Further Studies on Aggregation and Insolubilization of Soybean 11S Globulin with Humidity during Storage

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When soybean lyophilized 11S globulin was stored at RH 96% and 50°C, the redispersibility of the protein as measured over a 1 hr period drastically decreased after 4 hr. When the redispersing time was prolonged to 24 hr, 11S globulins stored for up to 12 hr redispersed similarly to the control, but became insoluble after 24 hr of storage. A gel filtration study showed that the stored 11S globulin had already polymerized mainly through disulfide bonds after 12 hr of storage.

Scanning electron microscopic observation showed that a globular structure of the control 11S globulin changed to an aggregated structure during storage. Polymerized subunits linked with disulfide bridges were observed using gel filtration in a sodium dodecyl sulfate (SDS) solution and SDS polyacrylamide gel electrophoresis and the number of the polymerized subunits increased during storage. The 11S globulin, however, did not polymerize with humidity as much as in heat denaturation at 100°C (ionic strength 0.5, protein concentration 0.5%).

Many studies on the storage of soybeans have recently been reported.^{1~13)} Okamoto *et al.*⁷⁾ and Saio *et al.*^{2,3)} have reported that relative humidity seems to be important for the deterioration of soybean during storage. We have made studies from the standpoint that soybean protein isolates will be increasingly utilized as processing materials for foods and that it is necessary to obtain basic data on the changes of soybean proteins during storage.^{14~18)}

Soybean proteins consist of 7S globulin (beta-conglycinin) and 11S globulin (glycinin) as their major components.¹⁷⁾ We have reported the effect of relative humidity (RH) on soybean 7S and 11S globulins¹⁴⁾ and acid precipitated protein,¹⁵⁾ which consists of 7S and 11S globulins, and on the role of disulfide bonds in the polymerization of 7S globulin during storage.¹⁶⁾ This paper describes the aggregation and insolubilization of 11S globulin in more detail.

MATERIALS AND METHODS

Preparation of 11S globulin. Soybeans (var. Raiden,

1981 crop.) were defatted with *n*-hexane at room temperature. A crude 11S globulin fraction was prepared from defatted soybean meals according to the method of Thanh and Shibasaki¹⁸⁾ and purified by ammonium sulfate fractionation.¹⁸⁾ The protein was lyophilized and stored in a desiccator at 5°C.

Humidity condition of 11S globulin. The lyophilized 11S globulin was placed in a desiccator containing a saturated K₂SO₄ solution at 50°C, which gave an RH level of 96%.

Determination of protein redispersibility. When the redispersibility was examined in a phosphate buffer (0.0026 M KH₂PO₄, 0.0325 M K₂HPO₄ and 0.4 M NaCl, pH 7.6), the buiret method was used as described previously.¹⁴⁾ To examine the redispersibility in a 0.5% SDS solution, the following method was used: Three ml of the 0.5% SDS solution was added to about 30 mg of 11S globulin. The mixture was stirred for 1 hr or stood for 24 hrs at room temperature and submitted to centrifugation (25,400 × *g*, 20°C, 15 min). The supernatant was filled up to 50 ml and the absorbance was measured at 280 nm.

Scanning electron microscopy. Scanning electron microscopic observation was carried out with a Hitachi S-700. The 11S globulin stored for 3 days was kept in distilled water for 24 hr, then frozen and lyophilized. The dried protein samples were mounted on an aluminum stub and coated with gold. The specimens were observ-

ed at an accelerating voltage of 20 kV.

SDS polyacrylamide gel electrophoresis (SDS-PAGE). SDS polyacrylamide gel electrophoresis was carried out using a slab gel apparatus according to Swank *et al.*²⁰⁾ All gels were stained with Coomassie Blue and destained by diffusion in 7.5% CH_3OH -5% CH_3COOH -water. The destained gels were scanned at 570 nm (reference 720 nm) with a Shimadzu CS-900 Dual-Wavelength TLC Scanner.

RESULTS

Changes in redispersibility during storage

Figure 1 shows the changes in redispersibility of the lyophilized 11S globulin stored at RH 96% and 50°C. When the redispersing time was set at 1 hr, the redispersibility lowered to 19% after 4 hr and the 11S globulin became completely insoluble beyond 24 hr of storage. On the other hand, when the redispersing time was prolonged to 24 hr, no marked decrease in redispersibility was observed up to 12 hr of storage. The 11S globulin, however, became almost insoluble after 24 hr of storage.

