Table V. Amino Acid Analysis of Sesame Seed Protein Products[a]

	meal	concentrate	isolate	req of human adults[b]
lysine	3.28	2.49	2.67	5.5
histidine	2.81	2.49	2.87	
arginine	14.10	12.80	12.20	
threonine	4.08	2.69	4.20	4.0
valine	4.36	3.46	5.45	5.0
methionine	2.64	2.80	3.10	
cystine	1.29	1.16	1.30	3.5
isoleucine	4.36	2.97	4.70	4.0
leucine	6.91	5.17	5.83	7.0
phenylalanine tyrosine	4.89	3.24	5.13	6.0

[a] Amino acids were calculated as grams/16 g of nitrogen. [b] FAO/WHO *Energy and Protein Requirements*, FAO Nutrition Meeting Report Series No. 52; Food and Agricultural Organization: Rome, 1973.

concentrate, and sesame protein isolate and an increase in the protein content (27.2 and 71.2%, respectively) for sesame protein concentrate and sesame protein isolate over the sesame meal. Values for oil in the three products are negligible. The protein isolate showed negligible ash and fiber contents. Naturally, with the increase in the protein content of the concentrate and isolate, the nitrogen-free extract decreased. Sesame meal contains 14.5 mg of phytate P/g of meal, whereas sesame protein concentrate contains only 1.81 mg of phytate P/g of concentrate. The sesame protein isolate can be considered almost free of phytate (0.65 mg of phytate P/g of isolate). The procedures used for the preparation of the sesame protein concentrate and isolate resulted in 87.5 and 95.5% reduction of the phytate content, respectively, over the meal.

Table V gives the amino acid analysis of the three sesame protein products as well as the requirements of human adults recommended by the FAO for comparison.

The amino acid analysis pattern shows that the three sesame protein products are first limiting in lysine but are rich sources of both methionine and cystine, which are primarily limiting amino acids in many vegetable protein sources. Johnson et al. (1979) and Lyon (1972) reported the same findings. Sesame meal is second limiting in valine, while the sesame protein isolate is second limiting in leucine. Sesame protein concentrate shows a poor amino acid composition when compared to the FAO requirements for human adults. It is limiting in lysine, threonine, valine, isoleucine, and leucine.

From the previous findings it is concluded that sesame seed protein isolate prepared by the given procedure provide a promising source of protein for incorporation in food, for either chicken or human consumption. It has the advantage of containing a high bland protein rich in sulfur amino acids and a negligible amount of phytate. Sesame protein concentrate has the disadvantage of a poor amino acid pattern, but this can be overcome through its supplementation with other plant proteins.

Registry No. NaOH, 1310-73-2; phytate, 83-86-3.

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Mössbauer Study of Iron in Soybean Seeds

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Chemical states of iron in soybean seeds cultivated with a nutrient solution containing enriched ^{57}Fe have been studied by Mössbauer spectroscopy. The Mössbauer spectra showed that iron in the seeds was in both trivalent high-spin and divalent high-spin states. Most of the iron in mature seeds was found to be in the ferric state. In immature seeds, the relative area of Mössbauer absorption due to ferrous ions was much larger than in ripe ones. The relative area of ferrous ions increased in the process of germination of the ripe seeds. The Mössbauer parameters were compared with those of ferric phytates, ferritin, and other iron compounds isolated from plants. The ferric ion in the soybean seeds was not identified as a ferric phytate as has been reported for wheat. More data with model compounds are required to specify the iron complexes in the seeds.

Although a number of biogenic iron compounds have been isolated and characterized, little is known concerning the distribution of the whole iron among different chemical species in living organisms. Oxidation–reduction reactions between ferric and ferrous ions are known to be associated with a number of biochemical phenomena such as absorption of iron by roots, transport of iron across inner

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membranes, and metabolism and biosynthesis of various compounds. However, our present knowledge on the chemical states of iron in vivo is still much limited.

