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Biochemical Characterization of Canavalin, the Major Storage Protein of Jack Bean[1]

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ABSTRACT

The structure of canavalin, a jack bean (*Canavalis ensiformis*) protein homologous to phaseolin, the major seed storage protein of *Phaseolus vulgaris*, has been investigated by x-ray crystallography and found to be a hexamer composed of three identical pairs of similar but nonidentical subunits related by a perfect 3-fold axis and pseudo dyad axes (strict C_3 and pseudo D_3). One member of each pair of subunits is derived from the amino terminal half of a precursor polypeptide of molecular weight 49,000 and the other from its carboxy terminal half. Thus, the crystallographic evidence indicates that the precursor polypeptide is a tandem duplicate and is structurally redundant (McPherson A. 1982 J Biol Chem 255: 10472). A number of physical and chemical properties of the protein in both the uncleaved and the cleaved form were investigated. These included the native molecular weights, amino acid analyses, number of exposed sulfhydryl groups, carbohydrate content, metal ion analysis, crystallization behavior, and the fate of the protein in developing seeds. It was also found that the purified precursor protein possesses a substantial level of α-D-mannosidase activity and seems to share a number of other physical and chemical properties with that enzyme.

Canavalin was first isolated from the jack bean (*Canavalis ensiformis*) by J. B. Sumner in 1919, along with concanavalins A and B (20), and was crystallized by Sumner and Howell in 1936 (21). The protein, which Sumner reported to have a mol wt of about 115,000 (22), is found in large amounts in jack bean seeds, making up nearly half per cent of the total soluble protein. It comprises the classical 'vicillin' protein fraction of the seed (1) and demonstrates most of the properties associated with that class of seed proteins.

Canavalin, as first described by Sumner (20), was an amorphous protein preparation of indeterminate purity. By chance, however, this amorphous material was allowed to stand for several days under septic conditions. Sumner and Howell subsequently observed large rhombohedral crystals in the container. Deducing that this crystallization resulted from the action of bacterial proteases, they investigated the effect of systematic digestion of the amorphous material with trypsin, pepsin, and ficin (2). The function of canavalin was never determined nor was it clearly demonstrated whether the crystalline material was a cleavage product of the reaction, or whether it was formed from a stable native seed protein after elimination of contaminants by digestion.

Although soluble both in salt solutions and in low ionic strength buffers, canavalin is insoluble in, and crystallizes from, physiological saline. Once the crude canavalin has been acted upon by trypsin and crystallization occurs, the molecule appears to be resistant to further proteolytic digestion. It can be recrystallized repeatedly or treated further with trypsin with no discernible alteration in its properties. Crystallization takes place at 37°C and the crystals remain intact at this temperature, suggesting that the molecules and their arrangement possess a high degree of stability.

The biochemical literature contained only one reference to canavalin (16) from 1938 until 1973 when McPherson and Rich (13) reproduced the crystallization of canavalin via digestion with trypsin and undertook a preliminary investigation of some of its molecular properties. Additional crystallographic data was presented 2 years later (14), showing that the protein was composed of six identical or nearly identical subunits of mol wt, 24,000 which are arranged in a hexamer having nearly exact 32 point group (D_3) symmetry. The structure of canavalin has now been solved using x-ray diffraction analysis to 3.0 Å resolution and a tentative course of the polypeptide chain determined. That analysis (15) confirmed the symmetry properties deduced in the earlier studies and provides a structural basis for interpreting the results presented here.

This investigation of the biochemical and biophysical properties of canavalin was undertaken in order to expedite the crystallographic analysis and to make possible a correlation between the structural features present in the protein and some of its solution properties. In addition, we sought to ascertain whether canavalin possesses any enzymic functions and what role it might play in the germination of the jack bean seed.

MATERIALS AND METHODS

Preparation of Precanavalin. The standard preparation of precanavalin was according to the method of Sumner and Howell (14), using jack bean (*Canavalis ensiformis*) meal purchased from Sigma. One hundred g of jack bean meal was extracted with 500 ml 32% acetone by stirring for 30 min at 4°C. The mixture was filtered in the cold, and the filtrate was discarded. The residue on the filter paper was scraped off, stirred with 500 ml 30% ethanol for 30 min at room temperature, and filtered overnight at room temperature. The filtrate was discarded. The residue on the filter was scraped off and stirred with 400 ml 1% NaCl containing 0.1% K_2HPO_4 (adjusted to pH 7.0) for 30 min at room temperature, and filtered. The filtrate was set aside; the residue on the filter was stirred with 250 ml 5% NaCl for 1 h at room temperature and filtered. The combined NaCl filtrates were dialyzed for 2 d against several changes of distilled H_2O, with toluene or sodium azide as a preservative. The precipitate of concanavalin A was removed by centrifugation after 24 h, and again after 48 h.

After the second centrifugation, the supernatant was brought to pH 5.1 by addition of 1 N acetic acid. The precipitate was allowed to settle overnight in the cold, and centrifuged. The supernatant was discarded, and enough 0.1 N NaOH was added to the precipitate to dissolve it and bring the pH back to 7.0. The solution was allowed to stand overnight in the cold to facilitate the crystallization of concanavalin B. The concanavalin B crystals were centri-

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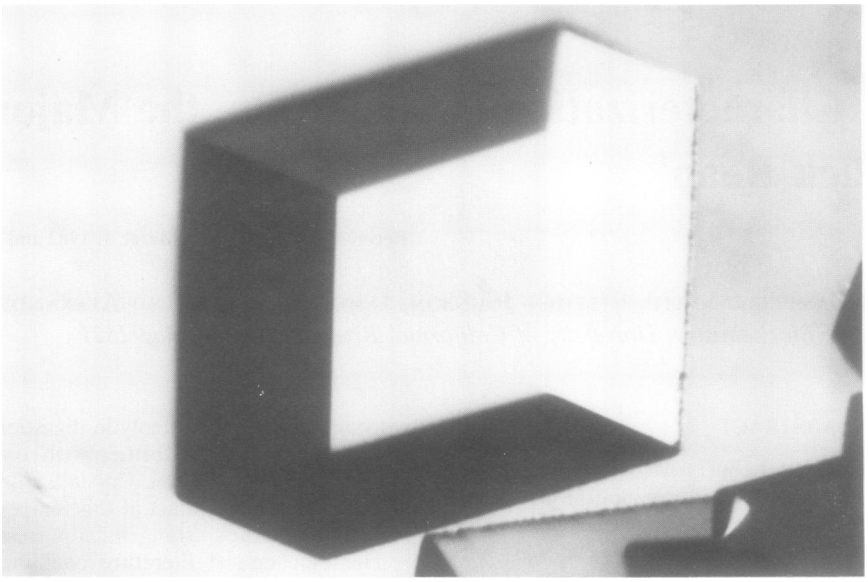


