Electrophoretical and Differential Thermal Analysis of Soybean 11S Globulin Heated in the Presence of *N*-Ethylmaleimide

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Eleven-S globulin heated in the presence of *N*-ethylmaleimide (NEM) retained its buffer-soluble state. Its gel filtration gave two peaks. Two kinds of electrophoresis indicated that the first peak contained soluble aggregates consisting of intermediary subunits as the major component, and the second peak contained a monomer of acidic subunit and a monomer of intermediary subunit. With an increase in heating time, soluble aggregates polymerized through disulfide bond occurred a little. The temperature of the endothermic peak of 11S globulin heated in the presence of NEM was slightly higher than that in the absence of NEM.

With the purpose of acquiring basic knowledge as to the thermal aggregation of 11S globulin, we previously studied the thermal aggregates electrophoretically¹⁾ and proposed a mechanism of thermal aggregation.²⁾

According to the mechanism, each various degree of the polymerization of oligomer and polymer constituting thermal aggregates is settled through the addition and release of subunits by disulfide exchange. In order to investigate the role of secondary force on thermal aggregation in a state of repressed disulfide exchange, we tried to block sulfhydryl residues in the presence of NEM on heating. Wolf and Tamura³⁾ reported that 11S globulin heated in the presence of NEM was not precipitated on continued heating for 30 min. We have been interested in this phenomenon, and we have previously studied the state of aromatic amino acid residues which participate in hydrophobic region.⁴⁾

In the present investigation, we studied the dissociation and rearrangement of 11S globulin molecules by heating in the presence of NEM in order to discuss the relationship between the hydrophobic region and its subunit structure.

MATERIALS AND METHODS

Materials. The Raiden variety of soybean was used throughout this work. Defatted meal was prepared from soybean by grinding, defatting by *n*-hexane and removing the solvent at room temperature.

All the reagents were of the highest grade. Sepharose CL-2B and TOYOPEARL HW-65 were obtained from Pharmacia Fine Chemicals and Toyo Soda Mfg., Co., Ltd., respectively.

Purified 11S globulin was prepared by the method described in the previous paper.¹⁾

Procedure of heat treatment. The purified 11S globulin was kept in solution and used for heat treatment. In experiments with NEM, a portion of stock protein solution was made 0.02 M with NEM by adding the solid reagent.

Heating studies were carried out with 2 ml portions of 0.5% protein solution (in standard buffer, phosphate buffer of pH 7.6 and 0.5 ionic strength) in an oil bath which was kept within the desired temperature of 100°C. Test tubes were loosely stoppered with glass marbles to reduce water evaporation. At the end of the desired heating time, the test tubes were removed and immediately cooled in ice.

Gel filtration: The heated protein solution was gel-filtrated on a column (2 × 90 cm) of Sepharose CL-2B or TOYOPEARL HW-65 by elution with standard buffer or 0.5% SDS in deionized water, respectively. Column effluents were collected in 3.5 ml fractions and monitored at

280 nm. Protein-containing fractions were pooled, concentrated by ultrafiltration, dialyzed against deionized water and lyophilized.

Electrophoresis. Acetic acid-gel electrophoresis in 6 M urea and 9% acrylamide gels was performed according to the method of Kitamura *et al.*⁵⁾ Sodium dodecyl sulfate (SDS)-urea gel electrophoresis was conducted with 0.1 M Tris-phosphate buffer (pH 6.8)⁶⁾ and 8 M urea in 5% acrylamide gels. All gels were stained with Coomassie blue and destained by diffusion in water-methanol-acetic acid (73:20:7).

Molar ratios of the subunits of soluble aggregates and dissociates. Their ratios (basic/acidic) were determined by the method described previously.¹⁾

Ultracentrifugal analysis. Sedimentation velocity measurements were conducted with a Hitachi Model UCA-1 ultracentrifuge equipped with an optical system. Svedberg unit (S) was calculated as the apparent sedimentation coefficient.

Differential thermal analysis (DTA). Thermal denaturation was measured by the DTA instrument, a Shimadzu Micro Thermal Analyzer type DT-20B with attached thermocoupled unit type MDM-20. The differential signal was recorded with a Riken Denshi SP-H recorder. Samples were heated at 5°C/min and recorded at chart speed of 1 cm/min. The temperature differential between samples and reference scale (ΔT) on the chart was 0.05°C/cm.

RESULTS

Fractionation of 11S globulin heated in the presence of NEM

As shown in Fig. 1, gel filtration indicated that thermal products induced in the presence of NEM consisted of two components. Judging from each peak area, buffer-soluble aggregates (first peak in Fig. 1-B to D) tended to decrease slightly, whereas buffer-soluble dissociates (second peak in Fig. 1) increased on a time course within 5 to 30 min.