Mössbauer absorption spectroscopy is useful in studying the chemical states and reactions of iron in living organisms (Dickson, 1984). We found that this method is applicable to the examination of a single grain of soybean seed harvested from a plant grown with a hydroponic culture medium containing iron enriched in ^{57}Fe, which is the stable isotope of iron sensitive to Mössbauer spectroscopy. Chemical states of iron in immature and ripe seeds were compared, and the change in the valence states of iron was followed in the course of germination. Measurement was also performed on leaves and roots dried after ripening of the seeds. The Mössbauer parameters of the soybean samples were compared with those of ferric phytates, ferritin, and other iron compounds identified so far in plants.

EXPERIMENTAL SECTION

Culture of Soybean Plants. Soybean (*Glycine max* L. Merrill cv. Okuharawase) seeds were obtained commercially. Iron content of the seeds was determined by neutron activation analysis. Soybean plants were cultivated hydroponically without soil in a greenhouse in the absence of root nodule bacteria. The nutrient solution contained per 1 dm^3 4 mmol of KNO_3, 4 mmol of $Ca(NO_3)_2 \cdot 4H_2O$, 1.5 mmol of $MgSO_4 \cdot 7H_2O$, 1.33 mmol of $NaH_2PO_4 \cdot 2H_2O$, 0.01 mmol of $MnSO_4 \cdot 4H_2O$, 0.001 mmol of $ZnSO_4 \cdot 7H_2O$, 0.001 mmol of $CuSO_4 \cdot 5H_2O$, 0.05 mmol of H_3BO_3, 0.0005 mmol of $Na_2MoO_4 \cdot 2H_2O$, 0.1 mmol of NaCl, and 0.05 mmol of ferric ethylenediaminetetraacetate (Fe^{3+}–EDTA) (Hewitt and Smith, 1975). In the present work, iron was enriched with $^{57}Fe^{3+}$ (90.24%) (for Mössbauer measurement) and with 4 μCi of $^{59}Fe^{3+}$ (as a radiotracer).

Soybean seeds were germinated among small pieces of filter paper soaked with distilled water. After 3 days, each seedling was transplanted into a glass vessel containing 1 dm^3 of an iron-deficient nutrient solution, which was the same as the normal nutrient solution described above except that no iron had been added. After 2 weeks from the transplantation, the iron-deficient solution was replaced by 1 dm^3 of the normal solution containing 0.05 mmol of $^{57}Fe^{3+}$ and 4 μCi of $^{59}Fe^{3+}$. About 200 cm^3 of distilled water was supplied once a week and 50–100 cm^3 of the iron-deficient nutrient solution once a month. Neither ^{57}Fe nor ^{59}Fe was replenished. After 3 months of cultivation, immature seeds were taken out from some of the pods, when both the pods and the seeds were still green. The seeds were immediately quenched with liquid nitrogen and were stored in a freezer until the Mössbauer measurement. After 4 months, when the remaining pods became yellowish brown and split spontaneously, the ripe, greenish yellow seeds were harvested.

Some of the ripe seeds harvested were germinated with distilled water to obtain cotyledons in different growth stages for Mössbauer study. After 3 days, the seedlings were exposed to 9000-lx light of fluorescent lamps 12 h a day for 1–7 days.

The γ-rays of ^{59}Fe in each part of the plant were measured with a 2.5 × 2.5 in. NaI scintillation counter with an aluminum cover in a definite geometry. Iron content of each part of the plant was determined from the ratio of the counting rate of the sample to that of an aliquot of the nutrient solution, whose content of iron was known.

Preparation of Ferric Phytates. Mono-, di-, and tetraferric phytates were prepared according to the methods described in the literature (Lipschitz et al., 1979; Ellis and Morris, 1979).

