Homology among the Acidic Subunits of Soybean 11S Globulin

Keisuke KITAMURA and Kazuo SHIBASAKI

*Faculty of Agriculture, Department of Food Chemistry,
Tohoku University, Sendai, Miyagi 980*

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Similarities and dissimilarities of amino acid sequences among the acidic subunits were analyzed by quantitative immunological precipitin with antibodies to the subunits, gel filtration of the CNBr-fragments and ion exchange chromatography of the tryptic peptides.

The gel chromatograms of the CNBr-fragments and the ion exchange chromatograms of the tryptic peptides of A₁ and A₂ subunits have demonstrated that they are two distinct polypeptide subunits which have very similar primary structures. Quantitative immunological precipitin revealed that A₁, A₂ and A₃ subunits were strongly immunologically related, therefore indicated that they shared a considerable degree of sequence homology. In fact, the analyses of the tryptic peptides of A₁, A₂ and A₃ subunits showed the sequence homology among the three subunits. Although A₄ subunit was shown to be serologically distinct polypeptide subunits from the other three subunits, some similarities of amino acid sequences were observed by the analyses of the tryptic peptides.

Soybean 11S globulin is the main storage protein which makes up 25~30 per cent of the total seed proteins. We have reported on the isolation of the four acidic subunits (A₁, A₂, A₃ and A₄) of soybean (var. Raiden) 11S globulin,¹⁾ and these were found to be present in the approximate molar ratio of 1:1:2:2 in the 11S globulin molecule.²⁾ A preliminary study on comparison in the primary structures among the four acidic subunits by the gel electrophoresis of the CNBr-fragments and by the double gel immunodiffusion using antibodies to the subunits has been reported.³⁾ In this paper, we report the study on comparison of the amino acid sequences among the four acidic subunits by quantitative precipitin by means of antibodies to the subunits and by analyses of the CNBr-fragments and the tryptic peptides using an amino acid analyzer.

MATERIALS AND METHODS

Seed. Soybean (var. Raiden) harvested at Furukawa, Miyagi, Japan in 1974 was used throughout this work.

Protein sample. Wolf's fraction VI was prepared from defatted soybean meal as described previously.²⁾

Isolation of subunits.* Isolation of the four acidic subunits (A₁, A₂, A₃ and A₄) of 11S globulin was performed according to the previous procedure¹⁾ with some modifications. Namely, 2.0 g of RCAM-Wolf's fraction VI dissolved in 100 ml of 0.12 M sodium phosphate buffer (pH 6.5) containing 6 M urea was applied to a column (3.4×30 cm) with DEAE-Sephadex A-50 equilibrated with the buffer. After non-adsorbed fraction was eluted, a linear gradient elution was made by adding 0.5 M NaCl solution to a mixing chamber containing 2000 ml of the buffer. Subsequently, a similar gradient elution was made on a DEAE-Sephadex A-50 column (1.8×25 cm) to isolate the acidic subunits from the partially purified fractions by the gradient elution in large scale. There was no detectable cross contamination among the isolated subunits, except between A₁ and A₂ subunits (the cross contamination was less than 10%), on the disc gel electrophoresis in 7 M urea.

Quantitative precipitin. Antigens (the subunits) were prepared by the dialysis method as described previously.³⁾ Increasing amounts of the subunit antigens in 0.4 ml of pH 7.6 phosphate buffer ($\mu=0.5$) were added to test tubes containing 0.2 ml of the antiserum. The reaction mixtures were incubated for 30 min at 37°C and then stored overnight at 4~6°C. The resultant

Abbreviations: RCAM, reduced S-carboxyamide-methylated; CNBr, cyanogen bromide.

* Subunits mean RCAM-subunits in this investigation.

** A_{1,2} represents a mixture of A₁ and A₂ subunits.

precipitates were centrifuged, washed twice with 0.035 M phosphate (pH 7.5) buffer (cold), and dissolved in 2.0 ml of 0.1 N NaOH. The absorbance of the solutions was measured at 280 nm by a Hitachi 124 Spectrophotometer. Per cent cross-reactivity was calculated from the absorbance at 280 nm at the point of maximum protein precipitation. There would be no significant error in obtaining per cent cross-reactivity from the absorbance at 280 nm, because $E_{1\text{cm}}^{0.1\%}$ values at 280 nm of A_1 , A_2 , A_3 and A_4 subunits were approximately the same (0.80, 0.80, 0.72 and 0.74, respectively).

