Components of Storage Protein in Hypocotyl-radicle Axis of Soybean (*Glycine max.* cv. Enrei) Seeds

Toshio SUGIMOTO, Michiko MOMMA, Kazumoto HASHIZUME
and Kyoko SAIO

National Food Research Institute, Yatabe, Tsukuba,
Ibaraki 305, Japan

Received November 4, 1986

Protein components in the hypocotyl-radicle axis of soybean seeds were compared with those in the cotyledon. The hypocotyl-radicle axis was rich in 7S and 2S proteins but poor in 11S protein on the profiles of analytical ultracentrifugation and of SDS-PAGE. Among the 7S-protein constituent polypeptides, α' -polypeptide was abundant but α - and β -polypeptides were less abundant than in the cotyledon. Several isomers of 7S protein were found and their main constituents were α -, α' -, or both polypeptides on two-dimensional PAGE. These results indicated that the hypocotyl-radicle axis contained different types of the 7S protein than the cotyledon.

An embryo of a dicotyledonous plant seed is made up of two cotyledons, a plumule, a hypocotyl and a radicle. The hypocotyl and radicle of soybean seeds are joined to each other in a structure called the hypocotyl-radicle axis (Hypo) or hypocotyl, which is about 5 mm long and flattened both on the seed coat and on the cotyledon.¹⁾ The tissue is connected with two cotyledons at hypocotyl side and the hypocotyl and cotyledons develop during the maturation of seed from one zygote.²⁾ The Hypo is one of the most important parts of a seed because the tissue develops into the stem and roots after germination, but the protein components in the tissue have not been well characterized.

The storage proteins of soybean seeds are classified into 11S (glycinin) and 7S (β -conglycinin) proteins by their different sedimentation coefficients on ultracentrifugation. The proteins were well characterized.^{3,4)}

The aim of this study is to identify the protein components in the Hypo of soybean seed. We found that the tissue is rich in 7S protein but poor in 11S protein and that the

polypeptide components of the 7S protein are different from those in the cotyledon.

MATERIALS

Materials. Soybean seeds (*Glycine max* cv. Enrei) were the 1984 crop in Nagano Prefecture, Japan. Agarose was purchased from LKB and other chemical reagents used were all extra-pure reagent grade.

METHODS

Preparation of defatted powders of Hypo and cotyledon of the seed. Seeds were cracked with a pair of pliers and Hypos and cotyledons were separated by hand. Tissues were ground in a mortar with a pestle. The powders were defatted with cold acetone (-20°C) three times and centrifuged. The precipitates were dried under reduced pressure.

Fractionation of the proteins in Hypo and cotyledon. The proteins were fractionated by the method of Thanh *et al.*⁵⁾ Duplicate samples of powder (0.5 g) of the Hypo and cotyledon were separately extracted in 7.5 ml of 63 mM Tris-HCl, pH 7.8, containing 10 mM 2-ME for 2 hr by continuous mixing at room temperature. The extracts were centrifuged at 12,000 rpm for 15 min at 20°C . The supernatants (5 ml) were dialyzed against 63 mM Tris-HCl, pH 6.6, at 4°C for 4 hr and then centrifuged at 12,000 rpm

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol; Hypo, hypocotyl-radicle axis; BSA, bovine serum albumin.

for 20 min at 2°C. The precipitate was a fraction of 11S protein. The pH of the resultant supernatants was adjusted to 4.8 with 0.02N HCl (about 2 ml) and centrifuged. The supernatant was a fraction of whey protein. The precipitate (crude 7S-protein fraction) was suspended in 3 ml of the Tris-HCl, pH 6.6, mixed thoroughly at 0°C, and then centrifuged. The supernatant thus obtained was a fraction of purified 7S protein.