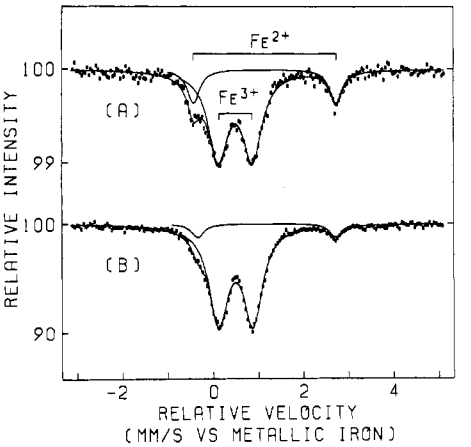

Figure 1. Mössbauer spectra of ^{57}Fe in (A) immature and (B) ripe soybean seeds at liquid nitrogen temperature. The relative velocity is given relative to metallic iron at room temperature.

Measurement of Mössbauer Spectra. The seed samples containing ^{57}Fe were subjected to Mössbauer measurement at room, liquid nitrogen, and liquid helium temperatures by means of conventional Mössbauer spectrometers (Austin S-600 and Ranger 700 series) against a 10–20-mCi $^{57}Co/Rh$ source. The 14.4-keV γ-rays were detected with a proportional counter filled with Kr + 3% CO_2 (Reuter-Stokes Inc.).

The spectra were least-squares fitted to lines of Lorentzian shape with a FACOM M380 computer at our institute. The isomer shifts are presented relative to metallic iron at room temperature.

RESULTS

Distribution of Iron in the Plant. The iron content of the whole seeds commercially obtained to be used in the present work was 95–110 ppm dry weight (neutron activation analysis). The content of iron (^{57}Fe 90.24%) in different parts of the ripe plant cultivated with ^{59}Fe and ^{57}Fe was determined to be 130 ppm dry weight for whole seeds, 140 for roots, 50 for leaves, 10 for stems, and 30 for pods by radioactivity measurement of ^{59}Fe.

Mössbauer Spectra of ^{57}Fe in Seeds. Since each harvested seed contained more than 10 μg of ^{57}Fe (the weight of each seed was in the range 100–300 mg), Mössbauer analysis of iron in a single seed was possible. In Figure 1 are shown the Mössbauer absorption spectra of ^{57}Fe in immature and ripe soybean seeds at liquid nitrogen temperature. As seen in the figure, the Mössbauer spectra of both immature and ripe seeds consist of four absorption peaks. The curves in the figure are the results of least-squares fitting of the data points, assuming two doublets of Lorentzian shape. The numerical results of the analysis are given in Table I. Although the ratio of area of the doublets is much different between the immature and ripe seeds, little difference is observed between the peak positions of them. The dominant doublet with the smaller isomer shift and quadrupole splitting is ascribed to high-spin ferric species, while the other one with larger isomer shift and quadrupole splitting to high-spin ferrous ions.

Figure 2 shows the Mössbauer spectrum of ^{57}Fe in a ripe seed measured at 7 K. The spectrum consists predominantly of a magnetically split sextet and a paramagnetic doublet, both assignable to ferric ions.

In vivo measurement on an immature green seed at room temperature gave no absorption peak within experimental uncertainties. On the other hand, Mössbauer absorption spectra were observed on ripe seeds at room temperature

