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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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In addition we report that numerous other (unidentified) soybean proteins are glycosylated.

MATERIALS AND METHODS

Preparation of Soybean Water Extract and Isolation of Protein Bodies. Soaked beans (Williams cultivar) were extracted with water as described by Lei et al. (1981).

Protein bodies of soybean seeds were isolated by the method of Saio and Watanabe (1966). The seeds were homogenized in cottonseed oil and fractionated by differential centrifugation; the density of the solvent was adjusted by adding carbon tetrachloride. Fractions of densities 1.360 and 1.390 were collected. By light microscopy, the particles in this fraction were found to correspond, as expected, with protein bodies purified by this method (Saio and Watanabe, 1966). The protein bodies were washed with carbon tetrachloride and dried under vacuum.

Isolation of Concanavalin A Binding Glycinin Polypeptides for Two-Dimensional Electrophoresis. Four grams of defatted soybean meal was suspended in 20 mL of 0.1 M potassium phosphate (pH 7.1)/1 M NaCl/0.01 M 2-mercaptoethanol and stirred for 1 h at room temperature. The protein extract was loaded onto a concanavalin A–agarose column (Bethesda Research Laboratories) that contained 70 mg of immobilized concanavalin A and had been equilibrated with the phosphate buffer. The proteins that did not bind to the column were removed by washing the column with the above phosphate buffer until the A_{280} of the effluent was less than 0.01. The proteins bound to the column were then eluted with 0.1 M α-methyl-D-mannoside in the standard phosphate buffer at pH 7.6 of Kitamura et al. (1974). The proteins thus eluted contained β-conglycinin, glycinin (in small amounts), lectin, and other glycoproteins. This set of proteins was subjected to one-dimensional SDS gel electrophoresis (Laemmli, 1970). Bands corresponding to the glycinin acidic and basic polypeptides were cut out from several gels. The polypeptides were eluted from the gels, acetone-precipitated according to the method of Hager and Burgess (1980), and subjected to two-dimensional electrophoresis.

Purification of Glycosylated Glycinin for Amino Acid Analysis. Glycinin was partially purified according to the pH precipitation method of Thanh and Shibasaki (1976). The glycinin was then chromatographed on a concanavalin A–agarose column as described by Kitamura et al. (1974). The flowthrough (which contained glycinin that was not glycosylated) and eluted proteins (which contained glycosylated species) were separately precipitated at 25% trichloroacetic acid (on ice), washed with acetone, and subjected to one-dimensional SDS gel electrophoresis (Laemmli, 1970). We excised 37–42-kDa (acidic) and 17–20-kDa (basic) polypeptide bands from the SDS gels. The polypeptides were eluted from the polyacrylamide and acetone-precipitated according to the method of Hager and Burgess (1980). The amino acid compositions of the polypeptides were determined by the method of Heinrikson and Meredith (1984).

Purification of Individual Soybean Proteins. For use as electrophoretic standards, glycinin and β-conglycinin were purified as described in Lei et al. (1983). In that procedure, we used concanavalin A–agarose chromatography as one step in separating glycinin and β-conglycinin. The glycinin thus purified (and used as a standard in Figure 6) was not retained by the concanavalin A–agarose column. Purified lectin was obtained from Bethesda Research Laboratories.

Two-Dimensional Gel Electrophoresis. Either isoelectric focusing (IEF) or nonequilibrium pH gradient electrophoresis (NEPHGE) was used as the first dimension, with slight modifications of the procedures given by O'Farrell (1975) and O'Farrell et al. (1977). These have been described elsewhere (Lei et al., 1983). Ampholines (LKB) with ranges of pH 4–6 and 5–7 were used in a 1:1 mixture for IEF and pH 5–7 ampholines were used alone for NEPHGE, and a total of 2800 V/h was applied.

