Determination of Sulfhydryl and Disulfide Contents of Soybean 11S Globulin and Their Change by Lyophilization

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Sulfhydryl (SH) and disulfide (SS) contents of soybean 11S globulin (var. Raiden) were determined by means of Ellman’s reagent. The amounts of surface SH, internal SH, and SS bonds of 11S globulin (not lyophilized) were 10.3, 4.6, and 17 mol/mol protein, respectively. On the other hand, when the 11S globulin was lyophilized, the surface and internal SH diminished to 5 and 3.6 mol/mol protein, respectively, and the SS bonds increased to 20.1 mol/mol protein. The result from sodium dodecyl sulfate polyacrylamide gel electrophoresis suggested the possibility that the newly formed SS bonds probably existed in each constituent subunit of 11S globulin and/or between the intermediary subunits which exist by nature. Differences and similarities between our result and those obtained by various workers were also discussed.

Eleven S globulin is the major storage protein of soybean seeds and has intermediary subunits (IS; MW 58,000~63,000), disulfide-bonded acidic (MW 37,000~45,000) and basic (MW 21,000~22,500) subunits, and is composed of 6 IS’s. We have studied the effects of relative humidity on soybean 11S globulin (var. Raiden), and it was found that SS bonds are closely related to the aggregation of lyophilized 11S globulin with humidity. Furthermore, sulfhydryl and disulfide groups have been implicated in the formation of SS polymers, heat denaturation, and intersubunit association of the protein. Mori et al. have reported that there are differences in SH contents among cultivars. Precise SH and SS contents on var. Raiden which we have used have seldom been reported. Therefore, we determined the SH and SS contents of 11S globulin (var. Raiden), examining the determining method. Furthermore, since we have used a lyophilized 11S globulin in a series of studies and the SH and SS contents were expected to change after lyophilization, the effects of lyophilization on the SH and SS contents of the proteins were also investigated.

MATERIALS AND METHODS

Materials. Soybean seeds (var. Raiden, 1981 crop) were stored at 5°C, ground with a coffee mill, screened through a 60 mesh sieve, and defatted with hexane. Bovine serum albumin (BSA, fraction five) was purchased from Nakarai Chemicals Co., Ltd. Soybean trypsin inhibitor (STI) was purchased from Sigma Chemical Co., St. Louis (U.S.A.). Dithiothreitol (DTT), 2-mercaptoethanol (2-ME), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), sodium dodecyl sulfate (SDS) and Coomassie Brilliant Blue G-250 were specially prepared reagents and all other reagents were of the highest grade, obtained from Nakarai Chemicals Co., Ltd. Guanidine hydrochloride was recrystallized from methanol. Sepharose 4B and Sephacryl G-25 were purchased from Pharmacia Fine Chemicals.

Preparation of 11S globulin. Defatted soybean meals were extracted with 0.03 M Tris-HCl buffer (pH 8.0) containing 0.01 M 2-ME and 0.001 M ethylenediaminetetraacetic acid disodium salt (EDTA–2Na) (meal: buffer, 1:20) for 1 hr and centrifuged (10,000 rpm for 20 min at 20°C). The extract was adjusted to pH 6.4 with 2 N HCl and stirred for 1 hr at 5°C. The suspension was centrifuged (10,000 rpm, 20 min, 5°C) to precipitate the crude 11S globulin and washed with 0.03 M Tris-HCl buffer (pH 6.4) two times. The crude 11S globulin was dissolved in a phosphate buffer (2.6 mM KH2PO4, 32.5 mM K2HPO4, 0.4 mM NaCl, 0.01 M 2-ME, 0.001 M EDTA–2Na, 0.05% NaN3, pH 7.6) and left overnight at 5°C (the standard
buffer hereafter means this buffer and the phosphate buffer means the standard buffer without 2-ME). The crude 11S globulin was further purified by ammonium sulfate fractionation essentially according to the method of Wolf et al. To the crude 11S globulin solution ammonium sulfate was added to 51% saturation. The supernatant was adjusted to 66% saturation with ammonium sulfate and the precipitate of 11S globulin was dissolved in the standard buffer (stock solution). An aliquot of the stock solution was dialyzed against the standard buffer overnight and submitted to gel filtration of Sepharose 4B in the phosphate buffer, and the rest was dialyzed against water and then lyophilized. After lyophilization, determinations were carried out promptly.