Table I. Mössbauer Parameters of Soybean Samples and Related Iron Compounds

samples	temp, K	isom shift,[a] mm/s	quadrupole spl, mm/s	rel area	assgnt
immature green	LN[b]	0.50 ± 0.03	0.74 ± 0.05	83	Fe^{3+}
seed		1.19 ± 0.05	3.09 ± 0.05	17	Fe^{2+}
ripe yellow	RT[c]	0.37 ± 0.03	0.74 ± 0.03	96	Fe^{3+}
seed		1.01 ± 0.05	2.56 ± 0.05	4	Fe^{2+}
	LN	0.49 ± 0.03	0.75 ± 0.05	92	Fe^{3+}
		1.17 ± 0.05	3.05 ± 0.05	8	Fe^{2+}
cotyledon	LN	0.49 ± 0.03	0.76 ± 0.03	86	Fe^{3+}
		1.18 ± 0.05	3.08 ± 0.05	14	Fe^{2+}
dry leaves	LN	0.47 ± 0.03	0.73 ± 0.05		Fe^{3+}
dry roots	LN	0.48 ± 0.03	0.72 ± 0.05		Fe^{3+}
monoferric	RT	0.43 ± 0.03	0.51 ± 0.03		
phytate	LN	0.53 ± 0.03	0.62 ± 0.03		
diferric	RT	0.42 ± 0.03	0.53 ± 0.03		
phytate	LN	0.54 ± 0.03	0.58 ± 0.03		
tetraferric	RT	0.42 ± 0.03	0.48 ± 0.03		
phytate	LN	0.54 ± 0.03	0.57 ± 0.03		
ferric citrate	77	0.50 ± 0.03	0.54 ± 0.05	*d*	
ferrous	295	1.27 ± 0.05	2.78 ± 0.05		
citrate	77	1.38 ± 0.05	3.38 ± 0.05		
ferritin	293	0.35 ± 0.05	0.68 ± 0.05	*e*	
	86	0.45 ± 0.05	0.70 ± 0.05		

[a] Relative to metallic iron at room temperature. [b] Liquid nitrogen temperature. [c] Room temperature. [d] Baggio-Saitovitch et al. (1972). [e] Fischbach et al. (1971).

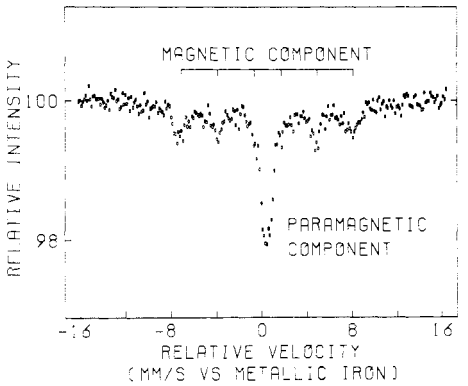

Figure 2. Mössbauer spectrum of ^{57}Fe in a ripe soybean seed at 7 K. The scale of the abscissa is different from that of the other spectra.

as shown in Figure 3A. Parts B and C of Figure 3 show the results of Mössbauer measurement on a ripe seed in the course of imbibing water and sprouting at room temperature. As the seed swelled, the intensity of Mössbauer absorption decreased gradually (Figure 3B), the spectrum vanishing completely in a few days (Figure 3C). When a swollen seed sample was subjected to measurement at liquid nitrogen temperature, a spectrum consisting of ferric and ferrous doublets was observed as shown in Figure 4A.

The spectra of cotyledons of the seedlings exposed to the light are also composed of both ferric and ferrous doublets as shown in Figure 4B. The relative area of the ferrous doublets was 7.8 ± 0.9% (data on six samples) and 14.1 ± 1.5% (data on five samples exposed to the light for 1–7 days) for the ripe seeds and for the cotyledons of the same crop, respectively. The change in the area with the duration of the light exposure was within the experimental uncertainties.

Mössbauer Spectra of ^{57}Fe in Leaves and Roots. The Mössbauer spectra of the leaves and roots dried over P_2O_5 consist of a doublet ascribable to trivalent high-spin iron at liquid nitrogen temperature (Figure 5). No absorption assignable to divalent iron is observed within experimental uncertainties.

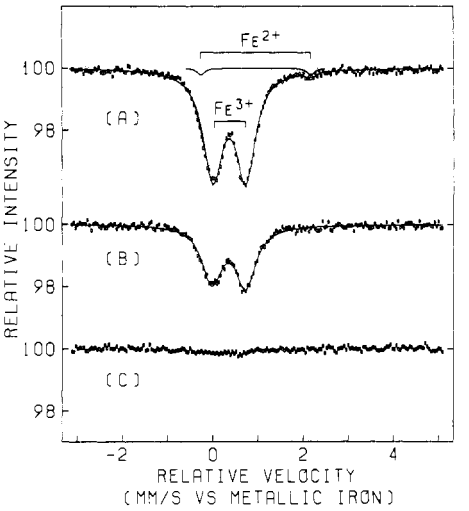

Figure 3. Mössbauer spectra of ^{57}Fe in (A) an untreated ripe soybean seed and in (B) and (C) one imbibing water and sprouting (measurement at room temperature).

