Spectroscopic Studies on the Aggregates and the Dissociates Induced by Heating Soybean 11S Globulin in the Presence of *N*-Ethylmaleimide

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Heating 11S globulin in the presence of *N*-ethylmaleimide (NEM) gave buffer-soluble aggregates and dissociates. The difference-second derivative absorption spectra indicated that the aggregates had larger internal hydrophobicity than 11S globulin, while that of the dissociates was conversely smaller. The protein's fluorescence spectra indicated that, compared with 11S globulin, the wavelength of the fluorescence peak of the aggregates was hardly shifted, while that of the dissociates was red-shifted. The circular dichroism of the aggregates was similar to that of 11S globulin.

These results suggest that the aggregates were formed with retention of the internal hydrophobic region of 11S globulin, while the dissociates released from 11S globulin were unfolded with increasing heating time.

Heating soybean protein causes structural changes^{1,2)} and modification of its rheological³⁾ and functional properties.⁴⁾ Accordingly, a basic knowledge about thermal aggregation and dissociation seems to be necessary for its utilization as a highly functional food protein. The thermal aggregation of soybean 11S globulin is mainly due to aggregation of the basic subunits.^{1,2,5~7)} We have proved that the thermal dissociation of subunits from the native oligomeric structure of 11S globulin and the thermal polymerization among basic subunits are attributable to sulfhydryl-disulfide exchange.²⁾ Furthermore, we have reported that the degree of polymerization among the subunits affects the quality of these thermal aggregates.⁸⁾

In order to investigate factors other than disulfide exchange on the thermal aggregation and dissociation, we tried to block the sulfhydryl residue on heating, which is an initiating factor for polymerization and dissociation among the subunits.⁹⁾ The heating of 11S globulin in the presence of NEM gave no precipitation, but formed buffer-soluble aggregates and dissociates.^{5,6,9)} We found that

the aggregates and dissociates consisted of intermediary subunits and a monomer of the acidic subunit or intermediary subunit, respectively.⁹⁾ The studies reported here were conducted to investigate the conformation of the aggregates and dissociates.

MATERIALS AND METHODS

Materials. Soybeans, cv. Raiden, were used throughout this work. Defatted meal was prepared from soybean by grinding the bean, defatting with *n*-hexane and then removing the solvent (at room temperature). All reagents were of analytical grade.

Purified 11S globulin was prepared by the method described in the previous paper.⁸⁾

Preparation of buffer-soluble aggregates and dissociates. Purified 11S globulin (0.5%) was heated at 100°C in a standard buffer⁹⁾ containing 0.02 M NEM. The heated protein solution was subjected to gel filtration on Sepharose CL-2B and the aggregates and dissociates were prepared by the method described in the previous paper.⁹⁾

Denaturation with urea. Stock solutions of 8 M urea in a standard buffer⁹⁾ were prepared just before use. Proteins were denatured by treating them in solution with urea for 24 hr at 6°C.

Protein fluorescence. Protein fluorescence measurements were made by using an excitation wavelength of 280 nm. The absorbance at 280 nm of samples examined did not exceed 0.1.

Fluorescence of protein-anilinonaphthalene-8-sulfonate (ANS) complex. Complexes of the proteins with ANS were prepared by adding the required volume of an ANS (Nakarai Chemical Co., Ltd.) solution in the standard buffer⁹⁾ to a protein solution prepared in the same buffer. The concentration of ANS solution was obtained by measuring the absorbance at 350 nm using a molar absorption coefficient of $5.9 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. In some measurements a solution of ANS of $0.2 \mu\text{g/ml}$ in dioxane, at room temperature, was used as a fluorescence standard. In these cases the results are expressed in relative fluorescence units. Relative fluorescence was calculated by dividing the fluorescence intensity of the sample at the wavelength corresponding to the emission maximum by the fluorescence intensity of the standard at 460 nm.

Instruments. Second derivative absorption spectra were measured in a Yanagimoto high order derivative UV-visible spectrophotometer. Output signals of the second derivative spectra by the wavelength modulation method¹⁰⁾ from the spectrophotometer were converted electrically to the derivative analog signals. The derivative wavelength difference, $\Delta\lambda$, was 3 or 5 nm.

Fluorescence spectra were measured with a Hitachi MPF-4 spectrophotofluorimeter. To obtain "true fluorescence spectra," the data obtained were corrected by measuring the fluorescence spectra of the standard substance, *i.e.* Rhodamine B, and using a revising spectra attachment and spectral data processor connected with the spectrophotofluorimeter.