Determination of sulphydryl groups. The method was essentially that described by Ellman. One ml of a protein solution (about 0.2% protein concentration) was mixed with 2 ml of the phosphate buffer. To this mixture 0.05 ml of DTNB solution (36.9 mg of DTNB dissolved in 10 ml of a phosphate buffer with ionic strength 0.1, pH 7.0) was added. In the case of the determination of the sulphydryl content in the presence of denaturants, 1 ml of a protein solution was mixed with 2 ml of the phosphate buffer containing 6 M guanidine hydrochloride. To this solution 0.05 ml of DTNB solution was added. Since the color developed within 5 min, the absorbance at 412 nm was measured 10 min later. The absorbance is expressed as SH mol/mol protein, using the molecular extinction coefficient (13,600 M/cm) for the calculation. Protein concentration (m) was determined from measurement of absorbance at 280 nm, using the value of 8.04 (E1% cm) and the molecular weight of 360,000. The SH value determined in the absence of the denaturant was defined as the surface SH and that in the presence of the denaturant was defined as the total SH. The SH value after subtracting the surface SH from the total SH was defined as the internal SH.

Determination of disulfide bonds. Two ml of a protein solution (about 0.2% protein concentration) was mixed with 4 ml of the phosphate buffer containing 9 M guanidine hydrochloride. To the mixture, 16 mg DTT was added, and then the protein was completely reduced for 4 hr under N2 gas at room temperature. After reduction, the solution was submitted to gel filtration on Sephadex G-25 equilibrated with 0.03 M Tris-HCl buffer (0.01 M EDTA–2Na, 0.05% NaN3, 4 M guanidine hydrochloride, pH 8.0) to eliminate DTT. The effluents were collected in 5 ml fractions and the absorbance of each fraction was monitored at 280 nm. The fractions containing protein were submitted to SH determination as described above. Half of the value after subtracting the total SH from this obtained value was defined as the SS content. All the glass vessels were washed with 0.001 M EDTA–2Na solution and then washed with distilled water before determining the SH or SS contents.

Electrophoresis. Disc gel electrophoresis was performed in a 6.5% polyacrylamide gel according to Ornstein and Davis. SDS polyacrylamide gel electrophoresis was carried out in a 8% gel according to Swank et al. All gels were stained with Coomassie Brilliant Blue G-250 and destained by diffusion in 7.5% CH3COOH–5% CH3OH–water.

RESULTS

SH Contents

Before determining the SH content of the 11S globulin, we examined whether the determining method described in MATERIALS AND METHODS was valid. Cysteine and BSA were used as standard materials. The results are shown in Table I. Cysteine (0.0129 g) was dissolved in 1000 ml of the phosphate buffer (1.06 x 10^-5 M). When this cysteine solution was determined with DTNB, the SH content was 1.06 x 10^-5 M in the absence of guanidine hydrochloride and was 1.05 x 10^-5 M in the presence of guanidine hydrochloride. Further, when BSA was determined with DTNB, the SH content was 0.57 mol/mol protein as surface SH and was 0.6 mol/mol protein as total SH. The molecular weight of 66,000 and E1% cm of 6.6 were used for calculation. The SH contents of BSA agreed with the reference. From these results, the validity of the determining method was verified.

An aliquot of the stock solution was submitted to a gel filtration on Sepharose 4B. Although 2-ME was used during the extraction and ammonium fractionation, 2-ME had to be completely eliminated before the determination of the SH groups of the 11S globulin.

Table I. Concentrations of SH and SS Groups Determined with DTNB

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<th>Cysteine (m)</th>
<th>BSA (mol/mol protein)</th>
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<tbody>
<tr>
<td>SH</td>
<td>1.06 x 10^-5</td>
<td>0.57</td>
</tr>
<tr>
<td>(+GuHCl)</td>
<td>1.05 x 10^-5</td>
<td>0.60</td>
</tr>
<tr>
<td>SS</td>
<td>16.8</td>
<td></td>
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* GuHCl, guanidine hydrochloride.
SH, SS Contents of Glycinin

Fig. 1. Gel Filtration Pattern of Mixture of 11S Globulin and 2-ME on Sepharose 4B.