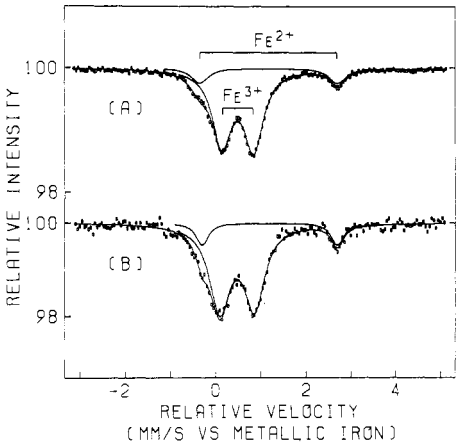

Figure 4. Mössbauer spectra of ^{57}Fe in (A) a soybean seed imbibing water and (B) cotyledon of the seedling exposed to light for 3 dyas (measurement at liquid nitrogen temperature).

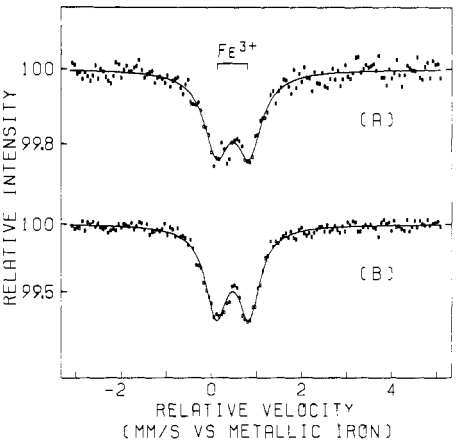

Figure 5. Mössbauer spectra of ^{57}Fe in (A) roots and (B) leaves of soybean dried over P_2O_5 (measurement at liquid nitrogen temperature).

Mössbauer Spectra of Iron Phytates. The Mössbauer parameters of the ferric phytate samples at room and liquid nitrogen temperatures are given in Table I. From chemical analysis, mono- and diferric phytate samples were found to contain about 10% of di- and 5% of tetraferric phytates, respectively. The three phytate samples give essentially the same Mössbauer parameters, suggesting that iron is in a similar chemical environment.

The parameters of mono- and diferric phytates at liquid nitrogen temperature are in agreement with those reported previously (May et al., 1980).

DISCUSSION

The Mössbauer spectra of iron in the root, leaf, and seed samples of soybean differ distinctly from those of solid Fe(III)–EDTA complex and also from its frozen solution (Ambe et al., 1985). It is evident that the $^{57}Fe^{3+}$–EDTA complex in the culture solution is converted into different chemical forms in the process of absorption by the plant.

It is reported that ferric ions in a nutrient solution are reduced by some reductants released by the roots and the resulting ferrous ions enter the root (Ambler et al., 1971), while ferrous ions are oxidized as they enter the metaxylem (Brown, 1977). The results of our Mössbauer measurement on dried whole root and leaf samples indicate that iron is exclusively in the trivalent state within experimental uncertainties. On the other hand, an absorption peak due to ferrous species was observed in every seed sample studied, the relative area being fairly large in immature seeds. No distinct absorption of ferrous ions has been observed in the plant samples studied so far (May et al., 1980; Goodman and DeKock, 1982; Goodman et al., 1982; Ambe et al., 1985; Huang et al., 1986) except for pea leaves (Goodman and DeKock, 1982) and green laver (Ambe et al., 1986).

Absence of an absorption peak in the spectrum of immature seeds at room temperature suggests that both ferric and ferrous species in the seeds are in a dissolved state or their recoilless fraction is very small at room temperature. In ripe seeds iron has an appreciable recoilless fraction yielding a distinct Mössbauer absorption at room temperature (Figure 3A). It is seen from Figure 3B,C that the recoilless fraction of iron species decreases remarkably as the seed imbibes water and swells.