The circular dichroic (CD) spectra were measured with a Yobin Yvon Dichrograph Mark III under constant nitrogen flushing. The protein concentration of a sample was 0.05%, and a cell of path length 0.01 cm was used. The data were recorded from 200 to 245 nm and reported in terms of mean residue ellipticity in $\text{deg} \cdot \text{cm}^2 \cdot \text{d} \cdot \text{mol}^{-1}$. The amino acid mean residue weight was set to a value of 114 daltons¹¹⁾ for the 11S globulin.

RESULTS

Second derivative absorption spectra of the aggregates and dissociates

The second derivative absorption spectra of the aggregates (the solid line in Fig. 1) showed that the positions of the peaks and troughs for the phenylalanine, tyrosine and tryptophan residues hardly changed with increasing heating time. On the other hand, their treatment with 6 M urea caused a blue shift (the broken

FIG. 1. Second Derivative Absorption Spectra of the Aggregates and Dissociates.

The aggregate and dissociate were induced by heating for 5 min in the presence of NEM. Solid line, in standard buffer (pH 7.0); Broken line, in 6 M urea. A, native 11S globulin (0.71 mg/ml); B, aggregate (0.82 mg/ml); C, dissociate (0.85 mg/ml). The derivative wavelength difference, $\Delta\lambda$, was 3 nm.

line in Fig. 1). The second derivative absorption spectrum of the aggregate denatured with 6 M urea was similar to that of native 11S globulin denatured with 6 M urea.

The second derivative absorption spectra of the dissociates (the solid line in Fig. 1) showed that the positions of the peaks and troughs in the region of 250 to 270 nm hardly changed with increasing heating time. On the other hand, heating caused a blue shift in the wavelength of the peaks and troughs due to the tyrosine and tryptophan residues above 271 nm (the broken line in Fig. 1). Treatment of the dissociates with 6 M urea caused a further slight blue shift.

FIG. 2. Difference-second Derivative Spectra of 11S Globulin, the Aggregate and the Dissociate.

The aggregate and the dissociate were induced by heating for 5 min in the presence of NEM. The two samples, such as either native or heated protein and its protein denatured with 6 M urea, were placed in the reference and sample compartment, respectively. A, native 11S globulin; B, aggregate (0.82 mg/ml); C, dissociate (0.85 mg/ml). The derivative wavelength difference, $\Delta\lambda$, was 5 nm.

Difference-second derivative absorption spectra of the aggregates and dissociates

The two samples for difference-second derivative absorption spectrophotometry, such as either native or heated protein, with and without denaturation by 6 M urea, were placed in the reference and sample compartments, respectively (Fig. 2). Difference-zero order absorption spectra indicate that native protein and heat-induced products underwent the typical blue-shift on urea denaturation (data not shown). The negative peaks in the difference-second derivative absorption spectra appeared at 279, 286 and 292 nm. The intensity between

FIG. 3. Fluorescence Spectra of Native 11S Globulin, the Aggregate and the Dissociate.

The aggregate and the dissociate were induced by heating for 5 and 30 min in the presence of NEM. (a), native 11S globulin (0.26 mg/ml); (b), the aggregate induced by heating for 5 min (0.31 mg/ml); (c), the dissociate induced by heating for 5 min (0.28 mg/ml); (d), the dissociate induced by heating for 30 min (0.33 mg/ml). Fluorescence spectra were measured in a standard buffer at an excitation wavelength of 280 nm. Arrows indicate fluorescence peaks.

the peak and trough depends on the degree of exposure of an aromatic amino acid residue buried in the protein molecule. When a denaturant was used as a perturbant, the intensity can be considered as the degree of burial of an aromatic amino acid residue remaining in the heat-induced products.

Protein fluorescence measurement of the aggregates and dissociates

Native 11S globulin, the aggregates and dissociates were excited at 280 nm and their emission curves were obtained (Fig. 3). The wavelength of the fluorescence peak of native 11S globulin was 333 nm. With increasing heating time, the wavelength of the fluorescence peak of the aggregates scarcely shifted, but that of the dissociates red-shifted (Fig. 3). The wavelength corresponding to the emission

FIG. 4. Circular Dichroism Spectra of the Aggregate Induced by Heating 11S Globulin in the Presence of NEM.

Solid line, native 11S globulin; broken line, the aggregate induced by heating for 5 min.

FIG. 5. Fluorescence Spectra of Protein-ANS Complexes in a Standard Buffer.

