Analysis of the State of Aromatic Amino Acid Residues in Heated Soybean 7S Globulin by Absorption Derivative Spectrophotometry and Spectrofluorimetry

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The heating of 7S globulin caused changes in the intensities, but hardly affected the positions of the peaks and troughs of the second derivative absorption spectra at wavelengths below 270 nm. On the other hand, above 271 nm, changes were reflected both in the intensities and in the positions of peaks and troughs. The difference-second derivative absorption spectra indicated that 60 and 70 percent, respectively, of phenylalanine and tyrosine residues buried in the native 7S globulin remained as the buried form even after heating.

A spectrofluorimetry and fluorescence-quenching study suggested that one residue of tryptophan in the 7S globulin tended to be transferred to the more hydrophobic interior on heating.

Heating contributes to the orientation of texture in a food system.1~4) Sulphydryl-disulfide interchange plays a great role in the formation of texture.5~7) Eleven S globulin, one of the major proteins in the soybean, contains more sulphydryl and disulfide residues than 7S globulin. Thus, the former was used as a model protein and its thermal aggregation was examined previously.8~11) Umeya et al.12) indicated the difference of hardening phenomena between 7S and 11S globulins: the apparent viscosity of 7S globulin increased considerably in the cooling process after heating, but that of 11S globulin scarcely changed. Japanese traditional foods such as tofu, soybean curd and aburage, fried soybean curd, are produced by heat processing. Saio et al.13) indicated the difference of physical properties (texture etc.) between foods made from 7S globulin and those from 11S globulin.

In order to investigate such differences, the thermal denaturation of 7S globulin, as well as of 11S globulin, needs to be investigated. We report here studies on the state of aromatic amino acid residues in the heated 7S globulin involving absorption-derivative spectrophotometry and spectrofluorimetry.

MATERIALS AND METHODS

Materials. The Raiden variety of soybean was used throughout this work. Defatted meal was prepared from ground soybeans by defatting with n-hexane and removing the solvent at room temperature. All reagents were of the highest grade. Sepharose 6B was obtained from Pharmacia Fine Chemicals.

Preparation of 7S globulin. Thanh's crude 7S globulin14) was subjected to ammonium sulfate fractionation and the precipitate was thoroughly suspended in 0.01 N HCl containing 0.6 M NaCl15) using a magnetic stirrer. After centrifugation of the suspension at 15,000 rpm for 30 min, the supernatant was dialysed against a phosphate buffer of pH 7.6 and 0.5 M ionic strength (standard buffer, hereafter, means this buffer solution). The dialysate was concentrated by ultrafiltration and gel-filtrated on a column (4 × 142 cm) of Sepharose 6B equilibrated with the standard buffer. The concentration of 7S globulin in the standard buffer was determined spectrophotometrically by using the value ε_1 cm^2 = 5.4716 (at 280 nm).

Procedure for heat treatment. Heating studies were carried out by the method described in the previous paper.8)

Denaturation with urea. Proteins were denatured by treatment with urea as described in the previous paper.10)

Measurement of tryptophanyl fluorescence. Fluorescence measurements were carried out with a Hitachi model...
MPF-4 spectrofluorimeter. All fluorescence measurements were made by using an excitation wavelength of 295 nm. The absorbance at 280 nm of samples examined never exceeded 0.1. Fluorescence-emission-quenching by KI was measured as described in the literature.17)

**Instruments.** Second derivative absorption spectra and fluorescence spectra were measured by the methods described in the previous paper.10)

**RESULTS**

The second derivative absorption spectra of heated 7S globulin

In the second derivative spectrum of native 7S globulin in the standard buffer, there were 6 peaks (detected as positive signals, \(+ d^2I/dx^2\) at 253, 258, 264, 268, 279 and 289 nm, and troughs (detected as negative signals, \(- d^2I/dx^2\) at 251, 256, 262, 266, 271 and 281 nm.

The second derivative absorption spectra of the heated 7S globulin (solid line in Fig. 1) showed that the optical densities at below 270 nm changed, but the positions of peaks and troughs scarcely did. With increased heating time, the optical density of phenylalanine residues in the region of 250 to 270 nm decreased. On the other hand, the optical densities of tyrosine and tryptophan residues above 271 nm changed little with an increase of heating time, but the positions of peaks and troughs were blue-shifted.

Treatment of 7S globulin heated for various times with 6 M urea caused a blue-shift in all wavelength regions measured, a decrease in optical densities in the region of 250 to 270 nm, and an increase in the region above 271 nm (broken line in Fig. 1). The second derivative absorption spectrum of heated 7S globulin in the presence of 6 M urea was similar to that of native 7S globulin in the presence of 6 M urea.