Seven S globulin, which is another component of soybean proteins, did not show an increase in redispersibility as with 11S globulin when the redispersing time was prolonged (redispersibility data not shown). The state of dispersion of 11S and 7S globulins is shown in Figs. 2 and 3, respectively. The 11S globulin

FIG. 1. Changes in Redispersibility of Stored 11S Globulin in the Phosphate Buffer.

One hour redispersing time (●) and 24 hr redispersing time (○).

FIG. 2. Photographs of 11S Globulin Redispersed after Storage in the Phosphate Buffer.

Photographs were taken before centrifugation. One hour redispersing time, a); 24 hr redispersing time, b).

FIG. 3. Photographs of 7S Globulin Redispersed after Storage in the Phosphate Buffer.

Photographs were taken before centrifugation. One hour redispersing time, a); 24 hr redispersing time, b).

stored beyond 4 hr formed particles and remained floating in the buffer over a 1 hr redispersing time (Fig. 2a). On the other hand, 11S globulins stored for up to 12 hr dispersed

in a 24 hr redispersing time (Fig. 2b). The 11S globulin stored for 24 hr did not disperse and showed a gel-like state. Furthermore, 11S globulins stored for 2 to 5 days showed a similar gel-like state to that of the 1 day stored 11S globulin (data not shown).

In the case of 7S globulin (Fig. 3b), the un-stored sample had little turbidity, while minor precipitation occurred during 4 hr of storage. Furthermore, the particles of lyophilized 7S globulins remained floating in the buffer beyond 12 hr of storage. Prolonging the redispersing time to 24 hr had little effect on the redispersal behavior (Fig. 3b).

Gel filtration with Sepharose 4B

After the 12 hr stored 11S globulin was redispersed in the phosphate buffer for 24 hr, the mixture was submitted to centrifugation $25,400\times g$, 20°C , 15 min). The supernatant was applied on a column of Sepharose 4B. The results are shown in Fig. 4. The main peak around fraction No. 42 corresponds to the 11S globulin. Fraction Nos. 21~23 are the void volume, in which the cause of turbidity of the 11S globulin solution was eluted. In the 11S globulin stored for 12 hr, the materials eluted in void volume fraction increased. Furthermore, the presence of polymers having various particle weights was observed in fraction Nos. 24~40. The polymers were sup-

posed to have approx. particle weights of $7\times 10^5\sim 10^7$ daltons and the species in the void volume had approx. particle weights more than 2×10^7 daltons using various standard proteins. Although the redispersibility did not change, it was observed that the 11S globulin had already polymerized during storage. Figure 4c shows the elution pattern of the 12 hr-stored 11S globulin which was dialyzed against a phosphate buffer containing 0.01 M 2-mercaptoethanol overnight at 5°C after it had been redispersed in the phosphate buffer for 24 hr. As shown in Fig. 4c, most of the polymers in Fig. 4b disappeared and the elution pattern was similar to that of the control, although a little polymer existed in fraction Nos. 30~35. It seems that disulfide bonds were related to the polymerization of stored 11S globulin with humidity.

Scanning electron microscopy (SEM)

Figure 5 shows the SEM micrographs of both the control and stored 11S globulins. The control 11S globulin had a globular micro structure. On the other hand, the stored 11S globulin had a highly aggregated structure.

Effects of SDS on redispersibility

It has been reported in the previous paper¹⁴⁾ that 2-mercaptoethanol had much effect on restoration of the redispersibility of the stored 11S globulin and SDS was not effective. The time course for redispersibility of the stored 11S globulin in an SDS solution was examined.

FIG. 4. Sepharose 4B Chromatography of Stored 11S Globulin.

A column of Sepharose 4B (1.7×90 cm) was eluted with the phosphate buffer (pH 7.6 ionic strength 0.5) in 3 ml fractions.

Control, a); 12 hr stored, b); 12 hr stored 11S globulin followed by dialysis against the phosphate buffer containing 0.01 M 2-mercaptoethanol, c).

FIG. 5. Scanning Electron Microscopic Observation of 11S Globulin.