FIG. 1. A large, single rhombohedral crystal of canavalin, like those used in the diffraction analysis, seen under a low power microscope. Crystals are obtained from physiological saline initially at pH 9 gradually equilibrated by vapor diffusion to neutrality. Crystals are frequently obtained with dimensions greater than 2 mm.

fuged off, and the precanavalin in the supernatant was reprecipitated with acetic acid, redissolved with NaOH, and centrifuged again.

A modified procedure for the preparation of precanavalin was developed with the aim of preserving or increasing its α-mannosidase activity, taking care to protect bound Zn^{2+} ions and reactive sulfhydryl groups. The entire isolation was carried out at 4°C. To all the solutions in the standard procedure described above, up to and including the dialysis step, 0.01 to 1.0 mM $ZnSO_4$ and 0.01 to 1.0 mM DTT were added. In each case, the pH of the extracting solution was adjusted to its value in the standard procedure. In the third step, extraction with 1% NaCl, Hepes was used as a pH 7.0 buffer instead of K_2HOP_4. The crude precanavalin preparation was dialyzed against water containing 0.01 to 1.0 mM $ZnSO_4$, 0.01 to 1.0 mM DTT and sodium azide or toluene (pH 7.0).

The precanavalin obtained with this modified method was slightly less pure, as judged by electrophoresis on SDS polyacrylamide, than precanavalin obtained by the standard method. Precanavalin could be further purified to complete homogeneity using isoelectric focusing as described below.

Preparation of Canavalin. A crude preparation of precanavalin obtained by the method described above was dissolved in water at a concentration of 25 mg/ml. This was incubated at 37°C with trypsin (Gibco) at a concentration of approximately 5 mg/ml. After about 2 h, rhombohedral crystals of canavalin began to settle out on the bottom of the flask. After 24 h, the crystals were harvested by centrifugation and stored at −20°C for recrystallization.

The canavalin was recrystallized by dissolving the crystalline material in distilled H_2O with enough concentrated NH_4OH added to give a clear solution. The solution was centrifuged to remove undissolved material and debris, then dialyzed *versus* DPBS[2] pH 7.2 at room temperature. After 24 h, the canavalin crystals were collected by centrifugation. The recrystallized canavalin seen in Figure 1 showed no contaminating bands when run on an SDS-polyacrylamide gel. Two other crystal forms, orthorhombic plates and hexagonal prisms, can be obtained under different conditions (13).

SDS-PAGE. SDS-PAGE was performed according to Laemmli (5), using a discontinuous Tris-glycine buffer system, with the following modifications.

Acrylamide, *N,N'*-methylene-*bis*-acrylamide and SDS were purchased from Bio-Rad and used without further purification. Coomassie brilliant blue and bromphenol blue were purchased from Sigma. In general, gels were poured to form an approximately linear gradient betwen 10% and 15% polyacrylamide concentrations. The gels were polymerized between glass plates, by the addition of 0.05% by volume of TEMED and of 0.33% to 0.67% by volume of a 10% solution of ammonium persulfate. The slab gels thus formed were 1.5 mm thick and 110 mm long by 155 mm wide.

Samples were prepared for electrophoresis by mixing each protein solution with a 'sample buffer' containing 0.014% bromphenol blue, 10% glycerol, 10% SDS, 2.5% 2-mercaptoethanol, and 0.61 M Tris base (pH 7.3). The final samples when applied to the gel (2-5 μl/well) contained approximately 0.15 to 0.20 μg protein. Metastable aggregates were eliminated by immersing the final samples for 2 min in boiling water.

Electrophoresis was carried out at a constant voltage of 150 v (25–50 mamp) until the tracking dye reached the bottom of the gel (about 3–4 h). The proteins were simultaneously fixed in the gel and stained, using a solution containing 10% acetic acid, 50% methanol, and 0.2% Coomassie brilliant blue. Gels were stained at 37°C for 1 h, then diffusion-destained by repeated washing in aqueous solution containing 10% acetic acid and 10% methanol.

Reactivation of Precanavalin and Canavalin. Samples (2 ml) of precanavalin and canavalin at 40 and 15 mg/ml, respectively, were dialyzed at room temperature for 8 h against 1 L 5 mM EDTA and 5 mM DTT in water. They were then dialyzed overnight against 1 L 0.1 mM $ZnCl_2$ (pH about 4).

α-D-Mannosidase Assays. One ml 6 mM *p*-nitrophenyl-α-D-mannoside (Sigma) dissolved in 50 mM sodium citrate buffer at pH 4.5 was added to each acid washed test tube. A graduated quantity of protein was added to each tube, except those used as controls, as quickly as possible. The tubes were incubated at 25°C for 30 min, at which time 3 ml 0.2 M sodium borate buffer at pH 9.8 was added to each tube to develop the *p*-nitrophenol color. The *A* at 400 nm was read on a Zeiss spectrophotometer against

[2] Abbrevations: DPBS, Dulbecco's phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; TEMED, *N,N,N',N'*-tetramethylethylenediamine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

a reagent blank. The activity was calculated as μmol *p*-nitrophenol produced per minute, using a value of 1.77×10^4 M^{-1} cm^{-1} for the molar extinction coefficient of *p*-nitrophenyl. The specific activity of the enzyme was computed using the calculated activities, measured amounts of enzyme, and the definition that 1 unit of activity produces 1.0 μmol *p*-nitrophenol/min at 25°C. The procedure described here is essentially that of Li and Li (9).

