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The Glycoproteins of Plant Seeds[1]

ANALYSIS BY TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS AND BY THEIR LECTIN-BINDING PROPERTIES

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ABSTRACT

Protein from the jack bean, peanut, soybean and kidney bean seeds were extracted with a solution containing 9.3 molar urea, 5 millimolar K_2CO_3, 0.5% dithiothreitol and 2% Nonidet P-40 and then subjected to two-dimensional gel electrophoresis. After electrophoresis, the slab gels were stained with a variety of ^{125}I-labeled lectins and the lectin-binding proteins were identified after autoradiography. Incubation of slab gels of jack bean with concanavalin A, peanut with peanut agglutinin, soybean with soybean agglutinin, and kidney bean with phytohemagglutinin showed that the majority of the polypeptides in each seed type were able to bind to their homologous lectins. Control slab gels in which incubations were carried out with identical amounts of proteins, ^{125}I-lectin and an appropriate sugar inhibitor showed little or no lectin binding to the polypeptides. Additionally, incubation of slab gels of peanut proteins with ^{125}I-ricin, ^{125}I-wheat germ agglutinin, ^{125}I-concanavalin A, and ^{125}I-soybean agglutinin each revealed a clearly distinct binding pattern compared to the one observed with the peanut agglutinin. The results demonstrate that a large number of legume seed polypeptides are glycoproteins and that the carbohydrate groups within a seed species are heterogeneous in structure, thus indicating the existence of complex glycosylating enzyme systems in legume seeds. It is suggested that the high degree of binding between seed proteins and their homologous lectins might have some functional significance in maintaining large aggregates of protein in compact, insoluble form.

Although legume seeds are known to contain glycoproteins (2–4, 15–18, 20) not a great deal of information is available about their structures, the function of the carbohydrate group and the species distribution. Here, a study was initiated to identify the glycoproteins of peanut and other legume seed proteins and to classify them according to their lectin binding properties. To overcome the problem of resolution encountered when analyzing complex protein mixtures, we have separated the polypeptides in the seeds using two dimensional polyacrylamide gel electrophoresis. After electrophoresis, the glycoproteins were detected using a variety of ^{125}I-labeled lectins which will bind specifically to different carbohydrate residues of the glycoproteins. This approach, which has been used for analysis of glycoproteins in mammalian cells (6–9), is considerably more sensitive than conventional procedures such as the Periodic acid-Schiff stain, and provides an insight into the structure of the carbohydrate chains and a possible role of lectins in the seed.

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MATERIALS AND METHODS

Radiochemicals. Sodium [^{125}I]iodide (carrier free) was purchased from Union Carbide, Oak Ridge, TN, and used within 1 month of purchase.

Seeds. Seed samples of jack bean (*Canavalia ensiformis L.*), soybean (*Glycine max L.*), kidney bean (*Phaseolus vulgaris L.*) were obtained from the Atlee Burpee Company, Burpee, IN. Peanut (*Arachis hypogaea L.*) seeds were a gift from Dr. Allan J. Norden of University of Florida, Gainesville, FL. After removing embryonic axes and testae, the cotyledons were ground to a flour using a mortar and pestle, and defatted with ethyl ether (1).

Lectins. Soybean agglutinin, ricin I agglutinin, wheat germ agglutinin, and peanut lectin were products of Vector Laboratories, Novato, CA. Concanavalin A was bought from Miles-Yeda, Elkhart, IN, and phytohemagglutinin from Difco Laboratories, Detroit, MI.

Protein Extraction. Seed proteins were solubilized by homogenizing the defatted meals in a 9.3 M urea, 5 mM K_2CO_3 (pH 10.2) solution, followed by the addition of Nonidet P-40 (to 2% v/v) and DTT (to 0.5% w/v) (7). This procedure solubilized approximately 95% of the total protein, most of which is considered to be storage material (1).

Two-dimensional Polyacrylamide Gel Electrophoresis. The protein extracts were subjected to two-dimensional polyacrylamide gel electrophoresis by a modification (1) of the method of O'Farrell (15). After electrophoresis the gels were stained with Coomassie blue.