Ultracentrifugal analysis of soluble aggregates and dissociates induced by heating in the presence of NEM

Figure 2 shows the ultracentrifugal pattern of soluble aggregate induced by heating for 5 min in the presence of NEM. The soluble aggregates induced by heating for 5, 10 and

30 min had the values of 41.3S, 43.5S and 44.3S, respectively. On the other hand, S values of soluble dissociates were similar to those obtained by Wolf and Tamura.³⁾

Electrophoretic analysis of soluble aggregates and dissociates induced by heating in the presence of NEM

Electrophoretic analysis of soluble aggregates and dissociates fractionated by gel filtration would have given the mechanism of rearrangement and dissociation of 11S globulin subunits. The fractionated samples were subjected to SDS-urea or acetic acid-urea polyacrylamide gel electrophoresis in the absence of 2-mercaptoethanol for the purpose of retaining the disulfide bonds in thermal products. Eleven S globulin has the intermediary subunits in which one mol of the acidic subunit linked with the specific partner of one mol of the basic subunit through disulfide bridges.⁵⁾ A time course of thermal aggregation and dissociation of 11S globulin analyzed by gel electrophoresis without reducing the disulfide bridges showed dissociation followed by rearrangement of the intermediary subunits.

Heating for 1 min did not induce such soluble aggregate as a macromolecule shown in Fig. 1. As shown in Fig. 3-a, the acetic acid-urea electrophoretogram of soluble aggregates indicated that the electrophoretic mobility of zone *a* coincided with that of the intermediary subunit. Furthermore, its electrophoresis showed the zones (zones *b* in Fig. 3-a) as having a lower electrophoretic mobility than zone *a* and those (zones *c* in Fig. 3-a) remaining in the origin of the gel. As shown in Fig. 3-b, acetic acid-urea electrophoresis indicated that zones *a*, *b* and *c* disappeared on the addition of 2-mercaptoethanol to each soluble aggregate, and acidic and basic subunits appeared. Each molar ratio (basic/acidic) of soluble aggregates induced by heating for 5 and 10 min was close to that of native 11S globulin. Even on continued heating for 30 min, the soluble aggregate had the molar ratio of 2.1 (about 3, in-

FIG. 1. Gel Filtration of 11S Globulin Heated in the Presence of NEM on Sepharose CL-2B in Standard Buffer (pH 7.6).

Periods of heating were: A, 1; B, 5; C, 10; D, 30 min. The absorbance at 280 nm was measured. Heating temperature and ionic strength; 100°C and 0.5.

soluble aggregates induced on heating in the absence of NEM).

Acetic acid-urea electrophoresis suggested the existence of covalently polymerized subunits (zone *c* remaining in the origin of the gel). For the purpose of detecting the polymerized subunits induced in the presence of

NEM, each soluble aggregate was gel-filtrated with a TOYOPEARL HW-65 column in the presence of 0.5% SDS. They were separated into two fractions as shown in Fig. 4. Each part was subjected to acetic acid-urea electrophoresis (in the absence of 2-mercaptoethanol). The first and second peaks contained

FIG. 2. Ultracentrifugal Patterns of Soluble Aggregate Induced by Heating of 11S Globulin in the Presence of NEM.

The soluble aggregate was induced by heating for 5 min and obtained by gel filtration on Sepharose CL-2B. Pictures A~E were taken after 6, 12, 18, 24 and 30 min of ultracentrifugation, respectively. Direction of sedimentation is from left to right. A speed of 34450 rpm was used. Buffer, standard buffer. Temperature, 16°C.

components corresponding to zone *c* and zone *a* as the major component, respectively (their electrophoretograms are not shown).

An acetic acid-urea electrophoretogram of soluble dissociates is shown in Fig. 3-a (D to F). Heating for 5 min gave a major zone identical to that of the intermediary subunit. With an increase in the heating time, an acidic subunit began to appear. Further heating for 30 min gave only an acidic subunit. By the addition of 2-mercaptoethanol to each soluble dissociate, the zone identical with that of the

intermediary subunit disappeared and an acidic and basic subunit appeared.

The results obtained by SDS-urea electrophoresis of soluble aggregates and dissociates were similar to those obtained by acetic acid-urea electrophoresis as described above.

Differential thermal analysis

The differential thermal analysis technique^{7,8)} consists of measuring the difference in temperature between a sample and reference material when temperature is linearly in-

FIG. 3. Acetic Acid-urea Polyacrylamide Gel Electrophoretogram of Each Fraction Isolated by Gel Filtration.