Determination of Sulfhydryl Groups. The number of exposed sulfhydryl groups in precanavalin and canavalin was determined by reaction of the proteins with Ellman's reagent, 5,5′-dithiobis-2-nitrobenzoic acid (3, 4). To 50 μl precanavalin at 28 mg/ml or 200 μl of canavalin at 8 mg/ml was added 450 μl or 300 μl, respectively, of 0.1 M phosphate buffer at pH 8.0 and 500 μl of distilled H_2O. Ten μl of 0.01 M DTNB in 0.1 M phosphate buffer (pH 8.0) was added to the protein solutions and the color was allowed to develop for 30 min. The *A* at 412 nm was measured against an Ellman reagent blank. For calculation of sulfhydryl content, a molar absorptivity value of 13,600 M^{-1} cm^{-1} was employed.

Protein Determinations. Total protein was determined by the method of Lowry *et al.* (11), using BSA as a standard. The *A* at 280 nm of parallel samples of precanavalin and canavalin was measured and the values of $OD^{1\%}_{280}$ calculated to be 0.71 and 0.68, respectively.

Determination of Carbohydrate Content of Precanavalin and Canavalin. The neutral sugar content of precanavalin and canavalin was estimated using the phenol-sulfuric acid method of Dubois *et al.* (1). Quantitation was achieved by comparison with a standard curve determined for an equimolar mixture of four sugars: fructose, dextrose, mannose and galactose. Amino sugars were determined on a Beckman 121 automated amino acid analyzer after hydrolysis in 6 N HCl for 24 h at 100°C.

Metal Ion Analysis. Metal ion analysis, specifically for Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}, was carried out by Galbraith Laboratories of Memphis, Tennessee on 25 mg samples of precanavalin and canavalin that had been extensively dialyzed against metal ion-free buffer.

Amino Acid Analyses. For amino acid analyses, 2 to 3 mg of protein was hydrolyzed under vacuum at 115°C for 24 h with 6 N HCl. For the purpose of tryptophan determination, the protein was hydrolyzed with 4 N methane-sulfonic acid (Eastman) containing 0.2% 3-(2-aminoethyl)indole (10, 12). At the end of hydrolysis 1.0 ml 3.5 N NaOH was added to the hydrolysate and this was then diluted to 5.0 ml with water. One-ml aliquots containing 0.1 mg protein were applied to a Beckman 121 automated amino acid analyzer.

Monomer Mol Wt Determinations. The monomer mol wt of precanavalin and of the constituent polypeptides of canavalin were determined by comparison with the migration distances of a series of standard proteins on SDS-PAGE. The buffer system was that of Laemmli (5), the entire slab gel was 10% polyacrylamide and the standards were BSA (68,000), ovalbumin (43,000), creatine kinase (41,000), pepsin (35,000), carboxypeptidase A (34,600), hemoglobin (15,500), chymotrypsin B chain (22,600) and Cyt *c* (11,700). Electrophoresis was carried out as described above.

Determination of Native Mol Wt by Gel Filtration and Liquid Chromatography. For native mol wt determinations, a Pharmacia column (1 × 100 cm) was packed with Sephadex G-200 equilibrated with 0.05 M Tris-HCl at pH 7.5. The elution profile was continuously recorded at 280 nm by a Pharmacia absorbance monitor and strip chart recorder. As standards, beef liver catalase, rabbit muscle lactate dehydrogenase and human hemoglobin were employed. Approximately 5 mg precanavalin and of each of the standard proteins was applied to the column and eluted with the equilibration buffer. The mol wt of precanavalin and canavalin was estimated by its elution volume relative to the standards.

The mol wt of native precanavalin and canavalin were independently determined by comparing their rates of migration through the column of a Waters Associates high pressure liquid chromatography system with those of a series of standard proteins of known molecular weight. The protein was dissolved at 2 mg/ml concentration in 25 mM Tris-HCl at pH 8.2.

Proteolytic Digestion. Trypsin-TPCK (Worthington Biochemicals) and α-chymotrypsin from bovine pancreas (Sigma) were dissolved in water at a concentration of 1.0 mg/ml. The precanavalin samples were in 10 mM Tris-HCl at pH 7.5. Samples of the protein were exposed to trypsin and chymotrypsin, both separately and in combination, over a period of 30 s to 24 h. The products of the digestion were sampled at expanding time intervals over the course of the experiment and subjected to SDS-PAGE. The reaction mixtures had a precanavalin to protease ratio of 100:1 and were carried out at 25°C and at 37°C in a volume of 100 μl. Proteolysis was stopped in the reaction aliquots by rapidly mixing the digestion solution with sample prep buffer containing 10% SDS and immediately boiling for 1 min. This procedure appeared to stop the proteolysis in these experiments as effectively as the addition of soybean trypsin inhibitor. The products of the reaction were then analyzed by SDS-PAGE as described above.

Fate of Precanavalin and Canavalin in the Developing Seed. One hundred dried jack beans (Sigma) were each placed in a disposable Petri dish, hydrated with 20 ml distilled H_2O, and incubated in the dark at 37°C. On day 3 after imbibition, the Petri plates were moved into the light at 25°C. On day 8 the germinated beans were planted in commercial sterilized potting soil in polystyrene containers. They were watered as necessary with distilled H_2O.

On day 2, and at intervals of 2 d thereafter, 5 healthy seedlings were selected, separated into roots, stems, cotyledons, and leaves, and the parts extracted by grinding in a mortar and pestle with 1 to 10 ml 2% SDS in distilled H_2O. The extracts were heated to 100°C for 5 min, centrifuged at 4,000 rpm, and the supernatants frozen.

Samples of the extracts were prepared for running on a 10% to 15% SDS-polyacrylamide gel by thawing, centrifuging the opaque extracts at 10,000 rpm and adding 25 μl of each supernatant to 50 μl of sample prep buffer. These final samples were heated at 100°C for 1 min, and 5 to 30 μl (usually 10 μl) of each sample was loaded on the gel.