Control, a); 3 day-stored, b).

FIG. 6. Changes in Redispersibility of Stored 11S Globulin in 0.5% SDS solution. One hour redispersing time (●) and 24 hr redispersing time (○).

FIG. 7. Gel Filtration Patterns of 11S Globulin Stored for 2 Days on Sephacryl S-300 in 0.5% SDS solution.

A column of Sephacryl S-300 (1.7 × 90 cm) was eluted with a 0.5% SDS solution in 3 ml fractions. After dissolving the stored 11S globulin for 24 hr, the mixture was submitted to centrifugation (25,400 × *g*, 20°C, 15 min) and the supernatant was applied on the column.

Control, a); 2 day-stored, b).

The results are shown in Fig. 6. In the case of a 1 hr redispersing time, the redispersibility began to decrease markedly after 1 day and became constant beyond 3 days (about 21%) as reported in the previous paper.¹⁴⁾ On the other hand, a slow decrease in redispersibility was observed in the case of a 24 hr redispersing time. The redispersibility was about 70% after 5 days.

Gel filtration with Sephacryl S-300

Since redispersibility in a 0.5% SDS solution decreased with time and the presence of polymerized subunits of 11S globulin linked with disulfide bonds was expected, fractiona-

tion of the stored 11S globulin was carried out with Sephacryl S-300 in a 0.5% SDS solution. The gel filtration patterns of Sephacryl S-300 are shown in Fig. 7. In the control 11S globulin, Fr. 2 with higher molecular weights was observed, as well as Fr. 3 as a major peak. When the 11S globulin was stored for 2 days, Fr. 1 newly appeared and the quantity in Fr. 3 decreased.

SDS-polyacrylamide gel electrophoresis

The species in each fraction from the gel filtration with Sephacryl S-300 were examined with SDS-PAGE. The results are shown in Fig. 8. In Fr. 1, polymers (b-1) which could not penetrate into the gel existed as main components besides b-2. Fr. 2 gave mainly b-2 and b-3 components, and intermediary subunits of 11S globulin (IS) were contained in Fr. 3. When their molecular weights were determined using standard proteins, b-1 had more than 20×10^4 , b-2 13×10^4 , b-3 11.2×10^4 and the IS 6.4×10^4 , respectively. When 2-mercap-

FIG. 8. SDS Polyacrylamide Gel Electrophoretic Patterns for Each Fraction of Fig. 7b.

SDS polyacrylamide gel electrophoresis was carried out in 8% gels.

Fr 1, 1); Fr 2, 2); Fr 3, 3), in the absence of 2-mercaptoethanol (2-ME).

Fr 1, 4); Fr 2, 5); Fr 3, 6), in the presence of 2-ME. IS is the intermediary subunits of 11S globulin. A and B are the acidic and basic subunits of 11S globulin. b-1, b-2 and b-3 are shown in the text.

FIG. 9. Gel Filtration Patterns of 11S Globulin Stored for 2 Days on Toyo Pearl HW-75 in 0.5% SDS Solution.

A column of HW-75 (1.7×90 cm) was eluted with a 0.5% SDS solution in 3 ml fractions. Control, a); 2-day stored, b).

ethanol was added to each fraction, the acidic and basic subunits, which are the constituent subunits of 11S globulin, appeared.

Gel filtration with Toyo Pearl HW-75

The 2 day-stored 11S globulin was redispersed in a 0.5% SDS solution for 24 hr and the mixture was submitted to centrifugation ($25,400 \times g$, 20°C , 15 min). The supernatant was applied on a column of Toyo Pearl HW-75 and the results are shown in Fig. 9. In both the control and the 11S globulin stored for 2 days a single peak eluted in fraction Nos. 40~50, although the peak eluted from the fraction No. 36 in the latter.

Changes in the relative amount of polymerized subunits during storage

Changes in the amount of the polymerized subunits mentioned in Fig. 8 during storage were examined. Stored 11S globulins were kept in a 0.5% SDS solution for 24 hr and the mixture was submitted to centrifugation ($25,400 \times g$, 20°C , 15 min). The supernatant was subjected to SDS-PAGE and the relative amounts of the polymer fractions (b-1, b-2 and b-3) and IS were estimated densitometrically. The results are shown in Fig. 10. In the control 11S globulin, the IS was about 88% and the total polymer fraction (polymerized subunits) was about 12%. These total polymer fractions increased with storage time up to 1

FIG. 10. Changes in Relative Amounts of the Polymerized Subunits and Intermediary Subunits during Storage.