The difference-second derivative absorption spectra of heated 7S globulin

The two samples for difference-direct and second derivative absorption spectrophotometry, native and heated proteins, were placed in the reference and the sample compartment, respectively (Fig. 2). After increasing the heating time, 7S globulin showed the denaturation blue-shift, although the spectra tended to be disturbed by turbidity. Negative peaks appeared at 280.5 and 289.5 nm (the spectra are not shown). The correspond-
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Fig. 2. Difference-Second Derivative Spectra of Heated 7S Globulin.

The concentration of 7S globulin was $3.29 \times 10^{-6}$ M. The derivative wavelength difference, $\Delta \lambda$, was 5 nm. Native and heated protein were used for the measurement, as the reference and sample, respectively. Periods of heating were: A, 0.5; B, 1; C, 5; D, 10; E, 30 min. The spectra of the protein heated for 0.5 and 1 min were measured with five fold the sensitivity for those of the protein heated for 5, 10, 30 min. The heating temperature was 100°C.

ing peaks in the difference-second derivative absorption spectra to the negative peaks appeared at 280 and 289 nm, respectively.

Fig. 3. Difference-Second Derivative Spectra of Heated 7S Globulin Denatured with Urea.

The concentration of 7S globulin was $4.02 \times 10^{-6}$ M. The derivative wavelength difference, $\Delta \lambda$, was 5 nm. Heated protein and heated protein denatured with 6 M urea were used for the measurement, as the reference and sample, respectively. Periods of heating were: A, 0.5; B, 1; C, 5; D, 10; E, 30 min. The heating temperature was 100°C.

The two samples for difference-second derivative absorption spectrophotometry, heated protein without and with denaturation with 6 M urea, were placed in the reference
The concentrations of 7S and 11S globulin were $4.32 \times 10^{-6}$ M and $3.83 \times 10^{-6}$ M, respectively. Solid line, derivative spectrum; broken line, zero order spectrum. A, 11S globulin; B, 7S globulin. The derivative wavelength difference, $\Delta \lambda$, was 5 nm. Native protein and native protein denatured with 6 M urea were used for the measurement, as the reference and sample, respectively.

and the sample compartment, respectively (Fig. 3). Difference-zero order absorption spectra showed that native (Fig. 4) and heated 7S globulin (data not shown) showed the typical blue-shift on urea denaturation. The same negative peaks appeared at 281 and 288.5 nm as reported by Fukushima.\textsuperscript{18} The corresponding peaks in the difference-second derivative absorption spectra to the negative peaks appeared at 279.5 and 288 nm. With an increase of heating time, the negative peak at 279.5 nm did not shift, but that at 288 nm did, to 287 nm. The derivative difference intensities ($d^2 I/d\lambda^2$) of peaks in the spectra of heated proteins in the presence of urea were less than those of native protein in the presence of urea. That their intensities depend on the degree of exposure of buried aromatic amino acid residue caused by heating will be considered in the discussion.

**Tryptophanyl fluorescence measurement of heated 7S globulin**

Native and heated 7S globulin were excited at 295 nm and their emission curves were obtained (data not shown). The wavelength of the peak fluorescence of native 7S globulin was 334 nm. With an increase of heating time, the wavelength of heated 7S globulin blue-shifted slightly. The relative fluorescence intensities were measured at each maximum wavelength, using the fluorescence intensity of native 7S globulin in the standard buffer as a reference. Table I shows that heating caused its intensity to increase slightly.

**Quenching of the tryptophanyl fluorescence of native and heated 7S globulin by solute perturbation with the iodide ion**

In the present study, KI was used as a quencher of tryptophanyl fluorescence. A modified version of the Stern–Volmer law was calculated for a heterogeneous distribution of fluorophors in a protein ($F_0/I = 1/f_0 K_0[I] + 1/f_0$).\textsuperscript{17} For the studies of thermal denaturation of 7S globulin, plots of $F_0/I$ vs. $1/[I^-]$ were made to determine the effective Stern–Volmer quenching constants ($K_0)_{eff}$ and values for the fractional accessible fluorescence ($f_0)_{eff}$. Relative fluorescence intensities at the wavelength of the peak fluorescence were measured, using the fluorescence intensities of native 7S globulin in the standard buffer (pH 7.6) as a reference. For this system, it is assumed\textsuperscript{17} that the quantum yield is proportional to the peak fluorescence, since the spectral shape (emission spectra) does not vary significantly even though there are small wavelength shifts (data not shown), and the difference of absorbance at 295 nm is small enough to be neglected.

Plotting the data according to the modified Stern–Volmer relationship resulted in a linear
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Fig. 5. Modified Stern–Volmer Plot of the Quenching of Native and Heated 7S Globulin Fluorescence by Iodide. The concentration of 7S globulin was $1.03 \times 10^{-7} \text{M}$. The wavelength of excitation was 295 nm. Periods of heating were: (○), 0; (●), 1; (□), 5; (■), 10; (▲), 30 min. The heating temperature was 100°C.