Concentration of gel was 9%. 3-a, soluble aggregates (A~C) and dissociates (D~F) untreated with 2-mercaptoethanol. 3-b, soluble aggregates (A~C) and dissociates (D~F) treated with 2-mercaptoethanol. Periods of heating were: A and D, 5; B and E, 10; C and F, 30 min; G, native 11S globulin. Is, I and II indicate each position of the intermediary, acidic and basic subunits, respectively.

creased with time. Figure 5 shows the thermograms obtained from the denaturation of 11S globulin heated in the absence or presence of NEM. Both thermograms gave an endothermic peak, but not an exothermic peak. The endothermic change of 11S globulin heated in the presence and absence of NEM appeared in the temperature range of 81°C to 113°C and 77°C to 109°C, with the peak minimum at 99°C and 96°C, respectively.

DISCUSSION

As sulfhydryl blocking represses disulfide exchange on heating, the contribution of secondary force to thermal aggregation could be investigated. Wolf and Tamura³⁾ demonstrated ultracentrifugally that the heating of 11S globulin in the presence of NEM gave

soluble aggregates of 58S to 67S and soluble dissociates of 2.4S to 4.4S. If these aggregates and dissociates are separated and their subunit structure is known, we should be able to discuss the role of secondary force in thermal aggregation in addition to previous derivative spectrophotometric studies.⁴⁾

The molar ratio (basic/acidic) of soluble aggregates suggested that sulfhydryl blocking in the presence of NEM on heating repressed the excess releasing of an acidic subunit followed by precipitation.

Gel filtration (Fig. 1) followed by electrophoresis (Fig. 3) showed that each soluble aggregate induced by heating for 5 and 10 min consisted of intermediary subunits as the major component. On the other hand, soluble dissociates may be a monomer of the intermediary subunit, a monomer of the acidic subunit or mixture of them, because they seem to correspond to the 3S fraction of Wolf and Tamura.³⁾ The dissociate induced at the initial stage of heating (5 min) seems to be a monomer of the intermediary subunit, because it had the molar ratio (basic/acidic) close to 1. These facts suggest the mechanism of thermal aggregation in the presence of NEM to be as follows: while an intermediary subunit is released from native 11S globulin at the initial stage of heating, 11S globulin lacking a released intermediary subunit is polymerized by secondary forces (*i.e.*, hydrophobic, ionic and hydrogen bonds), and soluble aggregates are formed. Because conditions used here are at high ionic strength and at high temperature, ionic and hydrogen bonds seem unlikely to be involved in the polymerization reaction. Quite possibly, hydrophobic interactions are responsible for the polymerization at the initial stage of heating. A releasing of an intermediary subunit at the initial stage of heating may be an initiating factor for polymerization, because each S value of the soluble aggregates became markedly larger at the initial stage of heating, but hardly at all on further heating. As described above, it is interesting that a released intermediary subunit can exist as a monomer in standard buffer at the initial stage

FIG. 4. Gel Filtration of Soluble Aggregates on TOYOPEARL HW-65 in 0.5% SDS.

The absorbance at 280 nm was measured. Periods of heating were: A, 0; B, 5; C, 10; D, 30 min. Heating temperature and ionic strength; 100°C and 0.5.

FIG. 5. DTA Thermograms of 11S Globulin Heated in the Presence and Absence of NEM.

A 5% solution of proteins in standard buffer, pH 7.6 was used.

of heating, because $\overline{7S}^{9,10}$ as trimer of an intermediary subunit has been detected, but its monomer never has. It is not clear whether this released intermediary subunit is a specific intermediary subunit of five kinds⁵⁾ of intermediary subunits or not. Further studies on this released intermediary subunit will be reported elsewhere.

Electrophoresis (component *c* in Fig. 3) and gel filtration (in the presence of SDS, first peak in Fig. 4) indicated that soluble aggregates contained covalently polymerized subunits as a minor component, because each gel filtration of soluble aggregates in the presence of SDS and 2-mercaptoethanol did not give the first peak shown in Fig. 4 (data not shown). This fact suggests that disulfide exchange induced by further heating is more rapid than the NEM-blocking of sulfhydryl residue. We have reported previously that 11S globulin heated in the presence of NEM re-

tained a rigid conformation.⁴⁾ Generally, sulfhydryl and disulfide residues have been considered to be located in the hydrophobic region of the protein.^{11~13)} Accordingly, sulfhydryl residues, which is buried in the hydrophobic region, seems to be present in the soluble aggregates and hard for NEM to attack. Such a sulfhydryl residue may have a low reactivity. However, further heating may induce a disulfide exchange for the following reasons: (1) ionization of sulfhydryl residue to thiolate ion,^{14,15)} which causes the disulfide exchange to accelerate, may be achieved by a conformational change induced by further heating. This conformational change may be not large, but only favorable to its ionization. This inference seems to be supported by the derivative spectrophotometric studies reported previously.⁴⁾ (2) the distance between sulfhydryl and disulfide residue may be closer and the intrachange in the soluble aggregate may be induced, because soluble aggregate may appear as a macromolecule of intermediary subunits polymerized by secondary force. On the other hand, NEM may not be able to approach the sulfhydryl residue before disulfide exchange by steric factor in the neighborhood of the cysteine residue,¹⁶⁾ which is responsible for a rigid conformation of soluble aggregates.⁴⁾ Ultracentrifugation indicated that soluble aggregates (in standard buffer) appeared more uniform in particle size. While covalently polymerized subunits (first peak shown in Fig. 4) through the disulfide bond in the soluble aggregates increase with an increase of the heating time, this particle size hardly became larger at all even by heating beyond 5 min. This fact suggests that disulfide exchange does not contribute to the increase in the particle size, but secondary force does, particularly at the initial stage of heating.