Crystallization of Proteins from 9-Day-Old Jack Bean Seedlings. Thirteen dried jack beans (Sigma) were each placed in a disposable Petri dish, wetted with 20 ml distilled H_2O, and incubated in the dark at 37°C. On day 3 after imbibition, the Petri plates were moved to a light at 25°C. On day 9, the cotyledons were removed from the seedlings, ground in a mortar and pestle, and extracted by stirring for an hour at room temperature with 100 ml of 4% NaCl (pH 5.0). The extract was centrifuged at 4,000 rpm, then at 15,000 rpm (4°C).

The supernatant was dialyzed overnight against 2 changes of 1 L distilled H_2O with sodium azide as a preservative, then centrifuged at 15,000 rpm. The supernatant was dialyzed for 24 h against two changes of 1,000 ml DPBS (pH 7.2) with sodium azide, then concentrated by reduced pressure in a collodion bag *versus* DPBS. Crystals began to form as the extract was concentrated.

The concentrate was centrifuged at low speed to collect the crystals, and the collodion bag washed with distilled H_2O to collect those which had grown on the sides of the bag. These were kept separate from the first batch of crystals. Each batch of crystals was centrifuged, washed quickly with cold distilled H_2O, then centrifuged again. A small amount of crystalline material from each batch was added directly to 50 μl of sample prep, heated to 100°C for 1 min, and run on a 10% to 15% SDS-polyacrylamide gel. A sample of the crude extract of the 9-d-old seedlings in 4% NaCl (pH 5.0) was also run on the gel.

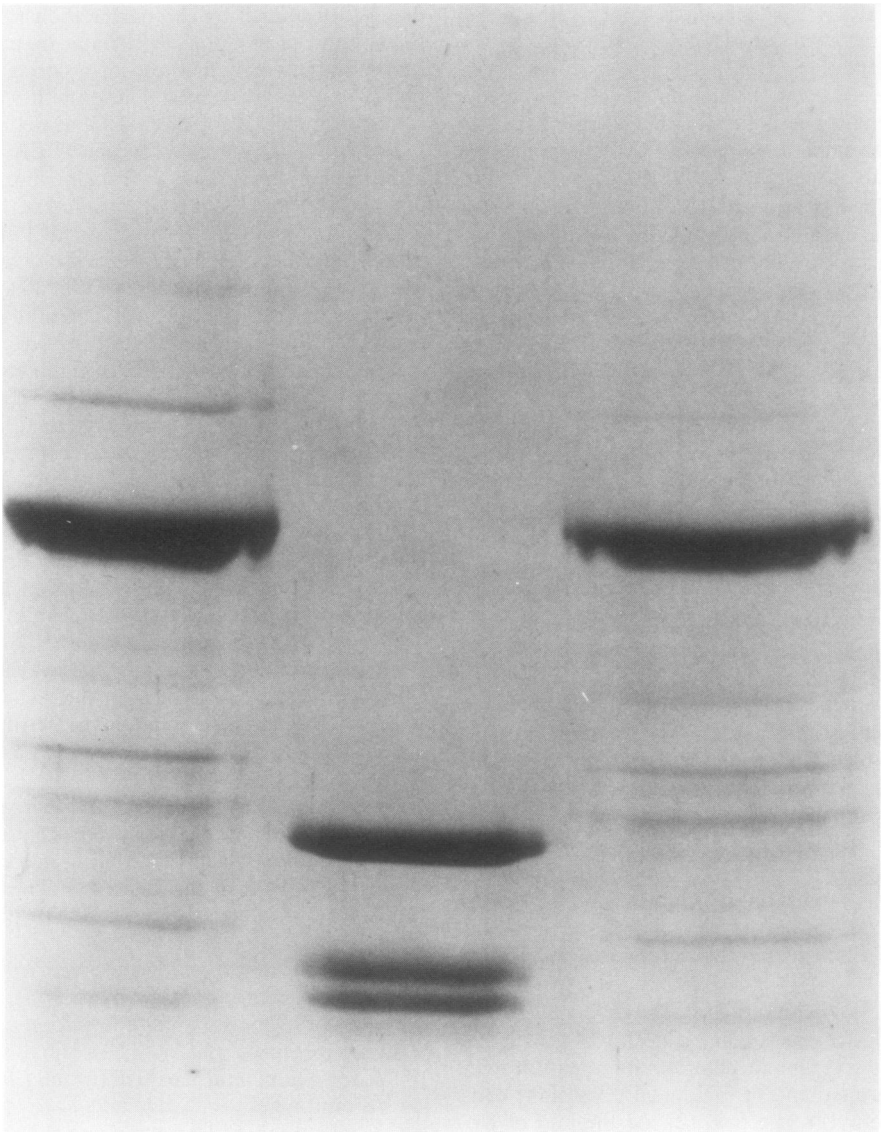


FIG. 2. Precanavalin and canavalin on a 5% to 15% SDS-polyacrylamide gradient gel. Two different preparations of precanavalin are on the right and left, and five times recrystallized canavalin is in the center. Canavalin, shown here, is produced from precanavalin by prolonged treatment with proteolytic enzymes.

Isoelectric Focusing. α-D-Mannosidase was electrofocused by loading 25 mg of the protein, which had been dialyzed against H_2O, on an LKB preparative IEF column of 110 ml capacity. The column was 2.5% carrier ampholytes stabilized in a sucrose gradient and ranged from pH 3.0 to 10.0. Focusing was carried out at 1,000 v and 23°C for 48 h after which 1.5 ml fractions were collected and both their pH and optical density at 280 nm were measured. The various individual peak fractions were pooled and the pools corresponding to each peak were run as above on SDS-PAGE. Precanavalin was also subjected to electrofocusing under the identical conditions described here except that it was predialyzed against 0.01 M NH_4OH.

Production of Antibodies to Precanavalin and Canavalin. For antibody production two white rabbits were employed. Each was bled four times over a 3-month period prior to immunization to provide control serum. Each animal was immunized with the appropriate antigen, either canavalin or precanavalin, and then bled once a week for 4 weeks using alternate ears. The rabbits were then boosted with antigen and again bled once a week for 4 weeks. The initial injections contained 0.1 μl Freund's complete adjuvant, 1.0 mg precanavalin, or 2.0 mg canavalin and DPBS in a final volume of 2.0 ml. The booster injections were the same, but 1.0 ml Freund's incomplete adjuvant was substituted. All injections were given on 3 consecutive days using about 0.3 ml antigen-adjuvant mixture each time.