A number of iron compounds have been isolated from plants and characterized by Mössbauer spectroscopy (Dickson, 1984). Mössbauer spectra of plant samples are expected to be a superposition of the spectra of different kinds of iron compounds. However, the observed spectra of iron in soybean seeds, leaves, and roots are all relatively simple as described above. The isomer shift and quadrupole splitting of the ferric and ferrous doublets observed in different states of the seeds as well as in roots and leaves are virtually the same at the same temperature. These observations suggest that the majority of iron in the samples is in limited chemical forms. The parameters of ferric species also show good agreement with those of frozen fresh soybean leaves in the literature (Goodman and DeKock, 1982).

Mössbauer parameters of ferric and ferrous ions in the seeds are distinctly different from those of heme proteins and iron–sulfur proteins (Dickson, 1984). The isomer shift of the ferric doublets is typical of ferric ions octahedrally coordinated by six oxygen atoms. But, coordination of other elements such as nitrogen is not ruled out. The isomer shift of the ferrous component is slightly lower than that for ferrous ions with six oxygen ligands as in $[Fe(OH_2)_6]^{2+}$ (Greenwood and Gibb, 1971).

It was shown that most of the iron in wheat seeds and bran is in the form of monoferric phytate (May et al., 1980). The isomer shift of the ferric component of the soybean seeds is in agreement with that of ferric phytates within the experimental uncertainties, but quadrupole splitting of the former is significantly larger than that of the phytates. It is concluded, therefore, that the ferric component of soybean seeds is not phytate.

It is reported that iron is translocated in the form of citrate in soybean plants (Tiffin, 1970). However, the Mössbauer parameters of neither ferric nor ferrous doublets of the soybean samples are in agreement with those of ferric or ferrous citrate (Baggio-Saitovitch et al., 1972).

Of the iron compounds found so far in plants, ferritin has the isomer shift and quadrupole splitting closest to those of the soybean seeds. The iron storage protein, ferritin, is a micelle of hydrated ferric oxide with some phosphate, $(FeOOH)_8(FeOOPO_3H_2)$, surrounded by a shell of protein (Crichton, 1973). The micelle core of ferritin isolated from horse spleen is about 70 Å in diameter and has a molecular weight of 418000 (Fischbach and Andergegg, 1965). Its Mössbauer spectrum is reported to consist of a paramagnetic doublet at 293 and 86 K with the isomer shift and quadrupole splitting given in Table I and to split to six lines at 11 K (Fischbach et al., 1971). This has been interpreted as resulting from antiferromagnetic ordering of the iron atoms.

Phytoferritin, ferritin in plants, was first found in pea embryos (Hyde et al., 1963). Later, it was also isolated from seeds of lentil and bean (Crichton et al., 1978; Van der Mark et al., 1983). If the dominant component of iron in soybean seeds is phytoferritin, the size of the hydrated ferric oxide unit must be smaller than that of horse spleen ferritin core, since a paramagnetic doublet is still observed at 7 K on them. A similar result is reported on duckweed (Goodman et al., 1982). It is reported that phytoferritin in plastids of the cambial zone of willow has a core comprising four to six subunits, each about 15 Å in diameter (Robards and Humpherson, 1967). More data with model compounds than is available at the present time are required to specify the iron complexes in soybean plant.

The observation that the immature seeds contain a much larger fraction of divalent iron than the ripe one (Figure 1) suggests the possibility that iron is transported to seeds as ferrous species and is then oxidized there to be stored as ferric species. Apoferritin, iron-free ferritin protein, is reported to catalyze the oxidation of ferrous to ferric ions to form ferritin (Crichton, 1973). An alternative possibility is that ferrous ions are needed for a certain biochemical reaction in the course of ripening of the seeds.