Table I. Effect of Heating Time on Relative Fluorescence Intensity and the Quenching of the Tryptophanyl Fluorescence of 7S Globulin by Iodide

<table>
<thead>
<tr>
<th>Heating time (min)</th>
<th>$\lambda_{\text{max}}$</th>
<th>RFI$^a$</th>
<th>$(f_a)_{\text{eff}}$</th>
<th>$(K_Q)_{\text{eff}}$ (M$^{-1}$)$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>334</td>
<td>100</td>
<td>0.65</td>
<td>4.56</td>
</tr>
<tr>
<td>1</td>
<td>333</td>
<td>105</td>
<td>0.60</td>
<td>4.26</td>
</tr>
<tr>
<td>5</td>
<td>332</td>
<td>113</td>
<td>0.56</td>
<td>5.06</td>
</tr>
<tr>
<td>10</td>
<td>331</td>
<td>117</td>
<td>0.54</td>
<td>3.02</td>
</tr>
<tr>
<td>30</td>
<td>331</td>
<td>125</td>
<td>0.47</td>
<td>3.15</td>
</tr>
</tbody>
</table>

$^a$ Relative fluorescence intensity. Relative fluorescence intensities were measured within the region in which the values change linearly with the concentration of each protein. The same legend and data as for Fig. 5.

$^b$ Fractional accessible fluorescence.

$^c$ Effective Stern–Volmer quenching constant $(K_Q)_{\text{eff}}$ indicates that heating represses the quenching of tryptophanyl fluorescence by iodide. However, it has not been recognized universally that its constant means the extent of exposure or burial.

dependence as shown in Fig. 5. From the intercept a value of $(f_a)_{\text{eff}}=0.65$ was obtained for native 7S globulin. Thus, about 65% of the tryptophanyl fluorescence is accessible for quenching by iodide. As shown in Table I, the heating of 7S globulin caused a slight decrease in the value for each fractional accessible fluorescence.

DISCUSSION

Conformational change of a protein reflects a change in either or both the absorption and fluorescence of the chromophoric side chain of the protein.$^{19}$ Recently, we investigated the state of aromatic amino acid residues in 11S globulin heated in the presence of N-ethylmaleimide using derivative absorption spectrophotometry.$^{10}$ This method enabled us to analyze not only the state of tyrosine and tryptophan residues but also that of phenylalanine residues, without being disturbed by the turbidity or masking by the absorption of tyrosine and tryptophan residues.

Soybean 7S and 11S globulin contain all types of aromatic acid residues and the contents of their phenylalanine residues are higher (73 and 130 residues) than those of tyrosine (37 and 80 residues) and tryptophan (8 and 24 residues), respectively.$^{18}$ The second derivative spectrum of 7S globulin clearly shows characteristic bands due to phenylalanine residues, which were also observed in the second derivative spectrum of 11S globulin.$^{10}$ The wavelength of the peak and the trough due to phenylalanine residues between 250 and 270 nm in the second derivative absorption spectrum of native 7S globulin was the same as that of native 11S globulin.$^{10}$ The intensities of phenylalanine bands of the heated 7S globulin were in between those of native 7S globulin and those of native or heated 7S globulin in the presence of 6 m urea, and the intensity of heated 7S globulin was closer to that of the sample in the presence of 6 m urea than that of native 7S globulin. These results suggest that phenylalanine residues on heating are less exposed to a hydrophilic environment than on...
urea denaturation. Peaks and troughs in the region of 290 to 295 nm, which are attributed to tryptophan residues, could not be detected. The large blue-shift above 271 nm suggests that heating caused some tyrosine residue to be exposed to the hydrophilic environment; and further blue-shift, by addition of urea to the 7S globulin heated for 30 min, suggests the exposure of more tyrosine residues in its hydrophobic region.