Electrophoresis. Slab SDS-PAGE was done by the method of Laemmli⁶⁾ in a 13% polyacrylamide gel. The defatted powders (0.01 g) were separately dissolved in 0.5 ml of a sample buffer (63 mM Tris-HCl, pH 6.8, 5% 2-ME, 8 M urea, and 2% SDS) and incubated for 2 hr at 100°C. Appropriate volumes of the sample solutions (60 µg of protein) were, after centrifugation, put into wells of the slab gel, and electrophoresed at a constant voltage of first 70 V for 1 hr and then at 100 V for 4 hr. For the fractionated samples, the proteins were mixed with an equal volume of an SDS-buffer to the same final concentration as the sample buffer, except for urea. For two-dimensional gel electrophoresis of the purified 7S protein from Hypo and cotyledons, 160 µg of protein was put into each tube. The first disc electrophoresis was done by a modification of the method of Davis⁷⁾ described by Thanh and Shibasaki⁸⁾ in a 6% polyacrylamide gel for 4 hr at a constant current of 1 mA/tube. Second-dimensional slab SDS-PAGE was done by the method of Laemmli in a 12.3% polyacrylamide gel. The disc gels were layered onto a stacking gel composed of 0.5% agarose, 1% 2-ME, 1% SDS, and 63 mM Tris-HCl, pH 7.8 and electrophoresed at a constant voltage of 70 V for 4 hr and then 100 V for 5 hr.

Analytical ultracentrifugation. The defatted flour of Hypo and cotyledon (0.2 g) was extracted with 2.0 ml of 1.0 M NaCl, 10 mM 2-ME, 0.1% NaN₃, and 50 mM Na-phosphate, pH 7.0, for 2 hr at room temperature and centrifuged at 12,000 rpm for 20 min at 20°C. The supernatants were dialyzed against the buffer for overnight at 4°C. Analytical ultracentrifugation was done with a Hitachi 282 Analytical Ultracentrifuge at 54,000 rpm and 20°C. The fractionated 7S protein was dialysed against 0.4 M NaCl, 16 mM K₂HPO₄, 3 mM KH₂PO₄, and 10 mM 2-ME at pH 7.8 overnight at 4°C and then centrifuged at 54,000 rpm, 20°C.

Estimation of protein content. The protein content of each fraction was estimated by the protein-dye binding method with BSA for the standard protein.⁹⁾

RESULTS

Protein components in the Hypo

Protein components in the Hypo were compared with those of cotyledons after analytical ultracentrifugation. The schlieren patterns of

FIG. 1. Profiles of Analytical Ultracentrifugation of Proteins Extracted from the Hypocotyl-radicle Axis and Cotyledon of Soybean Seeds.

The experiment was described in the text. Sedimentation is directed from right to left. (A) cotyledon, (B) hypocotyl-radicle axis, (C) mixture of (A) and (B). The pictures were taken after 54 min of centrifugation.

each solution are shown in Fig. 1. The profile of the protein in the Hypo had two clear peaks and one faint peak (Fig. 1B). The profile of the cotyledon had three peaks, which were identified as being 2S, 7S, and 11S proteins from right to left (Fig. 1A).¹⁰⁾ In a profile of a mixture of equal volumes of the two samples (Fig. 1C), three distinct peaks were observed and the positions of each peak were the same among the three samples, which indicated that the sedimentation constant of each protein in the Hypo was the same as that of the cotyledon. This result showed that sedimentation constants of the three peaks in the profile of the Hypo were 11S, 7S, and 2S from left to right. The area of each peak was proportional to the concentration of the respective protein in the extracts. Concentrations of the 7S and 2S proteins were higher in the Hypo than in

FIG. 2. SDS-PAGE of the Total Proteins in the Hypocotyl-radicle Axis and Cotyledon of Soybean Seed and Their Densitograms.

The experiment was described in the text. Electrophoresis was from left to right. (A) hypocotyl-radicle axis, (B) cotyledon. Each peak of polypeptides in the densitograms was numbered in the order of decreasing molecular weight.

the cotyledon and the reverse was true for the 11S protein.

Polypeptides components in the Hypo of the seed

An SDS-PAGE of the extracts of the Hypo was done in a slab gel along with that of cotyledon (Fig. 2). The densitograms of each lane showed the different amounts of each polypeptide. Bands 2, 3, and 8 had the same mobilities as the α' -, α -, and β -polypeptides of the 7S protein, respectively. Bands 12 and 14 were identified with the acidic subunit of 11S protein, and 24 and 25 were with basic subunit. Band 1 was the largest both in the Hypo and cotyledon fractions. The band might be a polypeptide of lipoxygenase, to judge from the mobility of the polypeptide.¹¹⁾ Bands 7, 9, 13, 15, 16, 17, and 27 were not observed in the

cotyledon fraction, whereas bands 12, 18, and 25 were observed in the cotyledon fraction only. The peak heights of the densitogram of each fraction indicated that the concentration of the α' -polypeptide of 7S protein was higher but that of α - and β -polypeptides of the 11S protein were also less abundant in the Hypo fraction. These results coincided with those of analytical ultracentrifugation and suggested that the components of the 7S protein in the Hypo were different from those in the cotyledon.