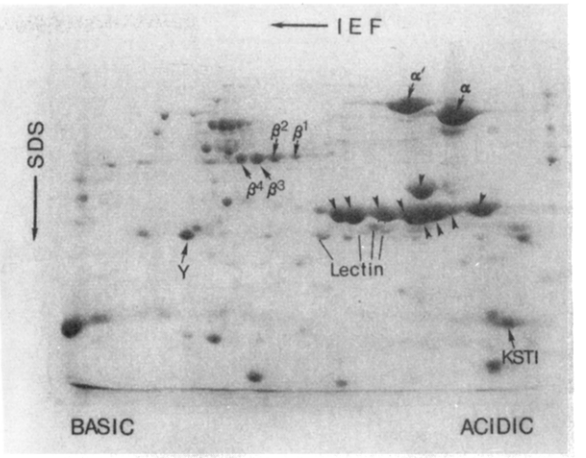

Figure 1. Proteins of isolated soybean protein bodies analyzed by two-dimensional electrophoresis with IEF in the first dimension. Second-dimension gel: 10% acrylamide/0.27% bis(acrylamide). The subunits of β-conglycinin are labeled α, α', β^1, β^2, β^3, and β^4. Soybean trypsin inhibitor (Kunitz) is labeled KSTI. The four components of soybean lectin are labeled Lectin. Arrowheads point to acidic subunits of glycinin. Y identifies a protein that is prominent in soybean whey. Those components are identified, based on previous studies (Lei et al., 1983).

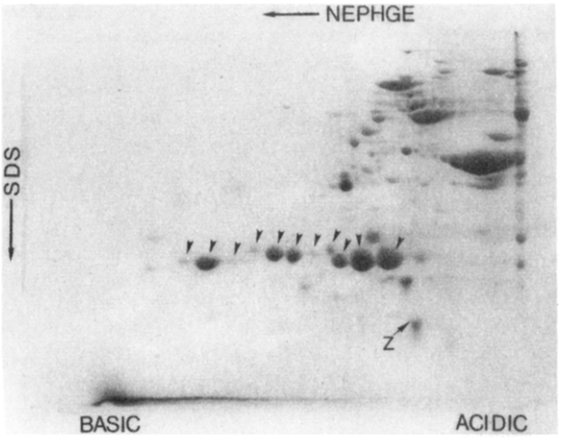

Figure 2. Proteins of isolated soybean protein bodies analyzed by two-dimensional electrophoresis with NEPHGE in the first dimension. Second-dimension gel: 12% acrylamide/0.32% bis(acrylamide). A total of 2800 V/h was applied in NEPHGE. Arrowheads indicate the basic subunits of glycinin. Identification of polypeptide spots is based on previous studies (Lei et al., 1983).

Detection of Glycoproteins by Fluorescence Method. Glycoproteins on the two-dimensional gels were detected with a sensitive dansylhydrazine fluorescence stain according to Eckhardt et al. (1976). The treated gels were photographed with Polaroid type 55 film and a Kodak Wratten 16 filter on a UV transilluminator (Ultraviolet Products, Inc.) of wavelength 366 nm.

RESULTS AND DISCUSSION

Electrophoretic Analysis of Proteins in Protein Bodies. Figure 1 shows an electrophoregram of neutral and acidic polypeptides present in our protein body

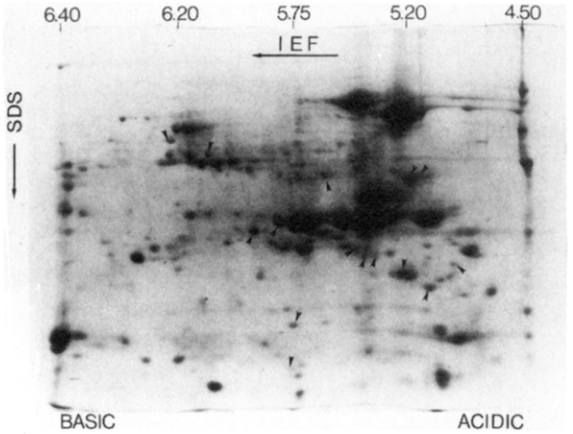

Figure 3. Total water-extracted soybean proteins analyzed by two-dimensional electrophoresis with IEF in the first dimension. The electrophoregram is adapted from Figure 1 of Lei et al. (1983) to indicate those soybean polypeptides (indicated with arrowheads) that are *not* detected in isolated protein bodies (cf. Figure 1).