The aggregate and the dissociate were induced by heating for 5 min in the presence of NEM. (a), native 11S globulin (0.56 mg/ml); (b), aggregate (0.47 mg/ml); (c), dissociate (0.98 mg/ml); (d), bovine serum albumin; (e), ANS in 50% ethanol; (f), ANS in standard buffer. Excitation wavelength was 408 nm. Arrows indicate fluorescence peaks.

maximum of the aggregates was approximately 5 to 10 nm shorter than that of the dissociates.

Circular dichroism of the aggregates

Figure 4 shows the circular dichroism (CD) of the aggregate induced by heating for 5 min in the presence of NEM and native 11S globulin in the region of 245 to 200 nm. The CD spectrum of the aggregates induced by heating for 5, 10 and 30 min were similar to that of native 11S globulin.

Fluorescence measurement of the protein-ANS complex

The emission spectra of the aggregate-ANS and the dissociate-ANS complexes under the same conditions are presented in Fig. 5. Heating caused an increase in the fluorescence intensity of the aggregate-ANS and the dissociate-ANS complexes. The wavelength corresponding to the emission maximum of the aggregate-ANS complexes was approximately 20 nm shorter than that of the dissociate-ANS complexes.

DISCUSSION

We reported previously that heating 11S globulin in the presence of NEM caused the formation of buffer-soluble aggregates consisting of intermediary subunits, and the release of a monomer of an intermediary or acidic subunit.⁹⁾ It appears that 11S globulin was transformed to the aggregates when the monomer (intermediary subunit) was dissociated at the initial stage of heating. When 11S globulin was heated in the absence of NEM, precipitation occurred and the localization of the acidic or basic subunit is markedly different in the supernatant and precipitate.²⁾ This indicates that blocking of the sulfhydryl residue by NEM did not cause rearrangement among the subunits and hence precipitation of the basic subunit. If there is a better understanding of the conformational basis of the aggregates and dissociates, this may suggest one mechanism of aggregation and dissociation.

The number or position of the peak and trough in the second derivative absorption spectra of the aggregates was similar to that of native 11S globulin. On the other hand, the

TABLE I. THE APPARENT INTERNAL HYDROPHOBICITY OF THE AGGREGATES AND DISSOCIATES INDUCED BY HEATING IN THE PRESENCE OF NEM

The positions of peaks (A, C, E and G) and troughs (B, D and F) shown are those measured in a standard buffer, pH 7.6. The positions scarcely differ after different heating times. Values in the table are averages for at least 5.

Heating time (min)	Apparent internal hydrophobicity					
	A ~ B (276.8 ~ 280.4 nm)	C ~ B (282.4 ~ 280.4 nm)	C ~ D (282.4 ~ 286.4 nm)	E ~ D (288.8 ~ 286.4 nm)	E ~ F (288.8 ~ 292.1 nm)	G ~ F (296 ~ 292.1 nm)
Aggregates	1	98.2	90.3	91.5	99.2	101.0
	5	93.5	130.0	137.4	180.0	212.0
	10	97.5	126.6	152.0	138.4	140.0
	30	45.5	113.1	122.1	127.5	136.8
Dissociates	5	80.5	117.1	79.5	85.3	130.5
	10	24.0	73.1	49.5	39.7	84.7
	30	33.5	55.1	44.4	48.1	75.8

spectra of the dissociates indicated that the number of peaks and troughs was similar to those of 11S globulin, but their position was close to that of native 11S globulin which had been denatured with urea, rather than that of native 11S globulin. These results suggest that the environmental change around the aromatic amino acid residues of the aggregates is smaller than that of the dissociates when heating 11S globulin induces these aggregates and dissociates.

When a denaturant such as urea was used as the solvent perturbant, difference absorption spectrophotometry offers the information about the change of state of aromatic amino acid residues buried in a protein. As native or heated protein, and its protein denatured with urea, were used as reference and sample, respectively, derivative difference maxima in the region between 270 and 300 nm must originate from the exposure of tyrosine and tryptophan residues buried in the native or heated protein. Each intensity, $\Delta(d^2AI/d\lambda^2)$, was measured as the difference between various peaks and troughs within a region where the value of $(d^2AI/d\lambda^2)$ changes linearly with concentration of each protein. The slope of intensity to protein concentration was measured. The slope represents the apparent internal hydrophobicity of a protein. Relative internal

hydrophobicity was calculated from the slope of the aggregates and dissociates relative to that for native 11S globulin. Table I shows that the relative internal hydrophobicity of the aggregates is larger than that of the dissociates. This suggests that the aggregates were formed while retaining the internal hydrophobic region of native 11S globulin, while the dissociates released from native 11S globulin were unfolded with increasing heating time.