Gel filtration of CNBr-fragments. Thirty mg of the lyophilized subunits was dissolved in 3.0 ml of 70% v/v HCOOH, and CNBr cleavage was performed essentially due to the procedure of Steer *et al.*⁴⁾ by adding 30 mg of CNBr to the solution. The reaction mixture was incubated for 24 hr at room temperature in the dark. The volume of reaction mixture was reduced to 0.5 ml in a rotary evaporator, diluted with water, and again concentrated to 0.5 ml. The sample was finally diluted to 1.5 ml with 10% HCOOH. A small amount of insoluble materials was centrifuged off. The supernatant was applied to a Bio-Gel P-100 column (1.8 × 190 cm) equilibrated with 10% HCOOH. The effluent tubes were analyzed by measurement of the absorbance at 280 nm and by Folin's method⁶⁾ after adjusting the effluents to neutral pH with 2.5 N NaOH.

Tryptic digestion of subunits. Typical tryptic digests of the acidic subunits were obtained by a following procedure. Forty mg of the lyophilized subunit was dissolved in 20 ml of 0.01 N NaOH and the solution was adjusted to pH 7.6–7.8 with 0.1 N HCl. Digestion was started by introduction of 0.4 mg of TPCK-treated trypsin (obtained from Worthington Biochemical Corp.) on a Hiranuma pH stat PS-11, the pH being kept at pH 8.0 with 0.02 N NaOH. After incubation for 4 hr at 30°C, an additional 0.2 mg of TPCK-treated trypsin was added. After 2 hr, the reaction mixture was adjusted to pH 2 with 1 N HCl and lyophilized. The tryptic peptides obtained by the above procedure gave reproducible elution profile in ion exchange column chromatography.

Gel filtration of tryptic peptides. Tryptic peptide mixture (40 mg) was dissolved in 2.0 ml of 5% HCOOH and a small amount of insoluble materials was centrifuged off. Ninety to ninety-five per cent of the total peptides was solubilized as being estimated by Folin's method. The peptide solution was applied to a Bio-Gel P-4 column (1.8 × 190 cm) equilibrated with 5% HCOOH. The elution patterns were determined by measurement of the absorbance of the effluent fractions at 280 nm and at 570 nm of ninhydrin color values. The fractions were pooled and lyophilized.

Ion exchange chromatography of tryptic peptides. Chromatography was performed essentially according to the procedure of Kawashima *et al.*⁶⁾ The tryptic peptide mixture (10 mg) and one thirds of the lyophilized F-I and II in Fig. 4 of each acidic subunit were dissolved in 1.0 ml of 0.2 M sodium citrate solution adjusted to pH 2.0. The soluble peptides (90–95% of the total peptides were soluble as being estimated by Folin's method and all peptides in F-I and II were perfectly solubilized) were applied to a 0.9 × 50 cm column of Hitachi No. 2612 strong acid resin equilibrated with the starting buffer, 0.2 N (Na^+), pH 3.1 (210 g citric acid · H_2O , 85 g NaOH (purity, 95%), 115 ml conc. HCl and 25 g BRIJ 35 to make 10 liters) in a Yanagimoto Amino Acid Analyzer Model LC-5. The column was maintained at 50°C throughout analysis. The first portion of elution was made using a linear gradient elution from the starting buffer, 0.2 N (Na^+), pH 3.1 to the limiting buffer, 2 N (Na^+), pH 5.2 (1050 g citric acid · H_2O , 214 ml glacial acetic acid, 480 g NaOH (purity, 95%), 1850 g sodium acetate · $3\text{H}_2\text{O}$ and 10 g BRIJ 35 to make 10 liters). The device of the gradient was similar to the simple gradient device described by Block and Ling.⁷⁾ Namely, the gradient was produced by placing 306 g of the starting buffer in the first mixing chamber and 306 g of the limiting buffer in a second connecting chamber of the same internal diameter. A buffer change was made at 360 min later; only the limiting buffer was pumped into the column to elute peptides tightly bound to the resin. The flow rate was 1 ml per min.

RESULTS

Quantitative precipitin of subunits

The results of quantitative precipitin of anti- A_3 and - A_4 sera with the four acidic subunits

FIG. 1-A. Quantitative Precipitin of the Acidic Subunits with Anti- A_3 Serum.

The detailed experimental procedure was described under MATERIALS AND METHODS. Absorbance at 280 nm represents amount of antigen-antibody complex.