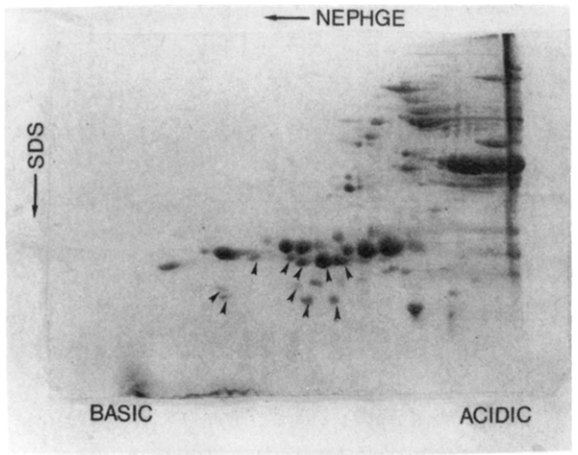

Figure 4. Total water-extracted soybean proteins analyzed by two-dimensional electrophoresis with NEPHGE in the first dimension. This electrophoregram is similar to that in Figure 2 of Lei et al. (1983). Second-dimensional gel: 12% acrylamide-/0.32% bis(acrylamide). A total of 2800 V/h was applied in NEPHGE. Arrowheads indicate the protein components that are not detected in the protein bodies (cf. Figure 2).

preparation. Figure 2 is an electrophoregram of the basic polypeptides in that preparation. On the basis of previous results (Lei et al., 1983), we identified, as shown in Figure 1, the acidic polypeptides of glycinin, the several subunits of β-conglycinin, lectin, Kunitz trypsin inhibitor, and the Y polypeptide (a predominant whey protein) and, in Figure 2, basic polypeptides of glycinin and the Z polypeptide [a small polypeptide of glycinin—probably a low molecular weight subunit that has been previously described by Moreira et al. (1979) and A5 of Staswick and Nielsen (1983)]. In addition, there are many polypeptide spots of unknown identity in the protein body preparation; we counted over 300 spots on the original gel.

The spots indicated by arrrowheads in Figures 3 and 4 (the electrophoregrams of *total* water-extracted proteins) show those polypeptides that are not found in isolated protein bodies. From the electrophoregrams in Figures 1–4, we can see that the protein composition of the isolated protein bodies is clearly different from that of total, water-extracted proteins. Thus, other protein-containing parts of the soybean cells are clearly absent from our preparation of protein bodies. Most basic polypeptides that are not part of glycinin do not occur in the protein