A column of Sepharose 4B (1.7 x 95 cm) was eluted with the phosphate buffer in 3 ml fractions. Eleven S globulin was detected by the absorbance at 280 nm and 2-ME at 412 nm, reacted with DTNB.

Fig. 2. Chromatogram of 11S Globulin by Gel Filtration.

As shown in Fig. 1, 11S globulin eluted in fractions 36 ~ 53 and 2-ME eluted in fractions 56 ~ 65. Thus, 2-ME could be completely removed from the 11S globulin. A representative elution pattern of the 11S globulin which was prepared from the defatted meals and not lyophilized during the preparation procedure is shown in Fig. 2. Fractions 41 ~ 48 (underlined part, M fraction) were collected. Polyacrylamide gel electrophoretic patterns of the M fraction are shown in Fig. 3. In the Davis system, bands of 11S globulin and 7S globulin were observed (Fig. 3a). Eleven S globulin is the dimer of 7S globulin and 11S globulin dissociates into 7S globulin in low ionic strengths. Because of the low ionic strength in the Davis system, 7S globulin occurred during electrophoresis. Figures 3b and c show SDS polyacrylamide gel electrophoretic patterns in the absence and presence of 2-ME, respectively. In Fig. 3b, an intermediary subunit band was observed as a predominant band besides two minor bands. One of the two minor bands might be an acidic subunit which Iyengar et al.\(^{14}\) have called the “free” acidic subunit and the other might be the dimer of basic subunits (BS\(_2\)) reported by Mori et al.\(^{15}\) Acidic subunits (A\(_1\) ~ A\(_4\)) and basic subunits (B\(_1\) ~ B\(_4\)) of 11S globulin newly appeared in the presence of 2-ME as shown in Fig. 3c. The minor band (minor) which was reported by Kitamura et al.\(^{16}\) was also observed. The SH contents of the purified 11S globulin are sum-

![Table II](https://www.journals.org/resource/0/0/0/7-17-01-00-84.png)

**Table II. SH and SS Contents of 11S Globulin**

<table>
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<tr>
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<th>11S Globulin (Not lyophilized)</th>
<th>11S Globulin (Lyophilized)</th>
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<tbody>
<tr>
<td>Surface SH</td>
<td>10.3</td>
<td>5.0</td>
</tr>
<tr>
<td>Internal SH</td>
<td>4.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Total SH</td>
<td>14.9</td>
<td>8.6</td>
</tr>
<tr>
<td>SS</td>
<td>17.0</td>
<td>20.1</td>
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</table>
Fig. 4. Chromatogram of Lyophilized 11S Globulin by Gel Filtration.
A column (Sepharose 4B, 1.7 x 95 cm) was eluted with the phosphate buffer in 3 ml fractions.

Fig. 5. Polyacrylamide Gel Electrophoretic Patterns of Lyophilized 11S Globulins.
Electrophoretic patterns of M fraction (a) and L fraction (b) in Fig. 4 in the Davis system. Electrophoretic patterns of the M fraction in the SDS system without 2-ME (c), with 2-ME (d) and L fraction without 2-ME (e).

SS Contents
Before determining the SS content of the 11S globulin, the validity of the determining method of SS content was examined with BSA as the standard protein. The results are shown in Table I. The SS content of BSA was 16.8 mol/mol protein and agreed well with the reference.17)

Determination of the 11S globulin was carried out. Two ml of the underlined parts in Fig. 2 was completely reduced and the solution was submitted to a gel filtration of Sephadex.
G-25. As shown in Fig. 6, DTT could be eliminated from STI (MW 21,500). Since the molecular weights of the subunits of 11S globulin were 21,000~45,000, DTT could be removed from 11S globulin subunits with this gel filtration. The SS contents of the 11S globulin were summarized in Table II. The SS content of the 11S globulin was 17 mol/mol protein and of the lyophilized 11S globulin increased to 20.1 mol/mol protein.