Polypeptide components and their distribution in the fractionated proteins of Hypo

The proteins in the Hypo were fractionated by use of their different solubilities at different pHs by the method of Thanh *et al.*⁵⁾ The protein content of each fraction is shown in

TABLE I. AMOUNT (mg) OF PROTEINS IN FRACTIONS OF THE HYPOCOTYL-RADICLE AXIS AND COTYLEDON

Sample	Fraction	Crude 7S	Purified 7S	11S	Whey	Total ^a
Hypocotyl-radicle axis	(A)	40.23 (70.6%)	16.00 (28.1%)	2.29 (3.9%)	11.94 (20.9%)	57.00
	(B)	45.11 (77.7%)	14.11 (24.3%)	3.35 (5.8%)	12.57 (21.7%)	58.04
Cotyledon	(A)	55.34 (48.1%)	33.85 (29.4%)	33.77 (29.3%)	11.91 (10.3%)	115.16
	(B)	56.59 (54.0%)	36.40 (34.8%)	37.53 (35.8%)	12.96 (12.4%)	104.72

^a Amounts of protein in 5 ml of the extracts (mg).

FIG. 3. SDS-PAGE of the Fractionated Proteins in the Hypocotyl-radicle Axis and Cotyledon.

The experiment was described in the text. (a)~(e), hypocotyl-radicle axis; (f)~(j), cotyledon; (a) and (f), total proteins; (b) and (g), whey protein fraction; (c) and (h), 11S-protein fractions; (d) and (i), insoluble part of crude 7S-protein fractions; (e) and (j), soluble part of crude 7S-protein fractions (purified 7S protein). The marks α , α' , and β indicate the position of the respective polypeptides of the 7S protein.

Table I. About 75% of the protein extracted from the Hypo was in the crude 7S-protein fraction, 5% was in the 11S-protein fraction, and 20% was in the whey protein fraction. About 50% of the protein extracted from the cotyledon was in the crude 7S-protein fraction, 30% was in the 11S-protein fraction, and 10% was in the whey protein fraction. Further purification of the 7S protein decreased the amount of protein in the fractions of both tissues. However, there was more protein

in the purified 7S-protein fraction of Hypo than in the 11S-protein fraction. These results indicated that there was little protein in the 11S-protein fraction of the Hypo, and that the 7S protein was one of the main Hypo proteins. Figure 3 shows the polypeptide components of the fractions of each protein. Profiles of the polypeptides in the extracts (lanes a and f) were the same as those of the total proteins in the Hypo and cotyledon (Fig. 2), which indicated that proteins in the tissues

were extracted satisfactorily in these conditions. In the 7S-protein fraction of the Hypo (lane e), two polypeptide bands were found that corresponded to Peaks 3 and 2 in Fig. 2, respectively, which showed the same mobilities with the α - and α' -polypeptides of 7S protein, whereas α -, α' -, and β -polypeptides were observed in the 7S-protein fraction of the cotyledon (lane j). From these results, we concluded that these two polypeptides in the Hypo are the α - and α' -polypeptides of the 7S protein. Although polypeptides of the 11S protein were observed in the 11S-protein fraction of the cotyledon (lane h), which showed the suitability of the methods for fractionation of the protein, such polypep-

tides were not clearly observed in the 11S-protein fraction of the Hypo (lane c). In the whey protein fractions of both the Hypo (lane b) and the cotyledon (lane g), the largest band (No. 1 in Fig. 2) was most intense; subunits of 11S protein were second in intensity in the cotyledon only. The profile of the insoluble part of the crude 7S-protein fraction of the Hypo (lane d) was similar to that of the whey protein fraction, whereas polypeptides of 7S and 11S proteins were observed in the profile of the cotyledon (lane i). These results also suggest that the amount of 11S protein in the Hypo was small and that the polypeptide constituents of the 7S protein in the Hypo were different from those in the cotyledon.