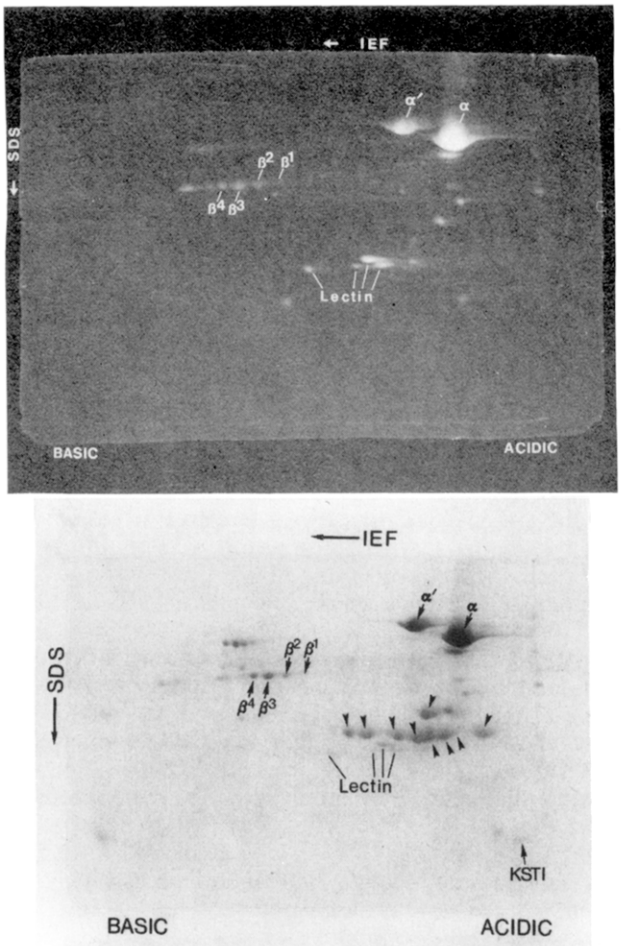

Figure 5. Total water-extracted soybean proteins analyzed by two-dimensional electrophoresis with IEF in the first dimension. Second-dimension: 10% acrylamide/0.27% bis(acrylamide). The gel was treated with dansylhydrazine to stain glycoproteins (A, top) and then stained with Coomassie blue R-250 (B, bottom). Polypeptides were identified on the basis of previous studies (Lei et al., 1983); they were labeled the same as in Figure 1.

bodies (see arrowheads in Figure 4). These non-glycinin basic polypeptides may have peculiar amino acid compositions, since they are not precipitated by 25% trichloroacetic acid (Lei, 1984).

Our electrophoretic analyses of isolated soybean protein bodies show that their polypeptide composition is a good deal more complicated than has previously been thought. In particular, soybean protein bodies contain numerous polypeptides that are evidently not storage proteins. These include lectin and trypsin inhibitors [as expected, on the basis of the work of Horisberger and Vonlanthen (1980) and Horisberger and Tacchini-Vonlanthen (1983a,b)] and many polypeptides that, as judged by their scarcity compared to glycinin and β-conglycinin, seem unlikely to function as storage proteins. Thus, from a physiological perspective, protein bodies should perhaps be thought of as more than sites of deposition of storage proteins.

Glycoproteins. Total, water-extracted soybean proteins were separated by two-dimensional electrophoresis. Glycosylated polypeptides were detected by a fluorescence staining procedure (Figure 5A). The same two-dimensional gel was then stained with Coomassie blue R-250 (Figure 5B). (Prior treatment with the fluorescence stain results in blurred patterns in the Coomassie blue staining. If the gel had not been treated with the fluorescence stain, the Coomassie blue pattern would have been very similar to that in Figure 3.) β-Conglycinin and lectin were

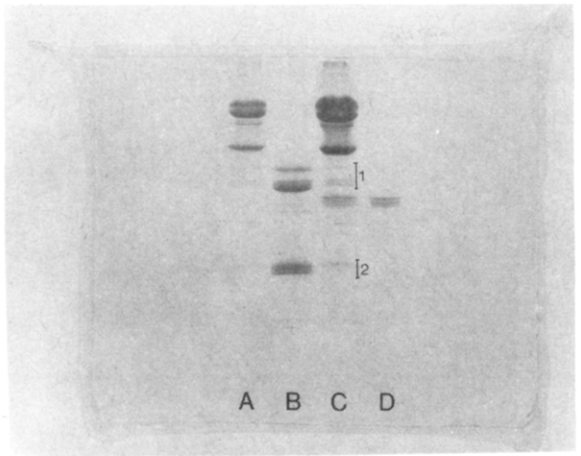

Figure 6. SDS polyacrylamide gel electrophoresis of soybean proteins retained by a concanavalin A–agarose column. The separating gel was polymerized from 12% acrylamide/0.32% bis(acrylamide). Lane A: β-conglycinin. Lane B: glycinin. Lane C: material eluted from concanavalin A–agarose column. Regions 1 and 2 correspond to acidic and basic subunits of glycinin, respectively. Lane D: soybean lectin.