The present fluorimetric study indicated that the maximum wavelength of the intrinsic protein fluorescence in the aggregates was hardly shifted, while that in the dissociates was red-shifted. Protein fluorescence intensity was measured within a region in which the value changes linearly with the concentration of each protein. The slope of fluorescence intensity to protein concentration was measured. Relative fluorescence intensity was calculated from the slopes of the aggregates and dissociates relative to that for native 11S globulin. As shown in Table II, the relative fluorescence intensity of the aggregates was higher than that of the dissociates. Even after heating for 30 min, the aggregate had a similar relative fluorescence intensity to native 11S globulin. These results suggest that, as 11S globulin is transformed into aggregates, the environment around their aromatic amino acid residues is retained, while

TABLE II. RELATIVE FLUORESCENCE INTENSITIES OF THE AGGREGATES AND DISSOCIATES INDUCED BY HEATING IN THE PRESENCE OF NEM

Heating time (min)	$\lambda_{\max}$	RFI ^a
Aggregates	0	333
	1	333
	5	334
	10	334
	30	334
Dissociates	5	339
	10	340
	30	345

^a Relative fluorescence intensity.

that of the dissociates is not.

The fluorimetric information corresponded with that obtained by the difference-derivative absorption spectra. As shown in Fig. 5, the CD spectrum of the aggregate after 5 min was similar to that of native 11S globulin. The aggregates induced with increasing heating time had similar CD patterns to native 11S globulin (data not shown). This result suggests that the aggregates retain the secondary structural conformation of native 11S globulin as 11S globulin is transformed into the aggregates.

To examine their affinities for ANS of the aggregates and the dissociates, the fluorescence intensity was measured within a region in which the value increased linearly with the concentration of each protein. The slope of fluorescence intensity to protein concentration was measured. The relative affinity for ANS was calculated from the slopes of the aggregates and dissociates relative to that for native 11S globulin. Table III summarizes the relative affinities of heat-induced products, taking the affinity of native 11S globulin as 1.0. The affinity of 11S globulin increased as the heating time increased, and that of the aggregates reached a constant value of about 5 after 5 min. With the dissociates, these after 5 min had the same affinity as that after 10 min, while the affinity of the dissociate after 30 min decreased to an extent similar to that of native 11S globulin. The wavelength of the

TABLE III. AFFINITIES OF THE AGGREGATES AND DISSOCIATES FOR ANS

Heating time (min)	$\lambda_{\max}$	Relative affinity
Aggregates	0	498
	1	486
	5	478
	10	476
	30	476
Dissociates	5	498
	10	498
	30	498
BSA ^a	480	
ANS ^b	498	
ANS ^c	524	

^a Bovine serum albumin in standard buffer.

^b ANS in 50% ethanol.

^c ANS in standard buffer.

fluorescence peak of native 11S globulin or the dissociates is the same as that for ANS in 50% ethanol, while that of the aggregates is the same as that for bovine serum albumin in a standard buffer. Table II indicates that the surface of the aggregates has a stronger hydrophobic character than that of the dissociates. These results also indicate that the formation of the aggregates follows the gain of hydrophobic region on their surface. Other spectroscopic studies used here indicate that the aggregates were formed while retaining the internal hydrophobic region of 11S globulin. Accordingly this ANS-fluorimetric study seems to conflict with other spectroscopic studies here, because binding between molecule-molecule interfaces may be due to loss of the hydrophobic region on aggregation or polymerization. These discrepancies may be attributed to some obscure properties of ANS as a hydrophobic probe; *e.g.*, whether the interaction with protein of this dye is only hydrophobic or not, because this dye not only has a hydrophobic moiety but also an ionic moiety.¹²⁾

The results obtained here suggest that NEM-blocking of the sulfhydryl residue on heating caused retention of the conformation of 11S globulin in the aggregates. The prevention of thermal aggregation by successive

SH-blocking on heating may be concerned with retaining the state of the intermediary subunit in the aggregates. Damodaran and Kinsella⁷⁾ have reported that the addition of highly negatively charged protein prevented thermal aggregation of 11S globulin by electrostatic interaction. According to their suggestion, we consider that the released form of acidic subunits is not sufficiently acidic to electrostatically interact with the basic subunit and has lower acidity than the bound form (intermediary subunit). We reported previously that heating 11S globulin caused release of the monomer of the acidic subunit as a major component.^{2,8)} Our results suggest that retention of the disulfide bonding between the acidic and basic subunits is due to preservation of the acidity of the acidic subunit in the intermediary subunit. Thus, we speculate that the conformation of the acidic subunit in the aggregates is similar to that of native 11S globulin.

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