Iodination of Lectins. Lectins (10 mg) were dissolved in 2 ml of 0.2 M sodium acetate buffer (pH 5.6) containing 1 mg lactoperoxidase (Sigma). After addition of Na^{125}I (0.5 mCi, carrier free, Union Carbide) the reaction was initiated by adding 0.06% (v/v) H_2O_2 (10 μl) and subsequent additions were made at 1-min intervals for 15 min. The reaction was terminated by addition of about 1 mg of solid sodium azide. Labeled lectins were then freed of salts by chromatography on a Bio-Gel P-2 column. In addition, concanavalin A was purified by affinity chromatography on Sephadex G-150. Labeled lectins ($\sim 10^8$ cpm/mg) were used directly or stored at −20 C in Tris buffered saline (50 mM Tris-HCl, [pH 7.4] containing 0.15 M NaCl).

Lectin Overlays. Destained slab gels were equilibrated with TBS[3] containing 0.01% sodium azide (five changes of 250 ml), and transferred to a plastic, heat seal bag (Kapak, 3M Co., Minneapolis, MN). Labeled lectin (3×10^7 cpm) was diluted with 20 ml TBS-azide (pH 7.4) containing 0.1 mg/ml hemoglobin and, when appropriate, a saccharide inhibitor. The overlay solution was added to the plastic bag which was then heat-sealed. The bag, containing the slab gel, was then rocked gently on a tilting platform (Bellco model 7730) operating at 0.2 Hz. After equilibra-

[3] Abbreviation: TBS, Tris-buffered saline.

tion for 15 h, a corner of the bag was cut, overlay solution drained off and replaced with 20 ml TBS-azide. The gel was rinsed, drained, removed from the bag, and soaked in about six to eight changes of TBS-azide (250 ml) until radioactivity in the buffer reached background. The gel was then dried and subjected to autoradiography with enhancing screens (10).

Control gels for concanavalin A were incubated in the presence of 0.5 M α-methyl D-mannoside and washed in the presence of 0.5 M α-D-glucose in TBS. Control gels for peanut agglutinin and ricin I agglutinin were incubated and washed in the presence of 0.5 M α-D-galactose. Control gels for soybean agglutinin, wheat germ agglutinin and phytohemagglutinin were not run due to the expense or unavailability of competing haptens.

RESULTS

Protein Extraction. Seed meals were defatted and extracted in solubilizing buffer. In the case of peanut (1) this procedure removes more than 95% of the seed protein. Comparable results were obtained with the other legumes. The advantages of the method for extraction and maintaining solubility of proteins has been discussed elsewhere (1, 7).

Electrophoretic Gel Patterns. Two-dimensional electrophoresis of the seed proteins of the four legumes used in this study revealed a very high degree of complexity which is not evident if electrophoresis is carried out in one dimension (Fig. 2A). In general, provided that large amounts of protein are loaded, between 100 and 200 polypeptides can be detected, although certain major ones predominated.

For the purpose of identifying specific proteins on such gels, and to aid comparisons between cultivars or species, we have found it useful to construct schematic diagrams showing the position of each polypeptide in relation to its apparent isoelectric point and molecular weight. In general those maps are based on observations made on at least 10 gels run at different times and after different amounts of protein had been loaded. For example, Figure 1, A and B, shows such maps for soybean and peanut. The area of the gel is divided into alphabetically labeled quadrants, the vertical lines of which are based on approximate pH values and the horizontal lines on particular molecular weights. Polypeptides within each quadrant may then be designated by number. The method we employed is capable of resolving polypeptide components with isoelectric points between pH 4.0 and 8.5 and mol wt between 17,000 and 300,000. By adjusting the mixture of ampholines or the polyacrylamide concentration in the slab gel, polypeptides with properties outside these ranges can be investigated. The above conditions were chosen because they were suitable for the great majority of legume seed proteins. Thus, for the above four species, most polypeptides had pI values between 4.4 and 8.0 and mol wt between 17,000 and 90,000. In all of the seed types, each of the major components, which we believe constitute the storage proteins of the seed, appear to exist as a series of isoelectric variants of similar molecular weight.

Staining of Gels ^{125}I-Lectins. Such two dimensional gels were then stained by means of ^{125}I-lectins in order to determine which of the polypeptides were glycoproteins. In Figure 2 we show the results using an homologous series of lectins. For example, jack bean proteins have been allowed to react with ^{125}I-concanavalin A, and peanut proteins with ^{125}I-peanut lectin.