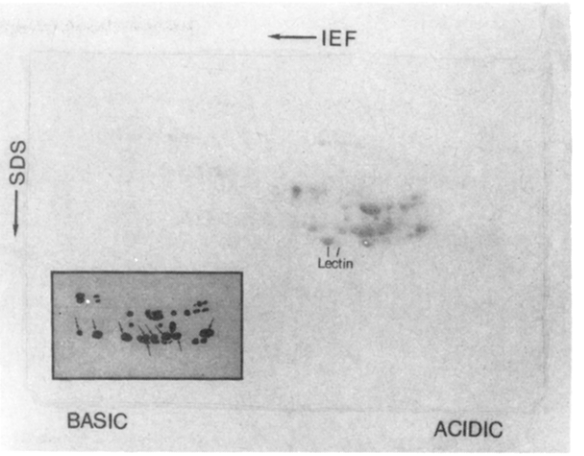

Figure 7. Two-dimensional electrophoresis (with IEF in the first dimension) of acidic polypeptides from glycinin that was retained by a concanavalin A–agarose column. The sample was polypeptides eluted from region 1 of one-dimensional SDS gels, such as that shown in Figure 6. Second-dimension: 10% acrylamide/0.27% bis(acrylamide). The electrophoregram shows the Coomassie blue staining pattern obtained on a gel that had previously been treated with dansylhydrazine. The inset is a schematic representation of the *glycinin portion* of the electrophoregram, which lies just above lectin in the pattern of stained spots. In the inset arrows indicate the acidic components of glycinin [identified on the basis of previous studied (Lei et al., 1983)], which *were not* fluorescent after exposure to dansylhydrazine; the rest of the components in the inset *were* fluorescent after dansylhydrazine treatment.

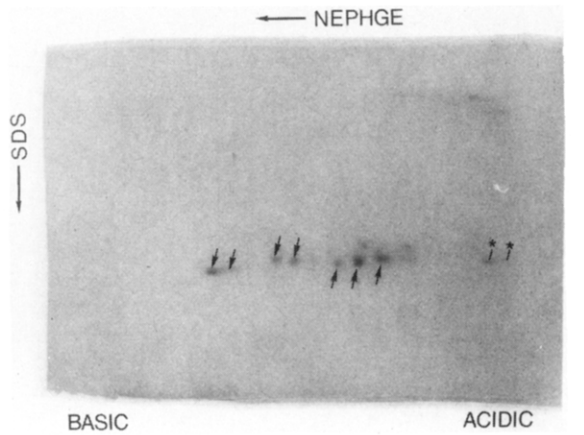

Figure 8. Two-dimensional electrophoresis (with NEPHGE in the first dimension) of basic subunits from glycinin that was retained by a concanavalin A–agarose column. The sample was polypeptides eluted from region 2 of one-dimensional SDS gels such as that shown in Figure 6. Second-dimension: 12% acrylamide/0.32% bis(acrylamide). A total of 2800 V/h was applied in NEPHGE. The electrophoregram shows the Coomassie blue pattern of a gel that had been treated with dansylhydrazine. Arrows indicate the basic polypeptides of glycinin [identified on the basis of previous studies (Lei et al., 1983)], which were not fluorescent after dansylhydrazine treatment. Stars indicate spots that were fluorescent after dansylhydrazine treatment.

fluorescent. This was expected, since they are known to be glycoproteins (Thanh and Shibasaki, 1977; Lis et al., 1970). In addition, however, there were several other polypeptides of unknown identity that were glycosylated. Note that glycinin was not fluorescent. Basic polypeptides were examined by NEPHGE–SDS electrophoresis, but no clear fluorescent spots were seen after treatment with dansylhydrazine (result not shown).

No glycosylated polypeptides were detected in a soybean whey preparation except the α subunit of β-conglycinin, which is a residual soybean curd protein present in low amounts in the whey (result not shown).

Glycosylation of a Subpopulation of Glycinin Molecules. The large majority of glycinin does not bind to a concanavalin A–agarose column. We confirmed the absence of carbohydrate in the protein fraction that does not bind to concanavalin A–agarose by the phenol–sulfuric acid method (Dubois et al., 1956) for total carbohydrate content. [We could have detected as little as 0.01% (w/w).] Thus, the unbound glycinin is not detectably glycosylated. Its polypeptides, as expected, are not fluorescent after treatment with the dansylhydrazine reagent (result not shown). There is, however, a small amount of glycinin (approximately 1% of the total) that is retained by a concanavalin A–agarose column even after very thorough washing with potassium phosphate (pH 7.1)/1 M NaCl/0.01 M 2-mercaptoethanol. This glycinin elutes from the column with β-conglycinin when α-methyl-D-mannoside is applied.