FIG. 1-B. Quantitative Precipitin of the Acidic Subunits with Anti- A_4 Serum.

are illustrated in Fig. 1-A and -B. Figure 1-A shows that the reaction of A_3 with anti- A_3 serum gives a typical precipitin curve and that per cent cross-reactivities of A_1 and A_2 subunits with anti- A_3 serum are 47 and 50%, respectively at the maximum precipitation. A_4 subunit gave substantially no precipitate with anti- A_3 serum at equivalence. It is observed from Fig. 1-B that the reaction of A_4 subunit with anti- A_4 serum gives a typical precipitin curve and that no cross-reactions of A_1 , A_2 and A_3 subunits with anti- A_4 serum occur. The results of the quantitative precipitin are consistent with those of the antigenic reactions observed previously on the double gel immunodiffusion.³⁾

Comparison of elution profiles of CNBr-fragments

In order to compare the amino acid sequences of the four acidic subunits, the CNBr-fragments were separated on a Bio-Gel P-100 column using 10% HCOOH as eluates. Each CNBr-treated subunit gave three to four peptide peaks, well consistent with the presence of two to three methionine residues per subunit (polypeptide chain) except A_3 subunit calculated from the amino acid compositions and the molecular weights of the subunits.¹⁾ It is observed from Fig. 2-A and -B that the elution profiles of the CNBr-treated A_1 and A_2 subunits are very similar to each other. This result is consistent with the gel electrophoretic patterns of the CNBr-fragments reported elsewhere.³⁾ The CNBr-treated A_3 subunit gave

FIG. 2. Gel Filtration of the CNBr-treated Subunits with Bio-Gel P-100.

—, absorbance at 280 nm; ----, absorbance at 750 nm (arbitrary units, Folin's method); column size, 1.4×190 cm; volume of fraction, 3.8~3.9 ml; flow rate, 9.0~10 ml per hour; eluate, 10% HCOOH ; temperature, room temperature; sample, 30 mg of the CNBr-treated acidic subunits (see MATERIALS AND METHODS).

a rather different elution profile from those of the CNBr-treated A_1 and A_2 subunits. Only one peptide (peak III in Fig. 2-C) emerged at a nearly identical position to that of one of the three peptide peaks in Fig. 2-A and -B. This result shows that most methionine residues of A_3 subunit are located at different positions in the polypeptide chain from those of A_1 and A_2 subunits. Elution profile of the CNBr-treated A_4 subunit is quite different from those of the other three subunits.

Comparison of ion exchange chromatograms of tryptic peptides of A_1 and A_2 subunits

Figure 3-A and -B show chromatograms of the total peptide mixture of A_1 and A_2 subunits, respectively. The number of peptide peaks resolved by ion exchange column chromatography was about a half of the number of peptides expected from the amino acid com-

FIG. 3. Ion Exchange Chromatograms of Total Tryptic Peptides of A₁ (A) and A₂ (B). The detailed experimental procedure was described under MATERIALS AND METHODS.

positions and the molecular weights (A₁ and A₂ subunits contain about 30 residues of lysine + arginine per mole). This means that more than one peptide are eluted in some of the resolved peaks. However, chromatograms were highly reproducible in duplicate experiments on each subunit. It is observed from Fig. 3-A and -B that the chromatograms of the tryptic peptides of A₁ and A₂ subunits are very similar to each other. In comparison to A₁ subunit, A₂ subunit also possessed the twelve peaks eluted at corresponding positions. While, one peptide peak (the peak 6) derived from A₁ subunit was not found in the chromatogram of A₂ subunit, and conversely, two peptide peaks (the peaks 2' and 14) derived from A₂ subunit were not found in the chromatogram of A₁ subunit. It seems to be very likely that the peptides emerged in the corresponding peaks are the same or at least very similar peptides which might come from homologous positions of the polypeptide chains of A₁ and A₂ subunits. It can be concluded from the above results that A₁ (or A₂) subunit is not an artifact of A₂ (or A₁) subunit, but has a very similar, almost identical primary structure to that of A₂ (or A₁) subunit.

FIG. 4. Fractionation of Tryptic Peptides of the Acidic Subunits by Gel Filtration on Biogel P-4 Column.

—, absorbance at 280 nm; ----, absorbance at 570 nm (ninhydrin color value); column size, 1.9 × 180 cm; volume of fraction, 5.0–5.1 ml; flow rate, 12–13 ml per hour; eluate, 10% HCOOH; temperature, room temperature; sample, 40 mg of tryptic peptides of A_{1,2} (A), A₃ (B) and A₄ (C).

Comparison of ion exchange chromatograms of fractionated tryptic peptides of subunits

In order to compare the amino acid sequences of the four acidic subunits, the tryptic peptides were chromatographed on the ion exchange column after fractionation of the total tryptic peptides on a Bio-Gel P-4 column (1.8×190 cm), because the chromatograms of the total tryptic peptides of a mixture of A_1 and A_2 ($A_{1,2}^{**}$), A_3 and A_4 subunits were not directly comparable (not shown here). A_3 and A_4 subunits were compared with $A_{1,2}$,

because of the high sequence homology between A_1 and A_2 subunits.