The soybean seeds are reported to contain sufficient iron to meet the plant's demand in the seedling stage (Brown, 1977) and two thirds of iron is shown to move from bean cotyledons to the axis of the seedling during the first week of germination (Biddulph, 1951). As seen in Table I, the ratio of peak area of the ferrous to ferric species is larger in the cotyledons compared with the untreated ripe seeds of the same crop. This can be interpreted to show that the ferric ions are transformed into a ferrous species to be transported to the other growing parts of the seedling. It is shown by in vitro experiment that the release of iron from ferritin is promoted by reduction of ferric ion to ferrous ion followed by chelation of the ferrous ions with compounds having oxygen and nitrogen as donor atoms (Crichton, 1973). Here again, the possibility of the ferrous ions taking part in another biochemical reaction in the cotyledon is not ruled out.

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Two-Dimensional Electrophoretic Analysis of the Proteins of Isolated Soybean Protein Bodies and of the Glycosylation of Soybean Proteins

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Two-dimensional electrophoresis was used to examine the protein components in isolated soybean protein bodies. We found that most individual, abundant polypeptides of soybean seeds occur in protein bodies. These include β-conglycinin, glycinin, lectin, and Kunitz trypsin inhibitor (as expected, based on previous work of other investigators) and many other, unidentified polypeptides. There are, however, numerous soybean polypeptides that are not present in protein bodies. Dansylhydrazine staining of water-extracted soybean proteins that had been separated on two-dimensional gels demonstrated that not only β-conglycinin and lectin but several other proteins of unknown identity are glycosylated. We also demonstrate that a small portion of glycinin is glycosylated.

Protein bodies in soybean seeds are nearly spherical particles with diameters of 2–10 μm (Saio and Watanabe, 1966) surrounded by a membrane (Tombs, 1967; Wolf, 1972). It has been revealed by immunoelectrophoresis (Catsimpoolas et al., 1968) and by ultracentrifugation (Wolf, 1970) that the major storage proteins, glycinin and β-conglycinin, are the most abundant components of protein bodies (Koshiyama, 1972). During germination, protein bodies disintegrate (Tombs, 1967; Catsimpoolas et al., 1968), and protein in them is degraded to serve as the source of nitrogen for nitrogen-containing compounds synthesized by the developing seedling (Derbyshire et al., 1976). Protein bodies can be isolated by differential centrifugation in cottonseed oil/carbon tetrachloride mixtures of various densities (Saio and Watanabe, 1966) or by sucrose density gradient centrifugation (Tombs, 1967). Employing two-dimensional electrophoresis on proteins of isolated protein bodies, we report here that protein bodies contain many other proteins besides glycinin and β-conglycinin. These include soybean lectin, Kunitz trypsin inhibitor, and a large number of other unidentified polypeptides.

We also report an electrophoretic analysis of glycosylation of soybean proteins. Certain soybean proteins are known to be glycosylated. The lectin of soybean seeds has been shown to consist of 4.5% mannose and 1.2% *N*-acetyl-D-glucosamine (Lis and Sharon, 1973). The β-conglycinin polypeptides are also found to have carbohydrate covalently attached: the α and α′ polypeptides contain 4 mol of glucosamine and 12 mol of mannose; the β polypeptides have 2 mol of glucosamine and 6 mol of mannose (Thanh and Shibasaki, 1977). The situation is not clear with glycinin. Fukushima (1968) reported that glycinin contains a low amount of carbohydrate (about 0.88%), and Wolf et al. (1966) reported 0.17–0.24% carbohydrate in glycinin but later Koshiyama and Fukushima (1976) reported that glycinin is not glycosylated.

Kitamura et al. (1974) used a concanavalin A–agarose column to purify glycinin by removing β-conglycinin, which is retained by the column. Using that method (Lei et al., 1983), we found that a small amount of glycinin binds to the concanavalin A–agarose column and coelutes with β-conglycinin. This suggests that a small portion of glycinin is glycosylated, and we confirm that in this paper.

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