Each bleeding collected about 5 to 6 ml serum; about 10 ml whole blood. The blood was allowed to clot at room temperature and then refrigerated overnight at 4°C. The clot was removed and the resulting serum centrifuged at 1,000*g* for 20 min. The supernatant was then stored frozen.

The Ouchterlony plates (18) consisted of 0.8% NaCl, 0.05% NaCN, 0.5% Difco Agar Noble, 50 mM Na_2EDTA and the final pH = 8.3 using NH_2OH. Microscope slides (1" × 3") were cleaned with 95% ethanol and 3.0 ml of this agar mixture was allowed to solidify on each slide. Wells were cut inthe agar and 10 μl of antigen, at 1 mg/ml, or 10 μl undiluted serum was placed in each well. The only exception to this was the *N*-acetylglycoseaminidase which was at a concentration of 0.19 mg/ml. The slides were incubated at 37°C for 5 h and read.

RESULTS

Figure 2 shows partially purified precanavalin from which canavalin is derived, and canavalin (5 times recrystallized) formed

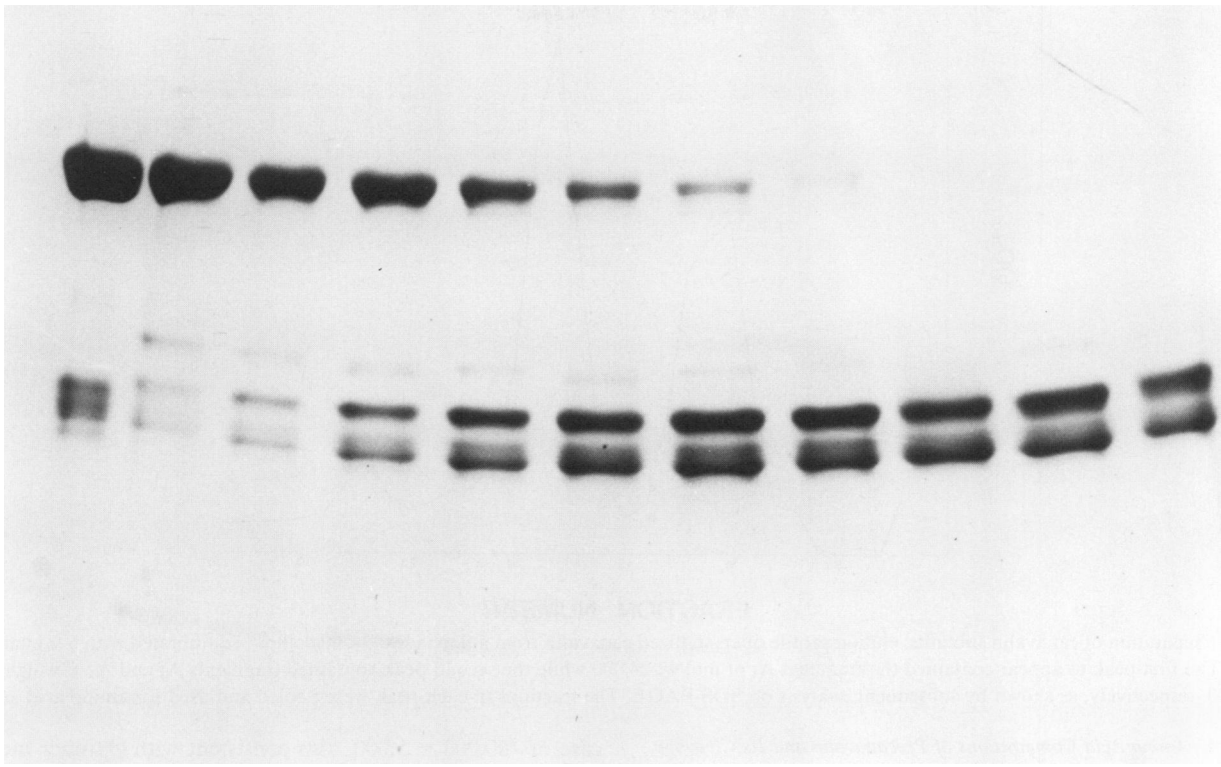


FIG. 3. Time course of digestion of precanavalin by α-chymotrypsin, shown on a 10% to 15% SDS-polyacrylamide gradient gel. The far left track is purified precanavalin (showing some proteolytic breakdown after storage at 4°C). The other tracks correspond, from left to right, to times of 0, 2, 5, 10, 15, 20, 30, 60, 90, and 120 min. This sequence shows the conversion of the single 42,000 dalton precanavalin subunit to the two canavalin fragments A_1 and A_2 without the appearance of other intermediates. The two polypeptides of canavalin have mol wt of 24,000 (A_1) and 25,000 (A_2).

by proteolysis of the precursor protein. Native precanavalin has a monomer mol wt, based on comparison with standards, of 49,000 daltons under denaturing conditions and exists as a single continuous polypeptide chain. The cleaved protein, canavalin, consists of three polypeptide chains of about 24,000, 13,000 and 12,000 D mol wt. Canavalin is obtained when precanavalin is incubated with 2.5% trypsin at 37°C for up to 24 h and can also be produced by a similar digestion with chymotrypsin.

Figure 3 shows a time course of digestion of the precursor protein with chymotrypsin. We show the pattern produced by that enzyme rather than trypsin because the digestion pattern is slightly less complex and because digestion occurs at about one-tenth the rate of the reaction with trypsin and is therefore more readily controlled. The gel demonstrates that the precursor protein is converted by a single cleavage into two polypeptides having mol wt of 24,000 and 25,000 which we have designated A_1 and A_2, respectively. This cleavage by chymotrypsin occurs quite rapidly, being complete under our conditions within 30 min. With trypsin, cleavage is essentially complete in about 3 min.