Figure 4-A, -B and -C show the elution profiles of the total tryptic peptides of $A_{1,2}$, A_3 and A_4 subunits, respectively on the Bio-Gel P-4 column. The three elution profiles are rather different from one another. However, most tryptic peptides were eluted in fraction 40 to 80. Fraction I (fractions 40~49, relatively large peptides) and fraction II (fractions 57~70, relatively small peptides) of each peptide mixture of the subunits were pooled and ana-

FIG. 5. Ion Exchange Chromatograms of F-I of the Tryptic Peptides of $A_{1,2}$ (A), A_3 (B) and A_4 (C) in Fig. 4.

FIG. 6. Ion exchange Chromatograms of F-II of the Tryptic Peptides of $A_{1,2}$ (A), A_1 (B) and A_1 (C) in Fig. 4.

lyzed by the ion exchange chromatography. The chromatogram of F-I of $A_{1,2}$, A_3 and A_4 subunits is shown in Fig. 5-A, -B and -C, respectively. It is observed that the chromatogram in Fig. 5-A is similar to that in Fig. 5-B; $A_{1,2}$ also possessed the peptide peaks 1, 2, 4 and 5 in accordance with A_3 subunit. On the other hand, the chromatogram in Fig. 5-C is rather different from those in Fig. 5-A and -B. However, two peptide peaks were observed at the nearly identical positions to those of the peaks 3 and 4 of A_3 subunit. The chromatogram of F-II of $A_{1,2}$, A_3 and A_4 subunit is shown in Fig. 6-A, -B and -C, respectively. It is observed that the three chromatograms are relatively similar to one another; in accordance with F-II of A_3 subunit, F-II of $A_{1,2}$ also possessed the peptide peaks 1, 2, 3, 4, 5, 6, 8, 10 and 11, and, also, F-II of A_4 subunit possessed the peptide peaks 2, 3, 4, 5, 6, 8, 9 and 11.

From the results of the ion exchange chromatography of the fractionated tryptic peptides of the acidic subunits, it can be said that there is a significant sequence homology among A_1 , A_2 and A_3 subunits and that there are some similarities in the primary structures among A_4 subunit and the other three acidic subunits.

DISCUSSION

Quantitative immunological techniques are capable of detecting similarities between amino acid sequences of proteins. Prager and Wilson⁸⁾ have reported that 50 per cent cross-reaction of animal lysozymes and bovine α -lactalbumins is equal to about 70 per cent of similarities of amino acid sequences. The fact that A_1 , A_2 and A_3 subunits are strongly immunologically related indicates that they share a considerable degree of sequence homology. The analyses of the tryptic peptides of A_1 , A_2 and A_3 subunits also demonstrate the sequence homology among the three acidic subunits. The lack of immunological cross-reactivity of A_4 subunit with the other three subunits has been shown by the quantitative precipitation, as well as the double gel immunodiffusion.³⁾

However, it is observed from the analyses of the fractionated tryptic peptides that there are some similarities in the primary structures among A_4 subunit and the other three acidic subunits. This means that the degree of similarity of the amino acid sequences among A_4 subunit and the other three subunits is not high enough to cross-react immunologically. From the above results and discussion, it can be concluded that there is a sequence homology among the four acidic subunits of soybean 11S globulin.

The sequence homology among the multiple acidic subunits implies a common evolutionary origin for these polypeptide subunits. It is most probable that gene duplication followed by point mutation has occurred in a similar fashion to those of hemoglobin⁹⁾ and lactate dehydrogenase.¹⁰⁾ Although the structural evolution of these biologically functional proteins has been intensively related to progressive improvement in the physiological system,^{11,12)} the significance of the structural evolution of 11S globulin is not clear. Recently, Boulter *et al.*¹³⁾ have proposed to define storage protein as follows; storage protein is laid down at one stage of the life cycle for future use at a metabolically more active stage. Furthermore, Miller¹⁴⁾ has suggested that molecular form of storage protein is related to the abilities to form stable protein bodies and to withstand desiccation. It can be thought that the term of progressive improvement of storage protein might mean that molecular form of storage protein is improved to more suitable form for transportation and deposition in protein bodies during seed development and for degradation to serve the source of nitrogen more effectively during seed germination. We can not, at present, relate the multiple subunits of 11S globulin to the improvement of the molecular form to get these abilities. However, it is reasonable to think that the multiple subunits of 11S globulin brought about as a result of the structural evolution might cause more efficient usage of nitrogen source, because oligomeric protein consisting of multiple polypeptide chains may be degraded at different rates,

supplying wide spectrum of necessary amino acids and peptides during seed germination. In fact, Yamada and Shibasaki¹⁵⁾ found that among the acidic subunits of 11S globulin, A₄ subunit was most rapidly degraded during seed germination, by means of the gel electrophoresis in urea and the immunological techniques.

Comparison of the amino acid sequences among the four basic subunits (B₁, B₂, B₃ and B₄ subunits)²⁾ of 11S globulin is under investigation.

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