Subunit Structure of Soybean 11S Globulin

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The acidic and the basic subunits were shown to be present in equimolar amounts in the 11S globulin molecule by the densitometric scanning of the SDS gel and the molecular weight consideration. The four acidic subunits (A_1 , A_2 , A_3 and A_4) were found to be present in the approximate molar ratio of 1:1:2:2. Four basic subunits separated and designated as B_1 , B_2 , B_3 and B_4 based on the relative mobilities in the acidic gel in 7 M urea were found to be present in the approximate molar ratio of 1:1:2:2. The four basic subunits were fractionated in approximately same amounts into three different peaks, peak I (B_1 and B_2), peak II (B_3) and peak III (B_4) by CM-Sephadex C-50 column chromatography in the presence of 6 M urea. Three kinds of intermediary subunits of 11S globulin were fractionated with DEAE-Sephadex A-50 in the absence of reducing agents in 6 M urea, and disulfide bonds appeared to participate in the binding between the acidic and the basic subunits in the molar ratio of 1:1 with the following combinations; A_1 and A_2 combined with B_3 , A_3 with B_1 and B_2 , and A_4 with B_4 . In view of the above results and molecular weight consideration, a new model of subunit structure was proposed for 11S globulin.

Catsimpooulas *et al.*¹⁾ reported that 11S globulin which is one of the main storage proteins in soybean seed consisted of the three kinds of acidic and the three kinds of basic subunits. However, Kitamura and Shibasaki²⁾ recently revealed that the four kinds of acidic subunits and at least three kinds of basic subunits of 11S globulin were separated by the disc electrophoresis in 7 M urea, and the former subunits were isolated with DEAE-Sephadex A-50 in the presence of 6 M urea. However, neither each acidic nor each basic subunit was present in equimolar amounts as estimated from the densitometric scanning of amido black 10 stain. A subunit composition of 11S globulin is still not clear.

In the present investigation, a modified procedure of the previous method³⁾ has been used for a purification of 11S globulin. The

subunit composition of 11S globulin has been investigated by ion exchange column chromatography in the presence of 6 M urea, SDS gel electrophoresis, the disc and the acidic gel electrophoresis in 7 M urea. Further, fractionation of intermediary subunits of 11S globulin has been done with DEAE-Sephadex A-50 in the absence of reducing agents in 6 M urea. In order to prevent sulfhydryl-disulfide exchange reactions during the fractionation, all free sulfhydryl groups in 11S globulin were blocked with iodoacetoamide in the presence of urea. Combinations between the acidic and the basic subunits were revealed by SDS gel and the acidic gel electrophoresis in 7 M urea after treatment of the fractionated intermediary subunits with 2-mercaptoethanol(2-ME) in the presence of urea or SDS. The data obtained led us to present a subunit structure of 11S globulin.

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Abbreviations: SDS, sodium dodecyl sulfate; 2-ME, 2-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid; CAM-11S, nonreduced S-carboxyamidomethylated 11S globulin; RCAM-11S, reduced S-carboxyamidomethylated 11S globulin; IS, intermediary subunit.

MATERIALS

Soybean (var. Raiden) harvested at Furukawa, Miyagi, Japan in 1974 was used throughout this work. Urea used for electrophoresis was specially prepared

reagent for biochemical research purchased from Nakarai Chemicals Ltd. All the other reagents were the highest grade. ConA-Sepharose 4B, Sepharose 6B, DEAE-Sephadex A-50 and CM-Sephadex C-50 were obtained from Pharmacia.

METHODS

Preparation of Wolf's fraction VI. Ten grams of defatted soybean meal were suspended in 1000 ml of 0.065 M NaCl solution containing 0.01 M 2-ME, adjusted to pH 8.0 with 1 N NaOH. The solution was filtered through several layers of gauze and then centrifuged. The supernatant was adjusted to pH 6.3 with 1 N HCl, stirred for 15 min and centrifuged. The above procedures were performed at 5°C. The precipitate (Okubo's crude 11S globulin)⁴⁾ was subjected to ammonium sulfate fractionation to prepare "fraction VI" described by Wolf *et al.*⁵⁾ The precipitation procedure at pH 4.0 between 26 and 40% saturation was omitted, since proteins (2S, 15S and >15S proteins) removed by this precipitation could be readily removed by a gel filtration with Sepharose 6B.