The purity of the purified 7S-protein fractions of both tissues was checked by analytical ultracentrifugation. Two peaks were observed in the 7S-protein fraction of the Hypo and the 7S protein were the main peak even though 2S protein still contaminated this fraction (Fig. 4B). In the 7S-protein fraction of the cotyledon (Fig. 4A), one main peak (7S) and two minor peaks (2S and 11S) were present. The mixture of the two fractions (Fig. 4C) showed one main peak (7S) and two minor peaks. The results showed that the main component of the 7S-protein fraction of the Hypo was 7S protein.

Polypeptide components of the 7S-protein fraction

The polypeptide components of the 7S proteins prepared from the Hypo were examined by two-dimensional gel electrophoresis. In the first disc gel, two main bands and some minor ones were observed that were different from the components of the cotyledon in their mobility (Fig. 5A). The disc gels were transferred onto a slab gel and electrophoresed. The electrophoregram of the second-dimensional PAGE showed polypeptide constituents of the 7S protein in both the Hypo (left side of the second gel) and cotyledon (right side of the 2nd gel; Fig. 5B). The parts of the gels corresponding to the α -, α' -, and β -polypeptides were magnified (Fig. 5C) and the densitograms of the eight lines descending from the numbers

FIG. 4. Profiles of Analytical Ultracentrifugation of Purified 7S Proteins of the Hypocotyl-radicle Axis and Cotyledon.

The experiment was described in the text. (A) cotyledon, (B) hypocotyl-radicle axis, (C) mixture of (A) and (B). The picture was taken after 54 min of centrifugation.

FIG. 5. Two-dimensional Electrophoresis of the 7S-Protein Fractions of the Hypocotyl-radicle Axis and Cotyledon.

(A) First-dimensional disc electrophoresis of the 7S proteins and their densitograms. Electrophoresis was from left to right. (a), hypocotyl-radicle axis; (b), cotyledon.

(B) Second-dimensional slab SDS-PAGE of the 7S proteins. Left side, hypocotyl-radicle axis; right side, cotyledon. Electrophoresis was from top to bottom. Methods are described in the text.

(C) Magnified images of part of the second-dimensional PAGE where polypeptides of 7S protein were found. Marks α , α' , and β indicate the position of the respective polypeptides of the 7S protein. Numbers 1~8 indicate the positions of scanning lines of densitometry in (D). (a) hypocotyl-radicle axis; (b) cotyledon.

(D) Densitograms of each line numbered in (C).

TABLE II. DISTRIBUTION OF THE THREE POLYPEPTIDES OF 7S PROTEIN IN THE LINES OF THE SECOND GEL IN TWO-DIMENSIONAL PAGE

Line No.	Polypeptides		
	α'	α	β
1	++	++	++
2	++	++	+
3	+	—	—
4	++	+	—
5	+	+	++
6	+	+	++
7	+	++	++
8	++	++	+

Line No. indicate positions of scanning lines of the densitometries on the gel shown in Fig. 6C.

Marks ++, +, and — indicate the intensity of the polypeptide bands.

on the top of the gel in Fig. 5C are shown in Fig. 5D. Intensities at each line of the three polypeptides of 7S proteins are summarized in Table II. The distribution of the three polypeptides of 7S protein in the Hypo protein of the

second gel was different from that in the cotyledon. These results showed that the 7S protein in the Hypo was composed from several isomers different from those in the cotyledon.

DISCUSSION

Meinke *et al.*¹²⁾ immunochemically detected the polypeptides of 11S and 7S proteins in the embryonic axis (Hypo) of soybean seeds and concluded that the kinds of polypeptides in the Hypo were different from those in the cotyledon. Our study showed that polypeptides with the same mobilities on SDS-PAGE as those of cotyledonous 7S protein formed a protein the sedimentation coefficient of which was 7S. To judge from the results of Meinke *et al.* and ours, we concluded that 7S protein is present in the Hypo and is formed from the same polypeptides as those in the cotyledon.