Intermediary subunits, as soluble dissociates induced at the initial stage of heating, may be cleaved into acidic and basic subunits by disulfide exchange on heating. Electrophoresis (Fig. 3-a) indicated that the continuous heating for 30 min gave only an acidic subunit as soluble dissociates. These

results suggest that the cleaved basic subunit is turned over soluble aggregates, which are retained in the hydrophobic region,⁴⁾ because the basic subunit is hydrophobic.¹⁾

Studies on thermal aggregation in the presence of NEM suggests that 11S globulin has sulfhydryl residues, which are located in a different environment. Draper and Catsimpoalas¹⁷⁾ reported that 11S globulin has 20 disulfide and 1.7 sulfhydryl residues and all of them were buried in the interior of the protein molecule. Assuming that 11S globulin has two sulfhydryl residues, one may be easily exposed to a polar environment by heating. Such a sulfhydryl residue may be blocked by NEM at the initial stage of heating. According to our results, this sulfhydryl residue may be an initiator in the thermal aggregation of 11S globulin in the absence of NEM, because its blocking represses precipitation and causes the retention of a rigid conformation even on heating.⁴⁾ On the other hand, the other may be hard to be exposed and remain in the hydrophobic region of the protein. This sulfhydryl residue may contribute to the stabilization of the subunit structure of the soluble aggregates, because local disulfide exchange, in which this sulfhydryl residue participates, may polymerize through a disulfide bond among intermediary subunits and stabilize its subunit structure against further heating.

In order to investigate whether the presence of NEM on heating gives heat-resistant properties to the conformation of 11S globulin or not, the DTA thermogram of 11S globulin was measured in the presence or absence of NEM. Although the difference of temperature between endothermic peaks formed in the presence and absence of NEM was not distinguishable, each thermogram suggested that the presence of NEM on heating stabilized the subunit structure of 11S globulin.

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REFERENCES

- 1) T. Yamagishi, F. Yamauchi and K. Shibasaki, *Agric. Biol. Chem.*, **44**, 1575 (1980).
 - 2) T. Yamagishi, F. Yamauchi and K. Shibasaki, in preparation.
 - 3) W. J. Wolf and T. Tamura, *Cereal Chem.*, **46**, 331 (1969).
 - 4) T. Yamagishi, F. Yamauchi and K. Shibasaki, *Agric. Biol. Chem.*, **45**, 459 (1981).
 - 5) K. Kitamura, T. Takagi and K. Shibasaki, *Agric. Biol. Chem.*, **40**, 1837 (1976).
 - 6) R. T. Swank and K. D. Munkres, *Anal. Biochem.*, **39**, 462 (1971).
 - 7) T. Itoh, Y. Wada and T. Nakanishi, *Agric. Biol. Chem.*, **40**, 1083 (1976).
 - 8) J. M. Steim, *Arch. Biochem. Biophys.*, **112**, 599 (1965).
 - 9) W. J. Wolf, J. J. Rackis, A. K. Smith, H. A. Sasame and G. E. Babcock, *J. Am. Chem. Soc.*, **80**, 5730 (1958).
 - 10) W. J. Wolf and D. R. Briggs, *Arch. Biochem. Biophys.*, **76**, 377 (1958).
 - 11) P. Heitmann, *Eur. J. Biochem.*, **3**, 346 (1968).
 - 12) R. Cecil, "The Proteins," Vol. I, ed. by H. Neuroth, Academic Press, New York, 1963, p. 379.
 - 13) M. F. Perutz, J. C. Kendrew and H. C. Waston, *J. Mol. Biol.*, **13**, 669 (1965).
 - 14) J. M. Wilson, R. J. Bayer and D. J. Hupe, *J. Am. Chem. Soc.*, **99**, 7922 (1977).
 - 15) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, Inc., New York, 1969, p. 7.
 - 16) R. Cecil and M. A. W. Thomas, *Nature*, **206**, 1317 (1965).
 - 17) M. Draper and N. Catsimpoolas, *Cereal Chem.*, **55**, 16 (1978).
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