Prolonged exposure, even at 37°C, to purified trypsin or chymotrypsin does not result in any further change in the band pattern shown in Figure 3. When the digestion is carried out under conditions where crystallization will occur, *i.e.* in physiologic saline for sufficiently long times (8–24 h), then we find that the higher mol wt band A_2 at 25,000 D is further cleaved to yield two lower mol wt fragments of 12,000 and 13,000 D which we have designated A_3 and A_4 (Fig. 3). The fragments do not dissociate from one another in either the $A_1 + A_2$ or in the $A_1 + (A_3 + A_4)$ state, as they can be repeatedly recrystallized and redissolved either as $A_1 + A_2$ or as $A_1 + (A_3 + A_4)$.

The course of cleavage leading to canavalin has an interesting consequence when considered in conjunction with the x-ray diffraction data from the rhombohedral crystals. The mol wt of canavalin was determined by Sumner in one of the earliest analytical centrifuge experiments to be about 115,000 in solution (22). Considering the instruments available at the time, this is, we feel, still consistent with a mixed hexamer of three A_1 subunits and three $A_3 + A_4$ subunits, of mol wt about 147,000 (*i.e.* $3 \times [25{,}000 + (13{,}000 + 12{,}000)]$). The asymmetric unit of the R3 rhombohedral crystals is one-third of the hexamer of 147,000, and the crystallographic demands of the space group, therefore, require that the hexamer contain a perfect 3-fold axis relating pairs of subunits. In other words, three sets of $A_1 + (A_3 + A_4)$ are combined around a perfect triad axis to form the 147,000 D entity found in the crystal and in solution. This conclusion is clear from the x-ray structural analysis already published (13, 14). The possibility that the crystals are disordered and that the two kinds of subunits A_1 and $A_3 + A_4$ are randomly distributed in the hexamer is ruled out by the diffraction analysis (15).

In addition to the crystallographic 3-fold axis, the diffraction patterns show an extraordinarily high degree of pseudo 32 symmetry, so high in fact that the space group of the crystals was initially reported incorrectly as R32 rather than R3 (13). The implication of this pseudo symmetry is that the two kinds of subunits A_1 and $(A_3 + A_4)$ must be extremely similar in structure and must be related by quasi 2-fold axes within the 147,000 D hexamer. Thus, the 49,000 D precanavalin monomer must be a repetitive polypeptide that can be cleaved by proteolysis to yield the two nondissociated and extremely similar subunits A_1 and A_2 having mol wt of 24,000 and 25,000, respectively, that we observe.

To provide further evidence, the fragments A_1 and $(A_3 + A_4)$ from three times recrystallized canavalin were separated and isolated on a Biogel P-100 chromatography column equilibrated with 6 M guanidinium chloride. The elution profile is shown in

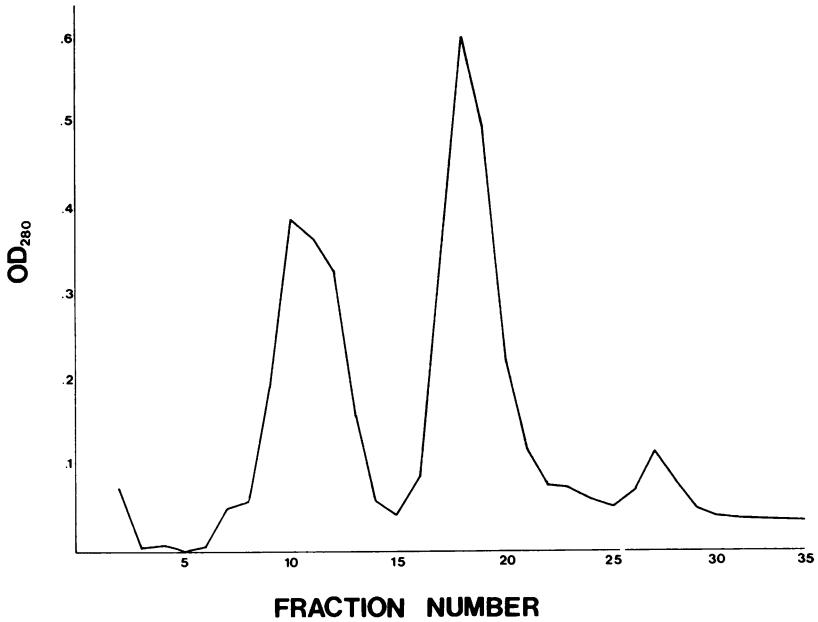


FIG. 4. Separation of canavalin subunits; elution profile of crystallized canavalin from a Sephadex G-200 column equilibrated with 6 M guanidinium chloride. The first peak to appear contained the fragment A_1 of mol wt 24,000 while the second peak contained fragments A_3 and A_4 of weights 12,000 and 13,000, respectively, as shown by subsequent analysis on SDS-PAGE. The fractions in each peak were pooled and used for amino acid analysis.

Table I. *Amino Acid Compositions of Precanavalin and Its Cleavage Products*

	Pre-canavalin	Canavalin	A_1	$A_3 + A_4$
Lysine	21	20	9	11
Histidine	8	7	4	3
Arginine	22	22	14	8
Cysteine	1	1	1	0
Aspartic acid	50	50	25	25
Threonine	17	16	8	8
Serine	27	25	12	13
Glutamic acid	58	56	21	35
Proline	21	18	9	9
Glycine	25	22	8	14
Alanine	24	22	8	14
Valine	18	18	8	10
Methionine	4	4	2	2
Isoleucine	18	18	7	11
Leucine	41	41	20	21
Tyrosine	14	14	7	7
Phenylalanine	18	18	8	10
Tryptophan	0	0	0	0

Figure 4. The amino acid analyses of the precanavalin monomer, the polypeptide chain A_1 derived from one half of the monomer and the polypeptide fragments $A_3 + A_4$ derived from the remaining half are shown in Table I. Although there are differences in amino acid composition between A_1 and $(A_3 + A_4)$, they are few in number. This would be expected if the two halves of the precanavalin monomer are nearly equivalent.