In Figure 6, it can be seen from one-dimensional SDS gel electrophoresis that concanavalin A binding soybean proteins contain bands in the positions of the acidic and basic subunits of glycinin (regions 1 and 2). These bands are fluorescent after treatment with dansylhydrazine (result not shown). They were eluted from several one-dimensional SDS gels, concentrated by acetone precipitation, and analyzed by two-dimensional electrophoresis. In Figures 7 and 8 we show the Coomassie blue patterns of two-dimensional gels that had first been treated with the dansylhydrazine stain to identify glycoproteins. The inset in Figure 7 is a schematic representation of the acidic glycinin polypeptide region of that electrophoregram. In that inset and in Figure 8, which shows basic polypeptides, arrows indicate polypeptides that *were not* fluorescent upon dansylhydrazine treatment. The rest of the spots in the inset *were* fluorescent. Two spots of intense fluorescence in the electrophoregram of Figure 8 are indicated by stars. There were, in addition, some diffuse and rather faint areas of fluorescence in that electrophoregram. [The actual fluorescence patterns (Lei, 1984) are not shown because they reproduce poorly.]

In Figures 7 and 8 we see both glycosylated and nonglycosylated spots. The patterns of the nonglycosylated spots resemble the patterns of acidic and basic polypeptides observed for a typical glycinin preparation (Lei et al., 1983). In the acidic glycinin polypeptide region

Table I. Amino Acid Compositions of Glycosylated and Nonglycosylated Glycinin[a]

amino acid[b]	37–42-kDa acidic polypeptides		17–20-kDa basic polypeptides	
	glycosylated	nonglycosylated	glycosylated	nonglycosylated
Asx	14	13	16	16
Glx	21	25	13	14
Ser	7.9	7.3	8.9	8.8
His	2.0	1.8	1.9	1.8
Arg	6.2	6.4	5.9	6.1
Thr	5.4	5.0	5.7	5.3
Ala	4.7	4.4	8.3	8.3
Pro	6.7	7.3	5.4	5.7
Tyr	2.8	2.7	3.8	3.7
Val	5.7	6.1	7.3	6.8
Met	1.1	1.0	1.0	1.0
Cys	1.3	1.3	0.6	0.6
Ile	4.8	4.4	4.6	4.3
Leu	7.0	6.6	10	9.8
Phe	4.4	4.1	5.1	5.0
Lys	4.2	4.3	3.0	3.0

[a] The values shown are averages of duplicate determinations (mol %). [b] Trp was not determined. Gly is excluded from these amino acid compositions because the SDS gel electrophoresis running buffer contained glycine. The polypeptides eluted from the SDS gels were therefore contaminated with glycine.

(Figure 7) the number of glycosylated spots is approximately the same as the number of nonglycosylated spots. There appears to be little, if any, glycosylation of the basic polypeptides of glycinin.

To obtain additional evidence that the glycosylated material with electrophoretic properties of glycinin was in fact glycinin, we obtained amino acid compositions of the glycosylated glycinin-like bands. In Table I, these compositions are compared to the compositions of the acidic and basic polypeptides of nonglycosylated glycinin. The excellent agreement between the compositions of the glycosylated material and nonglycosylated glycinin strongly supports our conclusion that the glycosylated material is glycinin.

We cannot exclude the possibility that there are particular glycinin polypeptides that occur in low amounts and that are glycosylated to a high extent. There appears to be a simpler interpretation, however. Given the structural similarities between glycinin and β-conglycinin (Argos et al., 1985; Lei et al., manuscript in preparation) and the similar complexities of nonglycosylated and glycosylated acidic glycinin polypeptides (Figure 7), the simplest interpretation of our observations is that most acidic glycinin polypeptides can be glycosylated (perhaps by the gl ycoslyation system that operates so efficiently on β-conglycinin) but that only a small fraction of any particular polypeptide is glycosylated in the total glycinin population. That is, the glycosylation of glycinin is inefficient. Even within the population of glycosylated glycinin molecules, only a portion of any particular polypeptide is glycosylated, since we find both glycosylated and nonglycosylated polypeptides in the concanavalin A binding glycinin molecules. It is of course unclear at this stage whether there is a special biological role for the small percentage of glycinin molecules that is glycosylated.

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