Although the polypeptide components in the proteins of the Hypo were similar to those of the cotyledon, the ratio of components was

different. The greatest point of difference was the concentration of 11S protein in each tissue. The shlieren pattern of the ultracentrifugation and the profile of the SDS-PAGE showed that there was little 11S protein in the Hypo. Although the 11S and 7S proteins are storage proteins of soybean seeds, only the 7S protein is abundant in the Hypo. Of the polypeptide components of the 7S protein, the α' -polypeptide was abundant in the Hypo and the α - and β -polypeptides were not. These findings might mean that the biosynthesis and accumulation of proteins in the Hypo was different from those in the cotyledon. Proteins in the cotyledon are synthesized and accumulate in protein bodies during the ripening of the seed. The 7S protein starts to accumulate earlier than the 11S.^{13,14)} In the cotyledon during ripening, α -polypeptide start to accumulate before β -polypeptide.¹⁵⁾ The biosynthesis and accumulation of these proteins in the Hypo are not understood in detail.

The 7S protein of soybean seed comprises six isomers^{8,16)}: $\alpha'\beta_2$ (B1), $\alpha\beta_2$ (B2), $\alpha\alpha'\beta$ (B3), $\alpha_2\beta$ (B4), $\alpha_2\alpha'$ (B5), and α_3 (B6). The α -polypeptide is the most abundant of the isomers B3 to B6. The 7S protein in the Hypo was composed of α - or α' -polypeptides, or both, as seen in the two-dimensional PAGE. The densities of each polypeptide (α and α') in the second gel suggested the ratio of each polypeptide in the isomers observed in the first disc gel (Fig. 5). The α' -polypeptide was most abundant the main isomer and minor ones comprised equal amounts of α and α' . This finding is inconsistent with previous reports,^{8,16)} and suggests that the isomers in the Hypo are different from those in the cotyledon in their polypeptide constituents. The isomers would have to be purified before their components could be identified.

Although various points such as the ultrastructure and storage protein of the cotyledon of soybean seed has been studied extensively, the Hypo has not. Details about the ultrastructure and protein components of this storage organ are not known.

Acknowledgments. We thank Dr. K. Harada, National Institute of Agrobiological Resources, for helpful discussion. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Agriculture, Forestry, and Fisheries of Japan (GEP-87-II-3-2).

REFERENCES

- 1) J. B. Carlson "Soybeans: Improvement, Production, and Uses," ed. by B. E. Caldwell, American Society of Agronomy, Inc., Madison, Wisconsin, U.S.A., 1973, pp. 17~95.
- 2) S. P. Bhatnager and B. M. Johri "Seed Biology," Vol. 1, ed. by T. T. Kozlowsky, Academic Press, New York and London, 1972, pp. 77~149.
- 3) A. Millerd, *Ann. Rev. Plant Physiol.*, **26**, 53 (1975).
- 4) T. J. V. Higgins, *Ann. Rev. Plant Physiol.*, **35**, 191 (1984).
- 5) V. H. Thanh, K. Okubo and K. Shibasaki, *Plant Physiol.*, **56**, 19 (1975).
- 6) U. K. Laemmli, *Nature*, **227**, 680 (1970).
- 7) B. J. Davis, *Ann. N. Y. Acad. Sci. U.S.A.*, **121**, 404 (1964).
- 8) V. H. Thanh and K. Shibasaki, *Biochim. Biophys. Acta*, **439**, 326 (1976).
- 9) M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
- 10) J. E. Hill and R. W. Breidenbach, *Plant Physiol.*, **53**, 742 (1974).
- 11) K. Kitamura, C. S. Davies, N. Kaizuma and N. C. Nielsen, *Crop Sci.*, **23**, 924 (1983).
- 12) D. W. Meinke, J. Chen and R. N. Beachy, *Planta*, **153**, 130 (1981).
- 13) S. O. Yanagi, C. Fukazawa and K. Harada, *Agric. Biol. Chem.*, **42**, 697 (1978).
- 14) K. Kondo, T. Sugimoto and K. Saio, *Agric. Biol. Chem.*, **50**, 581 (1986).
- 15) K. R. Gayler and G. E. Sykes, *Plant Physiol.*, **67**, 958 (1981).
- 16) V. H. Thanh and K. Shibasaki, *J. Agric. Food Chem.*, **26**, 692 (1978).