The amount of IS (○) and the amount of the polymerized subunits (●) which is the total of b-1, b-2 and b-3.

day (about 60%) and became constant beyond 2 days.

DISCUSSION

When soybean 11S globulin was stored at RH 96% and 50°C , redispersibility in the phosphate buffer decreased. Although the stored 11S globulin did not completely redisperse and remained floating in the buffer in the case of a 1 hr redispersing time, the 11S globulins stored for up to 12 hr redispersed similarly to the control 11S globulin when the redispersing time was prolonged to 24 hr. It was, however, observed from gel filtration with Sepharose 4B that 11S globulin stored for 12 hr had already polymerized in spite of its high redispersibility. When the 12 hr-stored 11S globulin was redispersed in the phosphate buffer for 24 hr and the solution was dialyzed against a phosphate buffer containing 0.01 M 2-mercaptoethanol, most of the polymeric species in Fig. 4b disappeared as shown in Fig. 4c. It seems that disulfide bonds were related to the polymerization of 11S globulin with humidity. On the other hand, prolonging the redispersing time to 24 hr had little effect on the redispersing behavior of stored 7S globulin.

When the 11S globulins stored for more than 1 day were kept in the buffer for 24 hr, the

insoluble masses presented gels. From the scanning electron microscopic observation, although the control 11S globulin had a globular structure, this structure collapsed and the 11S globulin formed an aggregated structure during storage. Scanning electron microscopic observation of soy protein isolates have shown that they possess globular structures as well.²¹⁾ Although the stored 11S globulin had an aggregated structure, it did not have a three dimensional network structure like tofu gels. This may be the cause of the massy gels of the stored 11S globulins. The lyophilized 11S globulin formed such an aggregated structure as to be redispersed with the phosphate buffer in a 24 hr redispersing time after up to 12 hr of storage. Prolonging the storage time, however, made the 11S globulin form a stronger aggregated structure which could not be redispersed with the buffer and could be solubilized with a 0.5% SDS solution in 24 hr but not in 1 hr.

When the stored 11S globulin was submitted to SDS-PAGE in the absence of 2-mercaptoethanol, b-1, b-2, b-3 and IS appeared. Each band was composed of equal moles of the acidic and basic subunits. Eleven S globulin consists of two monomers; each monomer contains three intermediary subunits (IS-1, MW = 5.8×10^4 ; IS-2, MW = 6.0×10^4 and IS-3, MW = 6.3×10^4) consisting of one mole of the acidic subunit and one mole of the basic subunit linked with disulfide bridges.²²⁾ These intermediary subunits appeared as one band in the SDS-PAGE used in this experiment. Although bands b-2 and b-3 are supposed to be dimers of these IS's, this is not yet clear.

Although the stored 11S globulin was fractionated into three fractions with gel filtration of Sephacryl S-300, only one peak existed in Toyo Pearl HW-75 chromatography. Yamagishi *et al.* have reported the heat denaturation of 11S globulin²³⁾; when 11S globulin is heated at 100°C—0.5 ionic strength—0.5% protein concentration, the precipitate is formed and gel filtration of the precipitate with Sepharose CL-2B in a 0.5% SDS solution gives two peaks. The first peak contains aggregates of

highly polymerized subunits, and the second peak contains a monomer of the basic subunit and seven kinds of oligomers, with various proportions of the basic subunits to the acidic subunit. In our study, gel filtration with Toyo Pearl HW-75 gave one peak. It is considered that the subunits of 11S globulin did not polymerize as much as they did in heat denaturation with disulfide bridges. The number of polymerized subunits increased during storage. It seems that disulfide bridges were mainly formed between 11S globulin molecules. Consequently, 11S globulin seems to polymerize (Fig. 4c), then, aggregate and finally become insoluble during storage with humidity. SDS probably cleaved bonds other than the disulfide bridges and increased the redispersibility, when the stored 11S globulin aggregates were kept in the 0.5% SDS solution.

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