Concanavalin A, which exhibits preferential binding to oligosaccharides containing α-D-glucosyl and for α-D-mannosyl residues (19) became bound to several polypeptides, including the major protein species on the gel. Interestingly, a minor neutral polypeptide with a mol wt of 67,000 (arrowed in Fig. 2B-a) showed particularly high binding of the lectin. Certain others (arrowed in Fig. 2A-a) failed to react with concanavalin A.

Control slab gels, containing identical amounts of protein, but in which the incubation with ^{125}I-concanavalin A and the subse-

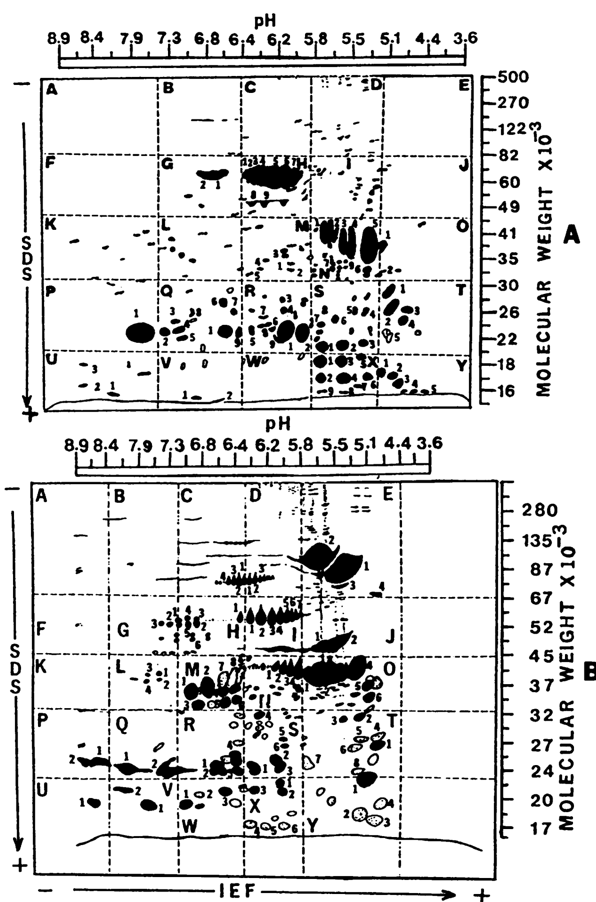

FIG. 1. Diagrams showing the positions of individual protein spots after two dimensional electrophoresis of peanut (A) and soybean (B) seed proteins. The map has been divided by lines into a series of lettered rectangles. Major proteins within a rectangle are given numbers. Black areas indicate major proteins noted on all gels. Dotted areas indicate proteins that are usually detectable, while open areas indicate minor proteins which are not always readily seen after Coomassie blue staining.

quent washing was carried out in the presence of α-methyl mannoside and D-glucose respectively, completely eliminated binding to all spots. We also tested a partial chitin hydrolysate, which is not considered to be a hapten for concanavalin A; this did not affect the binding of the lectin to the polypeptides. We therefore believe that the concanavalin A was binding specifically to either α-D-glucosyl or α-D-mannosyl residues and that the major storage proteins are glycoproteins bearing these residues.

Similarly, when peanut lectin binding to peanut proteins was tested, most of the major polypeptide species bound the lectin, again suggesting they were glycoproteins, possibly bearing the oligosaccharide sequence α-D-galactose-β-(1→3)-N-acetyl D-galactosamine (14). Only a few low molecular weight polypeptides (arrowed in Fig. 2A-b) failed to bind the lectin. Incubations carried out in presence of 0.5 M D-galactose reduced binding to about 5% of the experimental gel but did not eliminate it completely. All spots on the autoradiograph were similarly affected. When D-glucose was substituted for D-galactose, the autoradiographs were not reduced in intensity. Fetuin glycopeptides (20 mg) derived by pronase digestion of fetuin (see 7), also did not prevent binding.

