expense of the random coil. As temperature further rose to 95°C, the content of ordered structure of \(\alpha\)-helix and \(\beta\)-sheet both went up. In contrast, the random coil went down. It implied that the increase in \(\alpha\)-helix and \(\beta\)-sheet were converted from random coil.

2. Changes in microstructure

The corresponding microstructure is shown in Figs. 3a–c (fixed by glutaraldehyde) or Figs. 3d–f (without fixing). It showed that the micrographs were different with or without fixing treatments. However, the micrographs were no different with and without fixing treatments if the gel matrix formed at 90 and 95°C. The microstructure of gels formed at 90 and 95°C were well extended and integral, only containing some aggregates. However, the gel formed at 80°C had a lot of aggregated structure. Additionally, the gel strength of heat-induced gel at 90 and 95°C were greater than that of 80°C (Fig. 4). Especially at 95°C the gel strength was significantly higher.

The denaturation temperature of 11S globulin in ionic strength 0.5, pH 7.6 phosphate buffer was reported to be 89.6°C. \(^{32}\) Therefore, for 90 and 95°C, the temperature above the denaturation temperature resulted in a significant difference compared to 80°C. Furukawa et al., \(^{33}\) reported the gel hardness of aqueous soy isolate paste increased linearly with heating temperature. Aoki \(^{34}\) also reported an increase of gel strength and chewiness with the heat treatment of isolate protein paste. The phenomena of gelation was described as a two-stage process of initial denaturation of native protein into unfolded polypeptides, then gradual reassociation of those polypeptides to form the ordered gel matrix. \(^{35,36}\) Therefore, the soft gel formed at 80°C was due to the unfolding of the molecules being not yet adequate. The harder gel formed at temperatures above 90°C was due to the reassociation of the more extended unfolded molecules to form a more homologous gel matrix. The results was also accordant with the phenomena reported by Heertje and Klee \(^{37}\) in which an ovalbumin/water gel prepared at pH 10 or in a urea solution had a uniform and homologous microstructure, with the network formation occurring via flexible, unfolded protein chains.

3. The relationships among the secondary structure, microstructure, and gel strength

The relationships among the secondary structural changes (Fig. 2), microstructure (Fig. 3), and gel strength (Fig. 4) of heat-induced 11S globulin gel showed that the higher the temperature (90 and 95°C), the higher the homologous and extended network matrix were obtained, and the higher the gel strength resulted. Therefore, a stronger gel structure was formed by the molecules containing the more ordered structure of \(\alpha\)-helix and a larger axial ratio or stretched structure. Thus heating treatments transformed the unordered structure of random coil to the ordered structure of \(\alpha\)-helix. It made intermolecular entanglement easier, \(^{19}\) causing a well-formed microstructure. It implied that the ordered structure of \(\alpha\)-helix had a good relationship to the gel three-dimensional matrix. The results were in accordance with the report of Iwabuchi and Shibasaki, \(^{38}\) they reported that the ordered structure, especially \(\alpha\)-helix, is related to the formation of a three-dimensional structure in the gels.
obtained by alkali–alcohol treatment. On the other hand, for the 80°C one, the gel was soft, because the gel was constituted from molecules with a small axial ratio and a high percentage (63.8%) of random coil. Both results indicated that the shape of constituent 11S globulin still remained partially in the globular shape. Thus, there were no sufficient functional groups (especially the internal disulfide bonds which were buried in the interior) to interact. The results also suggested that the mechanism of gelation at 80°C was distinguished from that of 90 and 95°C.

Additionally, the shearing is another crucial factor on the conformation of 11S globulin during gel formation. The syneresis effects of shearing and temperature have been reported to have a significant influence on improving the formation of ordered structure when the ordered structure (α-helix and β-sheet) of sheared 11S globulin at 80 and 90°C, respectively, was 45 and 57%. Especially, the α-helix increased to 20 and 28% from 7.9% when sheared at 80 and 90°C, respectively. On the other hand, in this study, the ordered structure of 11S globulin at 80 and 90°C (without shearing) was 26.6 and 36.1%, respectively. It was lower than that of the sheared ones. The results reconfirmed that the shearing offered a strong driving force for stretching and aligning the unfolded molecules. The results also presented that the mechanism of formation of heat-induced gels is discriminated from that of shear/heat-induced gels.

4. The possible mechanism of coil–helix transition of 11S globulin
The torsion angles of φ and ψ of a protein basically determine the α-helix and β-sheet formation which was allowed only in some restricted domains as illustrated by the Ramachandran steric contour. For poly-L-glycine, the allowed domains was reported to be larger than poly-L-alanine and appear in each quadrant because the hydrogen atom of poly-L-glycine causes less steric hindrance than a methyl group of poly-L-alanine, indicating the allowed domain of φ and ψ is reduced not only by the intermolecular interactions but also by the adjacent units’ dependency rotation angles. For soy 11S globulin, the structure was found to be a compact globular protein, but, the actual torsion angles of φ and ψ are unknown, therefore, the actual secondary structure are still unknown.

Protein structure can be predicted from the amino acid sequence, although there are some restrictions and difficulties. However, based on local, short-range interactions and Chou and Fasman theory, protein secondary structure can be predicted. From the data of 15 protein crystal structures, Figs. 5a, b, and c show the frequency of α-helical, β-sheet, and random coil residues with their conformation parameters $P_α$, $P_β$, and $P_C$. From these parameters the structure tendencies were predicted. For example, with $P_α > 1$, there is a greater than average tendency to adopt the α-helix conformation, but $P_α < 1$ means a less than average frequency of occurrence of the α-helix conformation for that residue. The data showed a good agreement with the reports of the CD or X-ray methods. It also indicate the Glu, Ala, and Leu residues are favorable to the formation of α-helix, while Val, Met, and Ile residues are favorable to the formation of β-sheet. On the other hand, the Gly, and Pro residues are unfavorable to the formation of periodic structure and are favorable to the formation of random coil. For Soy 11S globulin, the secondary structure was reported to be composed of about 6% α-helix, 34% β-sheet, and 60% random coil. Therefore, theoretically it should contain more of the residues of Gly,
Pro, Tyr, and Asn, and few residues of Ghu, Ala, Leu, and His. However, from the amino acid composition reported (Figs. 5d-e) the result was just the opposite. The reason may be that the soy 11S globulin contains 23 mol of cystine, which restrict the formation of α-helix due to the steric hindrance even though the original amino acid residues are favorable to the formation of α-helix. The postulation is further supported by the result reported in Chen et al., 19, 22, which indicate the increase of α-helix content as the molecule is treated with heat and shearing. It implied that heat and/or shearing changed or removed the steric hindrance imposed by inter- and intra-disulfide bond interaction and then change the torsion angles of φ and ψ. Therefore, as the temperature rose to above the denaturation temperature such as 90 or 95°C, the energy supplied to the solution was high enough to split the disulfide bonds, which significantly increased the content of α-helix.

Acknowledgments. Appreciative thanks for the financial support from the National Science Council, Republic of China, Project No. NSC 79-0406-E019-04, and also thanks for the use of the fourier transform infrared spectroscopy in their instrument center.

References