Purification of 11S globulin. Twenty ml of the fraction VI (about 2% protein concentration) in the pH 7.6 standard buffer containing 0.1% NaN₃ was applied on a ConA-Sepharose 4B column (2.2 × 25 cm) equilibrated with the buffer and eluted at a flow rate of 10 ml per hour with the same buffer. After non-adsorbed fraction eluted, 30 ml of the buffer containing 0.1 M α -methyl-D-mannoside was added to the column and washed with the first buffer to elute adsorbed fraction. The non-adsorbed fraction was concentrated by ultrafiltration and chromatographed on a Sepharose 6B column. For further purification, ion exchange chromatography was performed, because faint bands which were unlikely to be subunit bands of 11S globulin were observed on a SDS gel of the 11S globulin purified with ConA-Sepharose 4B and Sepharose 6B from the fraction VI. The eluate in the main peak on the Sepharose 6B column was concentrated and dialyzed against 0.035 M phosphate buffer (pH 7.6) containing 0.2 M NaCl and 0.01 M 2-ME, and then applied on a DEAE-Sephadex A-50 column equilibrated with the buffer. Ionic strength gradient elution was achieved by adding 0.5 M NaCl solution to a mixing chamber containing 500 ml of the buffer.

Preparation of CAM-11S. 100 mg of the lyophilized 11S globulin was dissolved in 0.15 M tris-Cl (pH 8.6) in the presence of 8 M urea and 1 mM EDTA to a protein concentration of 1% under nitrogen gas. A small amount of undissolved materials was centrifuged off. The carboxyamidomethylation of free sulphydryl groups in the protein was performed by adding 0.135 g

of iodoacetoamide in the dark for 15 min. The modified protein was dialyzed against deionized water in the dark and lyophilized.

Electrophoresis of subunit. SDS gel electrophoresis was performed according to the method of Weber and Osborn⁶⁾ on 10% polyacrylamide gel. Molecular weights of the intermediary subunits were estimated by SDS gel electrophoresis in the absence of reducing agents. The standard proteins (bovine serum albumin, catalase, γ -globulin and ovalbumin) were electrophoresed in the presence of reducing agents on SDS gels. The disc gel electrophoresis in 7 M urea was performed according to the previous method.²⁾ Acidic gel electrophoresis in 7 M urea was performed by using a 6.0 × 100 mm glass tube in 10% CH₃COOH on 7.5% polyacrylamide gel. Gels were prepared as follows; for 10 gels, 5.0 ml of acrylamide solution (acrylamide 30.0 g, methylenebisacrylamide 0.8 g, and water to 100 ml) were mixed with 2.0 ml CH₃COOH, 4.0 ml of 5% N,N,N',N'-tetramethylethylenediamine and 1.4 ml of water, and 8.4 g of urea was dissolved. After deaeration, 2.0 ml of ammonium persulfate (10.5 mg per ml) was added. After further deaeration, each tube was filled with the solution up to 1 cm from the top of the tube. The gel was solidified after layering water on the top of the gel solution within a half hour at 25°C. Preelectrophoresis was carried out to remove excess ammonium persulfate and to get a constant resistance inside the gels. Electrophoresis was performed for 3.0 hr at a constant voltage of 150 V in 10% acetic acid. All gels were stained with Coomassie Blue and destained by diffusion in 7.5% CH₃COOH-5% CH₃OH-water.

Ion exchange chromatography of subunit. DEAE-Sephadex A-50 column and CM-Sephadex C-50 column chromatography were performed using 2.0 × 25 cm column in 0.12 M sodium phosphate buffer (pH 6.5) and in 0.09 M sodium phosphate buffer (pH 6.7) respectively in the presence of 6 M urea increasing the concentration of NaCl gradiently. A fractionation of the intermediary subunits was performed as follows; a column (2.0 × 25 cm) was prepared with DEAE-Sephadex A-50. 80 mg of CAM-11S dissolved in about 5 ml of 0.1 M sodium phosphate buffer (pH 6.5) containing 1 mM EDTA and 6 M urea was applied on the column equilibrated with the buffer. Ionic strength linear elution was achieved by adding 0.4 M NaCl solution to a mixing chamber containing 500 ml of the buffer. Fractions were monitored at 280 nm.

RESULTS

Purity of the purified 11S globulin

The underlined part of the elution profile

FIG. 1. DEAE-Sephadex A-50 Column Chromatogram of 11S Globulin.