We reported previously\(^\text{10}\) that difference-second derivative absorption spectrophotometry was useful for obtaining information on conformational changes of protein on denaturation, and for locating the chromophoric groups by means of the solvent perturbation method, irrespective of slight turbidity. Figure 2 shows that denaturation blue-shift occurs after heating even for a minute. As heated protein and heated protein in the presence of urea were used as the reference and sample, respectively, the derivative difference maxima at 279.5 and 289 nm must originate from the exposure of buried tyrosine and tryptophan residues which remained in the heated 7S globulin (Fig. 3). The ratio of each intensity, \(\frac{d^2A}{dl^2}\) in the difference-second derivative absorption spectra of heated proteins in the presence of urea to that of native protein in the presence of urea are listed in Table II. Each intensity is the difference between various peaks and troughs in the spectra and measured within a region in which the value of \(\frac{d^2A}{dl^2}\) changes linearly with the concentration of each protein. The negative peak at 289 nm may be contributed to by tryptophan residues, and so the difference-derivative absorption spectrum was measured under various conditions, namely measurement with a narrower derivative wavelength difference than 5 nm, but the tryptophanyl contribution could not be detected. Accordingly, the ratios based on each intensity between 276 and 296 nm may reflect mainly the changes of tyrosine residues, although their intensities originate from the exposure of both tyrosine and tryptophan residues. Table II shows that 70 percent of the tyrosine residues buried in native 7S globulin remains as the buried form in the heated 7S globulin on continued heating for as long as 30 min. The second derivative spectra of tyrosine and tryptophan residues in a protein have no influence on the spectrum of its phenylalanine residues over the range of 250 to 270 nm.\(^\text{10,20}\) Accordingly, the difference spectrum in the region of 260 to 270 nm must reveal a blue-shift due to an environmental change of phenylalanine residues. The ratios at the wavelength pairs of A–B (263–265 nm) and C–B (267–265 nm) are list-

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**Table II. The Ratios of Each Intensity, \(\frac{d^2A}{dl^2}\),* in the Difference-Second Derivative Spectra of Heated 7S Globulin Denatured with Urea to That of Native 7S Globulin Denatured with Urea**

<table>
<thead>
<tr>
<th>Heating time (min)</th>
<th>Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(263–</td>
</tr>
<tr>
<td></td>
<td>265 nm)</td>
</tr>
<tr>
<td>0.5</td>
<td>1.020</td>
</tr>
<tr>
<td>1</td>
<td>0.922</td>
</tr>
<tr>
<td>5</td>
<td>0.778</td>
</tr>
<tr>
<td>10</td>
<td>0.733</td>
</tr>
<tr>
<td>30</td>
<td>0.600</td>
</tr>
</tbody>
</table>

* Difference in \(d^2A/dl^2\) values between positive and negative peaks. The positions of peaks (A, C, E, G and I) and troughs (B, D, F and H) shown are those measured in the standard buffer, pH 7.6. The positions scarcely differ under different heating times. Values in the table are averages for at least 5 runs.
ed in Table II. Table II shows that with increased heating time, the decrease of the ratios due to phenylalanine contribution is parallel to that due to tyrosine contribution, and 60 percent of the total phenylalanine residues buried in native 7S globulin remains as the buried form in the heated 7S globulin after continued heating for as long as 30 min. Accordingly, it is noticeable that heating of 7S globulin caused more phenylalanine residues to be exposed than tyrosine residues.

Figure 4 shows the difference-second derivative absorption spectra of 7S and 11S globulin on denaturation by 6M urea. Native 7S globulin is free of the peak at 291–292 nm due to tryptophan residues, which was detected for 11S globulin. Fukushima\(^{18}\) reported that the tryptophan residues in 7S globulin were located on the surface of the molecule, based on the normal difference absorption spectrum (zero order spectrum). It is not clear, however, whether the failure to detect tryptophanyl difference absorption is due to hindrance by tyrosine difference absorption or to the absence of tryptophan residues buried in native 7S globulin. Accordingly, in the present study, spectrofluorimetry and fluorescence-quenching using a perturbant\(^{17}\) have been used to make up for the limitations of the absorption-spectroscopic technique. The fluorescence-quenching study suggests that 3 of 8 tryptophan residues are buried in the native 7S globulin. This result conflicts with that of a difference-second derivative absorption study. We consider that one of the causes of the lack of tryptophanyl contribution in the difference-second derivative spectrum of native 7S globulin is hindrance by a large tyrosinal contribution.

Sometimes, fluorescence can reveal structural changes in a protein that are too subtle to be observed by other techniques. Fluorescence spectra of non-conjugated proteins are essentially determined from tyrosine and tryptophan residues, of which 7S globulin contains 37 and 8 residues per molecule, respectively.\(^{18}\) On irradiation of a protein at 295 nm, most of the absorption of light is due to the tryptophan residues only, and the resultant emission spectrum is due mainly to these residues.\(^{21}\) The present fluorimetric study has provided evidence that the environment around tryptophan residues is affected by heating, as shown by the blue-shift of the maximum wavelength of the intrinsic protein fluorescence in heated 7S globulin, and also by the increase in fluorescence intensity. These alterations in the fluorescence characteristics can be accounted for by a conformational change with a decrease in polarity around the emitting tryptophan residues, related to the transfer of these residues from the exterior to the more hydrophobic interior of the protein molecule. This interpretation is consistent also with the results of the fluorescence-quenching experiments. With KI as the quencher, indication has been obtained of a more folded partial structure in the heated protein with respect to the native protein, since fractional accessible fluorescence decreases slightly with an increase of heating time. The fluorescence-quenching study suggests that heating causes one of 8 tryptophan residues of 7S globulin to be transferred to the hydrophobic environment.

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