Similar lectin staining experiments have been carried out with ^{125}I soybean agglutinin on soybean (Fig. 2c) and with ^{125}I-phytohemagglutinin on kidney bean (Fig. 2d). With the former, one major group of polypeptides (from quadrants J, N, and O of Fig. 2) failed to bind the lectin. Most of the others, including O_4 did

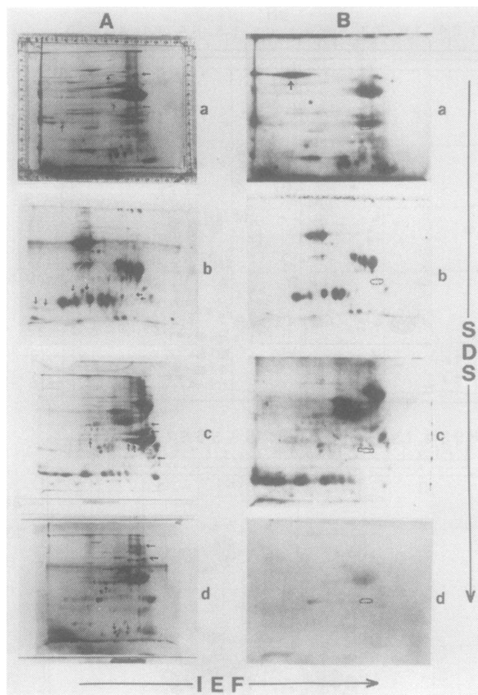

FIG. 2. Two-dimensional polypeptide maps of legume seed proteins (a, jack bean; b, peanut; c, soybean; d, kidney bean) stained with Coomassie blue (A) and ^{125}I-lectins (B). After two-dimensional gel electrophoresis, polypeptides in the slab gels were fixed and stained with Coomassie blue R-250. The Coomassie blue stained slabs were photographed (A) and then equilibriated with TBS (pH 7.4). Each slab gel was overlayed with 3×10^7 cpm of ^{125}I-lectin (a, jack bean with concanavalin A; b, peanut with peanut lectin; c, soybean with soybean agglutinin; d, kidney bean with phytohemagglutinin). After 24 h incubation with lectins, the slabs were thoroughly washed with TBS, dried, and autoradiographs were prepared. Outlined areas in B showed positions in which the homologous lectins migrate after two dimensional gel electrophoresis. Arrowed spots in A indicate some Coomassie blue stained components which fail to bind the homologous lectin.

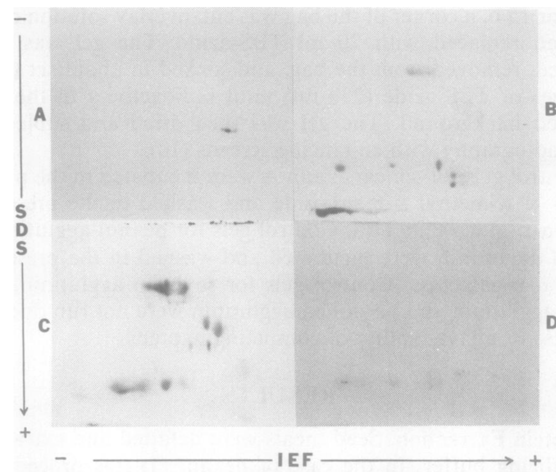

FIG. 3. Autoradiographs of two dimensional gels of peanut proteins prepared after the gels had been overlaid with ^{125}I-lectins to detect glycoproteins. A, peanut polypeptides stained by ^{125}I-ricin; B, stained by ^{125}I-wheat germ agglutinin; C, stained by ^{125}I-concanavalin A; and D, stained by ^{125}I-soybean agglutinin. The variety used in 3C was Florunner which, unlike Jenkin's Jumbo (A, B, and D), lacks polypeptides N_1, N_3, and N_5.

become radioactive suggesting that they were glycoproteins containing *N*-acetyl D-galactosamine (19). The specificity of phytohemagglutinin has not been well defined using low molecular weight saccharides, although it has been reported to bind preferentially to oligosaccharides containing the sequence D-galactose-*N*-acetyl D-galactosamine (13, 19), Again, however, several of the major polypeptide species of the kidney bean bound the lectin. Although appropriate controls have not been performed for either soybean lectin or phytohemagglutinin because either the cost or the difficulty of obtaining sufficient amounts of the possible competing sugars precluded such experiments, we believe that the observed binding does not represent a nonspecific association of the lectin with proteins in general, since several polypeptide components on the gels failed to bind the lectins at all. The positions of the lectin subunits themselves have been outlined (Fig. 2B). These were established by including an aliquot of commercially purified, ^{125}I-lectin with a sample of the total seed extract prior to two-dimensional electrophoretic analysis. Autoradiographs were then prepared of the dried gels without lectin overlays. There was no evidence that the ^{125}I-lectins bound to their own subunits. The lectins showed no signs of impurity in the sense that only the expected subunits were present.