Absorbance at 280 nm, —; NaCl gradient, ----; Sample, about 300 mg of the 11S globulin purified with ConA-Sepharose 4B and Sepharose 6B from the Wolf's fraction VI; Column size, 2.5×30 cm; Flow rate, 12~15 ml per hour; Volume of fraction, 9.4 ml; Temperature, 5°C ; Elution, 0.035 M potassium phosphate buffer (pH 7.6) with 0.2 M NaCl and 0.01 M 2-ME containing NaCl in gradient concentration of 0 to 0.75 M. Insets are sedimentation pattern (A) and polyacrylamide gel electrophoretic pattern (B) of the underlined part. Sedimentation is from left to right after 40 min at 52,000 rpm, at 0.7% protein concentration. The gel and electrode buffers were 0.035 M potassium phosphate buffer (pH 7.6). Electrophoresis was carried out for 3.5 hr at 4 mA per a gel (6.5% gel).

shown in Fig. 1 on the DEAE-Sephadex A-50 column was obtained as a purified 11S globulin. In the ultracentrifuge, a single symmetrical sedimentation peak was obtained and a single electrophoretic band was observed by the disc gel electrophoresis in 0.035 M phosphate buffer (pH 7.6). However, the faint bands which represented less than 1~2% of the total bands and were unlikely to be subunit bands of 11S globulin were also observed on the SDS gel of the purified 11S globulin. The faint bands may be contaminants attached to 11S protein in the mild buffers. A similar observation was also presented by Catsimpoolas *et al.*⁷⁾

Electrophoresis of subunit

The electrophoretic and the densitometric

patterns of the SDS gel electrophoresis of RCAM-11S shown in Fig. 2 show that the areas under the densitometric peak I, II and III are approximately 2:2:1. The previous results²⁾ showed that the peak I, II and III corresponded to the total basic subunits (MW 22,500), A_1 , A_2 and A_3 subunits (MW 37,000) and A_4 subunit (MW 45,000) respectively. Thus, the above results indicates that

FIG. 2. SDS Gel Electrophoretic Pattern and Densitometric Scanning of RCAM-11S.

Densitometric scanning was obtained with a Beckman ACTA CIII UV Visible spectrophotometer.

FIG. 3. Disc Electrophoretic Pattern in Alkaline Urea System and Densitometric Scanning of RCAM-11S.

the molar ratio of the total basic to the total acidic subunits in the 11S globulin molecule is approximate 1:1. The densitometric scanning of the disc gel electrophoresis of RCAM-11S in 7 M urea shown in Fig. 3 shows that the four acidic subunits (A_1 , A_2 , A_3 and A_4) occur in the stoichiometric proportions: 1:1:2:2. This ratio is in a good agreement with

the ratio of A_4 to the total amount of A_1 , A_2 and A_3 determined by the result of SDS gel electrophoresis. Figure 4 shows the electrophoretic and the densitometric patterns of the acidic gel electrophoresis of RCAM-11S in 7 M urea. Of six electrophoretic bands, the fast moving bands consisted of four basic subunits designated as B_1 , B_2 , B_3 and B_4 subunit based on these relative mobilities, the slow moving bands consisted of the acidic subunits. The four basic subunits (B_1 , B_2 , B_3 and B_4) exist in the approximate molar ratio of 1:1:2:2 in the 11S globulin molecule.

FIG. 4. Polyacrylamide Gel Electrophoretic Pattern in Acidic Urea System and Densitometric Scanning of RCAM-11S.

Fractionation of subunit

The fractionation profile of the acidic subunits was illustrated in Fig. 5. It can be seen from the electrophoretic patterns of the fractions that A_1 and A_2 elute in peak II, A_3 in peak III and A_4 in peak IV. Each peak area under the three peaks monitored at 280 nm and at 750 nm (the Folin-Lowry reaction⁸⁾) is approximately equivalent. This ratio is in a good agreement with the ratio of the total amount of A_1 , A_2 , A_3 and to A_4 subunit determined by the densitometric scanning of the disc gel electrophoresis of

FIG. 5. Fractionation Profile of the Acidic Subunits with DEAE-Sephadex A-50 in Urea.

Absorbance at 280 nm, —; Arbitrary absorbance at 750 nm (Folin-Lowry reaction), -----; Sample, 100 mg of RCAM-11S; Column size, 2.5×25 cm; Flow rate, 10~12 ml per hour; Volume of fraction, 7.9 ml; Temperature, 5°C; Elution, 0.12 M sodium phosphate buffer (pH 6.5) in the presence of 6 M urea containing NaCl in gradient concentration of 0 to 0.4 M. Insets are electrophoretic patterns of RCAM-11S(*) and the fractions (F-II, III and IV) in the alkaline urea system.