The native mol wt of precanavalin and canavalin under native conditions were estimated by gel filtration on Sephadex G-200 in comparison with the elution volumes of proteins of known mol wt. The results shown in Figure 5 indicate that precanavalin elutes at a mol wt of approximately 175,000. This is consistent with the trimer of 147,000 but would seem to rule out a higher oligomeric species under these conditions. Liquid chromatography of both canavalin and precanavalin yielded values for the mol wt in each case of 150,000 ± 5,000, also consistent with a trimer mol wt of 147,000. In light of the hexameric nature of its cleavage product, canavalin, we conclude that native precanavalin is very likely composed of three 49,000 D monomers.

Amino acid analysis of the polypeptide chains (Table I) shows a single cysteine residue in the precanavalin monomer. X-ray analysis shows that following cleavage this residue appears in the 24,000 dalton fragment A_1 (15). The fragments A_1 and $(A_3 + A_4)$ each contain two methionine residues. There is no tryptophan present in the molecule. Aside from reflecting the typical 'vicillin' protein fraction composition (1) the distribution is unremarkable. The 25,000 D subunit A_2 certainly must exhibit some other differences from A_1, inasmuch as it possesses an additional bond susceptible to trypsin cleavage in order to yield $A_2 + A_4$.

The phenol-sulfuric acid test for neutral sugars gave a barely perceptible positive reaction, implying no more than one carbohydrate residue for every three monomers of precanavalin and one for every eight or nine canavalin subunits. Because of these very low values, we conclude that the carbohydrate detected is likely a contaminant and that precanavalin has no covalently bound carbohydrate residues. The amino acid analyses show no amino sugars present.

Isoelectric focusing of precanavalin yielded the results shown in Figure 6. The major peak, which contains the precanavalin as demonstrated by subsequent SDS gel electrophoresis, appears at a pH value of 6.2. The minor peaks also contain predominently precanavalin and presumably are other isoelectric variants.

Using Ellman's reagent, we found that under nondenaturing conditions, the single sulfhydryl group of the 49,000 D precanavalin monomer is fully reactive. This group combined rapidly and completely in a 1:1 molar ratio with the reagent. In the case of the cleaved product, canavalin, however, we have reproducibly failed to observe more than about 50% reactivity of this group with Ellman's reagent. We cannot fully explain this partial reactivity.

Precanavalin, from which canavalin is derived, and which has on SDS-PAGE a monomer molecular weight of 49,000, was assayed for a wide variety of glycosidase activities, as was canavalin. Using the procedure of Li (3) with *p*-nitrophenyl-α-D-mannoside as substrate, both precanavalin and canavalin demonstrated α-mannosidase activity. No other glycosidase activity was

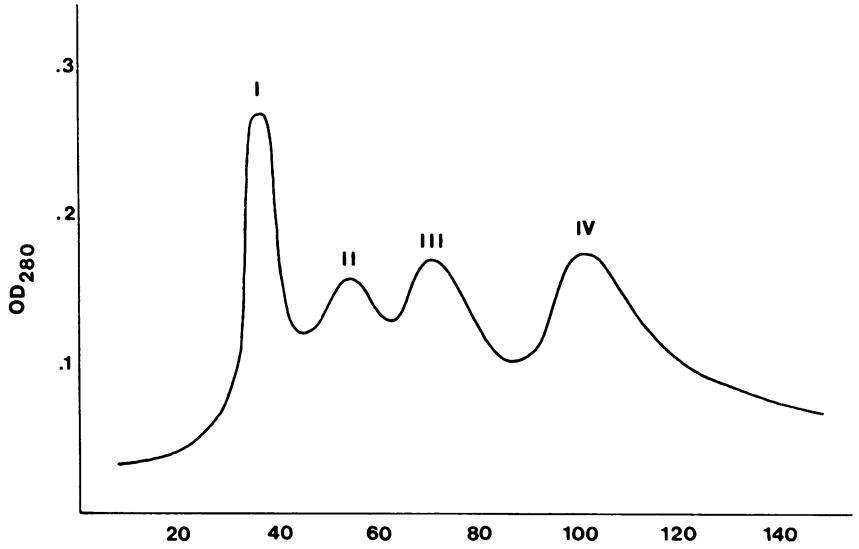


FIG. 5. Native mol wt of precanavalin; the elution profile form Sephadex G-200 of catalase (I), precanavalin (II), lactate dehydrogenase (III), and hemoglobin (IV). The apparent native mol wt of precanavalin implied by this experiment is about 175,000 and is consistent with the trimer structure.

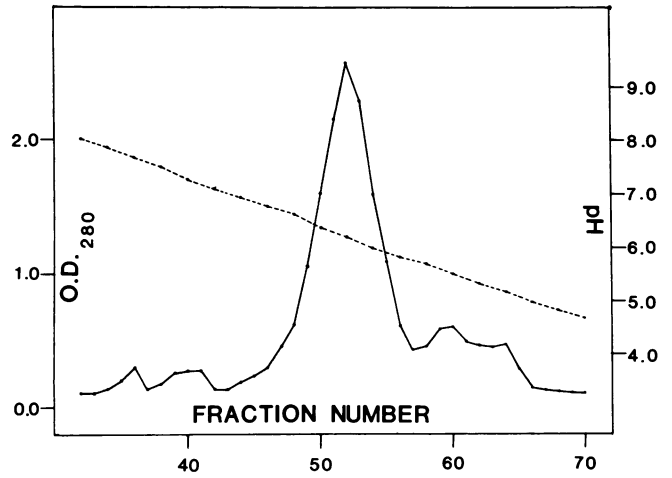


FIG. 6. Graph of the pH and optical density profile of precanavalin run on an isoelectric focusing column with ampholytes ranging from pH 3.0 to 10.0. The major peak consisted of about 90% precanavalin and 10% canavalin, presumably due to mixed oligomers of cleaved and uncleaved subunits as well as totally uncleaved trimers. This was demonstrated by subsequent electrophoresis on SDS-polyacrylamide gels.