Lectin Binding to Peanut and Soybean Seed Components. In Figure 3 the binding of various lectins to peanut seed components is illustrated. Unlike peanut agglutinin (Fig. 2b), ^{125}I-ricin which also binds more or less specifically to D-galactosyl residues (11, 13, 19) bound to only three minor components in the region of spot N_6 while soybean agglutinin showed no binding at all. Both these lectins were active when the same preparation were used to identify glycoproteins from plasma membrane of mammalian fibroblasts (8). Among the peanut polypeptides that bound ^{125}I-wheat germ agglutinin which is relatively specific for *N*-acetyl D-glucosamine were components P_1, Q_1, R_1, R_2, X_1, and X_2. A less intense staining of H_{1-6} and X_3 and X_4 was also observed. Very little ^{125}I was associated with other major protein components.

^{125}I-Concanavalin A also bound to several peanut polypeptides but the pattern was clearly distinct from that observed with either peanut agglutinin or with wheat germ agglutinin. We conclude that most of the major seed components of the peanut are glycoproteins but that they vary considerably in the structures of their carbohydrate groups.

Comparable experiments have been carried out with soybean, showing that a varied group of glycoproteins with considerable heterogeneity in their carbohydrate chains were present. For example, concanavalin A failed to bind to the major protein A_1, which stains strongly with soybean agglutinin itself, but did stain a number of streaked components in segments B and C. Soybean agglutinin, which runs as a series of subunits below N_4 and O_{1-4}, did bind concanavalin A, even though it is a relatively minor seed protein. Peanut agglutinin did not stain any of the soybean polypeptides strongly.

DISCUSSION

A large proportion of the proteins present in legume seeds, from which coat and embryonic axis have been removed, are believed to be storage materials localized in protein bodies. Here, we have demonstrated that many of the major seed meal proteins of four different legume species are glycoproteins. Moreover, a large proportion of these will bind to their homologous seed lectins. This binding appears to be specific inasmuch as for at least three of the lectins appropriate control experiments have been run to show that saccharide groups are involved. The failure to displace all of the peanut lectin by D-galactose is probably because the association constant of the lectin for this hapten is much lower than for the oligosaccharide chains on the glycoproteins. However, the apparent selectivity of lectin binding and the fact that each seed gives its own characteristic autoradiographic patterns with the different lectins suggests that the gel staining reaction is not

due merely to nonspecific, protein-protein associations. We have demonstrated elsewhere that soybean agglutinin, ricin I, wheat germ agglutinin, and concanavalin A do not bind generally to proteins of the plasma membrane of mammalian cells, but are highly specific (7, 8). Nonglycosylated proteins such as actin do not label, for example.

The results demonstrate that a large number of the polypeptides present in legume seeds are glycoproteins and that the carbohydrate groups even within the same seed species are heterogeneous in structure suggesting that, as in animal cells, a complex system of glycosylating enzymes exist which have different specificities and substrate requirements.

As pointed out earlier, it is likely that the horizontal rows of proteins noted for each seed type on the two dimensional gels are not artifacts resulting from charge modifications during and after extraction because: (*a*) they were consistent from experiment to experiment; (*b*) they were not progressive upon storage of the sample; and (*c*) different cultivars of peanut (1) and soybean (unpublished results) showed distinct and consistent differences in many of these protein spots. The inclusion of the protease inhibitor phenylmethanesulfonyl fluoride in the extraction medium did not influence the polypeptide patterns (unpublished results).

Very few plant glycoproteins have been thoroughly characterized with regard to carbohydrate structure. One of these is soybean agglutinin (12), which has high mannose chains linked *N*-glycosidically through a chitobiose core to L-asparagine. As expected, the subunits of this lectin bound concanavalin A, but not peanut agglutinin nor soybean agglutinin itself. Again, this emphasizes the likely carbohydrate specificities of the staining reactions.

One interesting feature of these experiments is that a large number of the major polypeptides in any seed species tended to bind their homologous lectin. Soybean lectin, which had a poor affinity for polypeptides of other plants, bound to several components of the soybean. Similar results were noted with peanut agglutinin. Thus, a lectin, being multivalent, might have the potential for aggregating or cross-linking many of the proteins present with it in the legume seed. It is possible that such associations might have some functional significance in the organization of proteins in storage form.

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