FIG. 6. Fractionation Profile of the Basic Subunits with CM-Sephadex C-50 in Urea.

Absorbance at 280 nm, —; Arbitrary absorbance at 750 nm, -----; Sample, the unadsorbed fraction (F-I in Fig. 5); Column size, 2.5×25 cm; Flow rate, 10~12 ml per hour; Volume of fraction, 7.9 ml; Temperature, 5°C ; Elution, 0.09 M sodium phosphate buffer (pH 6.7) in the presence of 6 M urea containing NaCl in gradient concentration of 0 to 0.3 M. Insets are electrophoretic patterns of RCAM-11S(*) and the fractions (F-I, II and III) in the acidic urea system.

FIG. 7. Fractionation Profile of Intermediary Subunit of 11S Globulin with DEAE-Sephadex A-50 in the Absence of Reducing Agents in Urea.

Absorbance at 280 nm, ●—●; NaCl gradient, -----; Sample, 80 mg of CAM-11S; Column size, 2.5×25 cm; Flow rate, 10~12 ml per hour; Volume of fraction, 6.0 ml; Temperature, 5°C ; Elution, 0.1 M sodium phosphate buffer (pH 6.5) in the presence of 6 M urea and 1 mM EDTA containing NaCl in gradient concentration of 0 to 0.4 M.

RCAM-11S in 7 M urea. The fractionation profile of the basic subunits was illustrated in Fig. 6. It can be seen from the electrophoretic patterns shown in Fig. 6 that B_1 and B_2 elute in peak I, B_3 in peak II and B_4 in peak III. Each peak area under the three peaks monitored at 280 nm is approximately equivalent, and this ratio is in a good agreement with the molar ratio of the total amount of B_1 , B_2 , B_3 and to B_4 in the 11S globulin molecule determined by the densitometric scanning of

the acidic gel electrophoresis of RCAM-11S in 7 M urea. But the peak area under peak III monitored at 750 nm is about a half of that under peak I and II respectively, and this result suggests that the amino acid composition of B_4 subunit may be different from those of the other three basic subunits.

Fractionation of intermediary subunit

The fractionation profile of the intermediary subunits was illustrated in Fig. 7. Four

FIG. 8-A. Acidic Urea Gel Electrophoretic Patterns of Non-reduced CAM-11S and Non-reduced Fractions in Fig. 7.

CAM-11S (a), F-I (b), F-II (c), F-III (d) and F-IV (e).

FIG. 8-B. SDS Gel Electrophoretic Patterns of Non-reduced CAM-11S and Non-reduced Fractions in Fig. 7.

CAM-11S (i), F-II (h), F-III (g) and F-IV (f).

fractions (F-I, II, III and IV) were obtained and freeze-dried after dialysis against deionized water. Proteins eluted in F-II, III and IV gave one major electrophoretic band respectively both on the acidic gels in 7 M urea (Fig. 8-A) and on the SDS gels (Fig. 8-B), and designated as IS-1, IS-2 and IS-3 respectively. IS-1, IS-2 and IS-3 migrated at relative mobilities corresponding to molecular weights of 58,000, 60,000 and 63,000 respectively on the SDS gels.

Polypeptide composition of intermediary subunit

The lyophilized intermediary subunits were dissolved in 0.2 M 2-ME solutions (pH 7~8) in the presence of 8 M urea or 0.1% SDS, and then applied on the acidic gels in 7 M urea and on the SDS gels. It is observed from the electrophoretic patterns shown in Fig. 9 that IS-1 consists of A₁, A₂ and B₃, IS-2 consists of A₃, B₁ and B₂, and IS-3 consists of A₄ and B₄ with the approximate molar ratio of the total acidic to the total basic subunits of 1:1, respectively. Proteins in F-I may be mainly the basic subunits of which disulfide bonds between the acidic and the basic subunits have already been cleaved

FIG. 9-A. Acidic Urea Gel Electrophoretic Patterns of RCAM-11S and Reduced Fractions in Fig. 7.

RCAM-11S (a), F-I (b), F-II (c), F-III (d) and F-IV (e).

FIG. 9-B. SDS Gel Electrophoretic Patterns of RCAM-11S and Reduced Fractions in Fig. 7.

RCAM-11S (i), F-II (h), F-III (g) and F-IV (f).

during the purification of 11S globulin by the action of 0.01 M 2-ME in the buffers for the purification.