DISCUSSION

Several authors have been reported the sulfhydryl, disulfide, and half cystine contents of 11S globulin by various methods.\(^4\)\(^,\)\(^5\)\(^,\)\(^18\)\(^~\)\(^21\) Results, however, are different among the authors and SH and SS contents have seldom been reported on var. Raiden\(^5\) which we have used in a study of the effect of relative humidity on soybean proteins.\(^2\)\(^,\)\(^3\) Therefore, we determined the SH and SS contents of 11S globulin (var. Raiden) by means of DTNB. Diez et al. have reported that DTNB was the most useful reagent for the determination of SH contents.\(^13\) The SH values obtained for cysteine and BSA agreed well with the calculated value and the reference.\(^13\)\(^,\)\(^17\) The SH groups of BSA are located on the surface of the protein\(^17\) and the SH content of BSA (fraction five) has been reported to be 0.57 mol/mol protein.\(^13\) Furthermore, the SS content of BSA is 17 mol/mol protein.\(^17\) The determining method we used was useful enough to determine accurate SH and SS contents of proteins.

When the 11S globulin was prepared from defatted soybean meal in the presence of 2-ME, the surface and internal SH contents were 10.3 and 4.6 mol/mol protein, respectively. When soybean 7S and 11S globulin are prepared, 2-ME has been usually used since 2-ME dissociates disulfide polymers and raises extractability. SS bonds which locate on the surface may be cleaved by 2-ME used in the protein preparation. Simard et al. have reported that 11S globulin (cultivar not given) has 1.1 mol/mol protein as surface SH, 4.5 mol/mol protein as internal SH, and 5 mol/mol protein as surface SS.\(^20\) If the surface SS (5 mol) is cleaved, the surface SH becomes 11.1 mol/mol protein and this value is similar to our result. Furthermore, the internal SH contents also agree with each other. On the other hand, Mori et al. have reported that there are differences in SH contents of 11S globulin among soybean cultivars and var. Raiden has 0.5 mol/mol protein as surface SH and 1.4 mol/mol protein as internal SH.\(^5\) Draper et al. have reported that 11S globulin (cultivar not shown) has 1.7 SH groups only in the interior region.\(^4\) These discrepancies may be due to the differences in preparation methods or cultivars. Furthermore, it may also be that the differences in determination procedures caused these discrepancies. In practice most of the surface SH, however, seemed to exist as SS bonds in a protein body. The SH groups were hard to reoxidize into SS bonds in our preparation method because of the EDTA–2Na contained in the preparation and determination system, when the SS bonds were once cleaved.

When the 11S globulin was lyophilized, the surface and internal SH diminished to 5 and 3.6 mol/mol protein, respectively. About 2.6 and 0.5 mol/mol protein SS bonds were newly formed on the surface and in the interior region, respectively. Differences between Figs. 3b and 5c were almost not observed. The newly formed SS bonds probably existed in each constituent subunit and/or between the intermediary subunits, which exist by nature. When the 11S globulin was lyophilized, gel filtration study showed that species with low molecular weights (L fraction) were formed (Fig. 4). SDS polyacrylamide gel electrophoresis showed that IS and acidic subunits (\(A_1 \sim A_3\)) were contained in the L fraction (Fig. 5e). This showed that a part of the 11S globulin dissociated into IS and its constituent subunits during lyophilization. Since the isoelectric points of basic subunits are near the pH value of the phosphate buffer, dissociated basic subunits were probably precipitated and not observed in SDS polyacrylamide gel elec-
trophoresis. The reason why the A₄ subunit was observed to such a small extent is not known yet. Figures 3a and 5a showed that there are no differences among the 11S globulins in each underlined part (Figs. 2 and 4).

The SS contents of the 11S globulin were 17 (not lyophilized) and 20.1 (lyophilized) mol/mol protein, respectively. The sum of the total SH and twice as many as the amount of SS bonds was 48.9 mol/mol protein. Catsimpoolas et al. have reported that 11S globulin has 44 ± 6 half cystine residues (MW 350,000). Draper et al. have also reported that 11S globulin has 41.8 half cystine residues (MW 320,000). Further, 42 half cystine residues have been reported by Badley et al. (MW 320,000). When these values are estimated on the basis of MW 360,000, 11S globulin should contain 45 ± 6, 47, and 47 half cystine residues, respectively. These values agree with our value. On the other hand, Fukushima and Simard et al. have reported that 11S globulin (Fukushima, var. Tsurunoko; Simard, not shown) have 36 and 43 half cystine residues (MW 360,000), respectively. These values are slightly lower than ours and this difference may be due to preparation procedures and/or cultivars.

REFERENCES