DISCUSSION

Catsimpoolas *et al.*¹⁾ supposed that the 11S globulin molecule consisted of two identical monomers having the three kinds of acidic and the three kinds of basic subunits. However, the present results showed that the four kinds of acidic (A₁, A₂, A₃ and A₄) and the four kinds of basic (B₁, B₂, B₃ and B₄) subunits presented in the molar ratio of 1:1:2:2 respectively in the 11S globulin molecule, and that disulfide bonds participated in the linkage of the acidic and the basic subunits in the molar ratio of 1:1. Although, the two acidic subunits A₁ and A₂, and also the two basic subunits B₁ and B₂ showed the very similar chromatographic and electrophoretic behaviours, it is unlikely that these subunits are artifacts resulted from the action of proteolytic enzymes or the chemical modification during purification, because the same two acidic and the same two basic subunits were shown to be present in freshly prepared 8 M urea - 0.2 M 2-ME extracts of soybean meals on the disc and on the acidic gel electrophoresis in 7 M urea (unpublished

results). The electrophoretic behaviours and the molecular weights of the fractionated intermediary subunits in the absence of reducing agents in 7 M urea or 0.1% SDS solutions can only be explained by postulating that the acidic and the basic subunits are linked by disulfide bonds in the approximate molar ratio of 1:1 in the 11S globulin molecule. Although, the presence of disulfide bonds in protein can lead to erroneous molecular weights estimated by SDS gel electrophoresis, the molecular weights of IS-1, IS-2 and IS-3 (58,000, 60,000 and 63,000 respectively) were in a relatively good agreement with the corresponding combined molecular weights of a pair of the acidic and the basic subunits and with the molecular weight (61,000) of 11S globulin determined by sedimentation equilibrium in 6 M guanidium hydrochloride solution adjusted to pH 2 with 1 N HCl (unpublished result).

Very recently, Badley *et al.*⁹⁾ reported that they succeeded in the preparation of the 11S protein which did not give the faster running component (the monomer of 11S globulin) on the polyacrylamide gel in Ornstein-Davis electrophoretic system (pH 8.9). However, we observed that the 11S globulin purified with ConA-Sepharose 4B and Sepharose 6B from the Wolf's fraction VI showed a single symmetrical peak on a Sepharose 6B column equilibrated with 1 M KH_2PO_4 buffer (pH 7.6) containing 0.01 M 2-ME (their gel chromatographic buffer), and that 11S protein eluted in a middle part of the single peak dialyzed against the pH 7.6 standard buffer gave two electrophoretic bands, the dimer band (major) and the monomer band (minor) as reported previously³⁾ on the ordinary polyacrylamide gel (pH 8.9). We can not evaluate clearly their results, because our purification procedure is different from theirs and they have not reported the yield of the 11S protein obtained by their method. The 11S globulin purified by our method at least showed a dissociation reaction as an intrinsic property at a slightly alkaline pH at a low ionic strength as observed by Wolf *et al.*¹⁰⁾ in sedimentation. Thus, we

agree that the most stable form of 11S globulin is the dimer form as suggested by Catsimpoolas *et al.*¹⁾

From the above results and the discussion, it is proposed that 11S globulin consists of two similar, but not identical monomers; either monomer containing three intermediary subunits consists of one mole of either A_1 or A_2 , one mole of A_3 , one mole of A_4 , one mole of either B_1 or B_2 , one mole of B_3 and one mole of B_4 . Thus, the dimer (11S globulin molecule) should exhibit a molecular weight of 362,000 due to the molecular weights of the intermediary subunits estimated by SDS gel electrophoresis, or a molecular weight of 373,000 due to the subunit molecular weights estimated by SDS gel electrophoresis.³⁾ These values are in a relatively good agreement with the value of 363,000¹¹⁾ reported.

The presence of at least six disulfide bonds in the 11S globulin molecule indicates that disulfide bonds also participate to maintain the subunit structure of 11S globulin in addition to hydrophobic, electrostatic and hydrogen bonds. A dissociation scheme for 11S globulin is proposed as follows;

Wright and Boulter¹²⁾ proposed the similar intermediary subunits for the legumin of *Vicia faba* (broad bean) from the comparison of the SDS gel electrophoretic patterns of reduced and unreduced legumin. However, the finding in this report that each acidic subunit (polypeptide chain) linked with the specific partner of the basic subunit (polypeptide chain) through disulfide bridges is the first example in storage proteins of legume seeds.

It will be interesting to study the mechanism by which the intermediary subunits are formed and assembled into the 11S globulin during seed development.

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