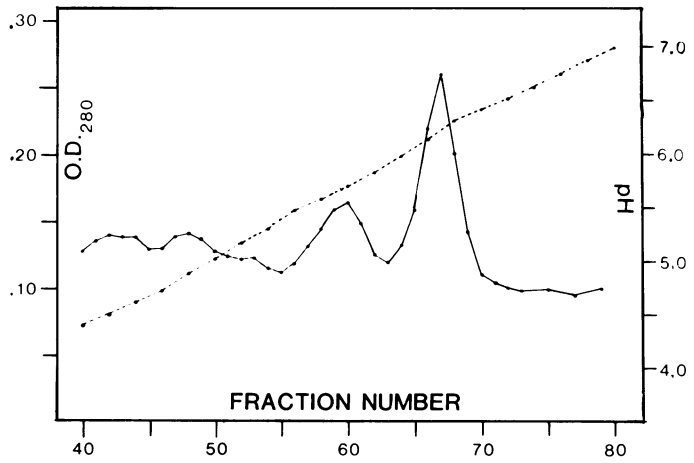


FIG. 7. Graph of the pH and optical density profile of α-D-mannosidase run on an isoelectric focusing column with ampholytes ranging from pH 3.0 to 10.0. No protein focused above the peak at pH 6.2. Samples of protein from the two peaks when run on SDS-PAGE were identical.

observed. In comparison to commercial α-mannosidase (Sigma and Boehringer) with a measured specific activity of 18 enzyme units/mg, our samples of the protein precanavalin yielded 5.6 enzyme units/mg. Canavalin had an activity of 2.4 enzyme units/mg. Whereas the precanavalin and canavalin demonstrate only about 30% and 15% the activity of the purified commercial enzyme, it should be borne in mind that our proteins were purified to homogeneity and crystallinity without regard to maintenance of α-mannosidase activity. Some of the purification steps involve drastic pH precipitations, filtrations, and lengthy dialysis. In light of the results of others that the activity of α-D-mannosidase is extremely labile (7, 9, 18, 19), the activity we observed is quite substantial. The protein samples used in these assays show a high degree of purity on SDS-PAGE; precanavalin showed no more than 5% contaminant on heavily overloaded SDS gels and no contaminant was detectable in three times recrystallized canavalin. There does not appear to be a high enough level of contaminant protein in either sample to account for the level of activity we find.

Commercial α-mannosidase (the Boehringer and Sigma products were identical) prepared by the method of Li (6) was examined by electrophoresis on a standard nondenaturing polyacrylamide gel and appeared as a single protein band even when heavily loaded. This protein we further subjected to isoelectric focusing on a pH gradient of 3 to 10. This resolved the α-mannosidase into two fractions, as seen in Figure 7, having pI values of 5.7 and 6.2. This result was nearly identical to that reported by Li (8) for α-D-mannosidase. When run on SDS-PAGE, however, both isoelectric focusing fractions appeared identically as two polypeptide bands of equal intensity having mol wt of about 60,000 and 49,000 D. Thus, we conclude that the jack bean α-D-mannosidase monomer consists of a complex of one 60,000 D subunit and one 49,000 D subunit or some multiple of this pair. If all reducing agent was omitted from the procedures, the same result was obtained, thus the complex is not disulfide linked.

The lower mol wt polypeptide of α-D-mannosidase co-migrates and is inseparable on SDS-PAGE from precanavalin. Interestingly, we further found that the higher mol wt protein co-migrated with β-*N*-acetylglucosaminidase (from Sigma). This protein is reported (6–8, 18) to be the primary contaminent of α-D-mannosidase preparations from jack bean and its activity is eliminated only in the final stage of purification by treatment of the α-D-mannosidase with pyridine (7).

Although it was not our intention to characterize jack bean α-D-mannosidase, it becoming necessary only to compare with precanavalin, we cannot help but be intrigued by these results. They suggest, but of course by no means prove, that the α-D-mannosidase and β-*N*-acetylglucosaminidase functions may be associated with independent polypeptides of 49,000 and 60,000 which complex and exist as a single oligomeric molecule. This would suggest as well, that precanavalin may be identical to or is closely homologous with the polypeptide chain bearing the α-D-mannosidase activity.

To pursue this further, antibodies to precanavalin and antibodies to five times recrystallized canavalin were raised in rabbits. As expected, the two antibodies reacted in tests against their corresponding antigen and also reacted with the opposite antigen, thus further confirming the precanavalin-canavalin relationship. Most strikingly, however, we were able to demonstrate on Ouchterlony plates that both precanavalin and canavalin cross reacted with the commercial preparations of α-D-mannosidase. The precanavalin antibody reacted significantly faster and more completely than did the canavalin antibody, but both showed substantial cross reactivity. Although we cannot rule out contamination as a source of this cross reactivity, the results would seem to be consistent with the close similarity or homology of precanavalin with the 49,000 polypeptide chain of α-D-mannosidase.

Preliminary metal ion analyses using atomic absorption were ambiguous, indicating the possible presence of Mg^{2+}, Ca^{2+}, and Zn^{2+} (13). Careful estimations on larger samples of both precanavalin and canavalin indicate that precanavalin contains 1 mol Zn^{2+}/mol protein and that canavalin, its cleaved product, contains 1 mol Zn^{2+}/mol protein. The implication is that every Zn^{2+} ion is bound to three 49,000 D precanavalin monomers or their cleaved equivalents in canavalin. It might be noted in passing that α-mannosidase from jack bean has been reported by Snaith (19) to be a Zn^{2+} containing oligomeric protein.

To determine the fate of precanavalin in the developing jack bean, seeds were germinated and groups of seedlings sacrificed for SDS-PAGE analysis at intervals of 2 to 22 d following imbibition. A number of other proteins obtained from the jack bean could be identified in extracts of the developing plant by comparison with standards run on the same slab gel. Thus, we could identify concanavalin A at 26,000 D and its two proteolytic fragments at about 13,000 and 10,000 D, concanavalin B at 32,000 D, precanavalin isolated by us as well as α-D-mannosidase and β-*N*-acetylglucosaminidase purchased from commercial sources, and canavalin fragments at 24,000, 13,000, and 12,000 D.

In Figure 8, we show the results of SDS extraction of jack bean cotyledons on days 0 through 22 after imbibition. It is apparent from the gels that precanavalin is the predominant protein component of the jack bean seed. At day 0, it exists entirely as the uncleaved 49,000 D form; virtually no canavalin, the cleavage product, is present in the dry seed. The second most prominent seed protein is concanavalin A. Even in the dry seed, a significant

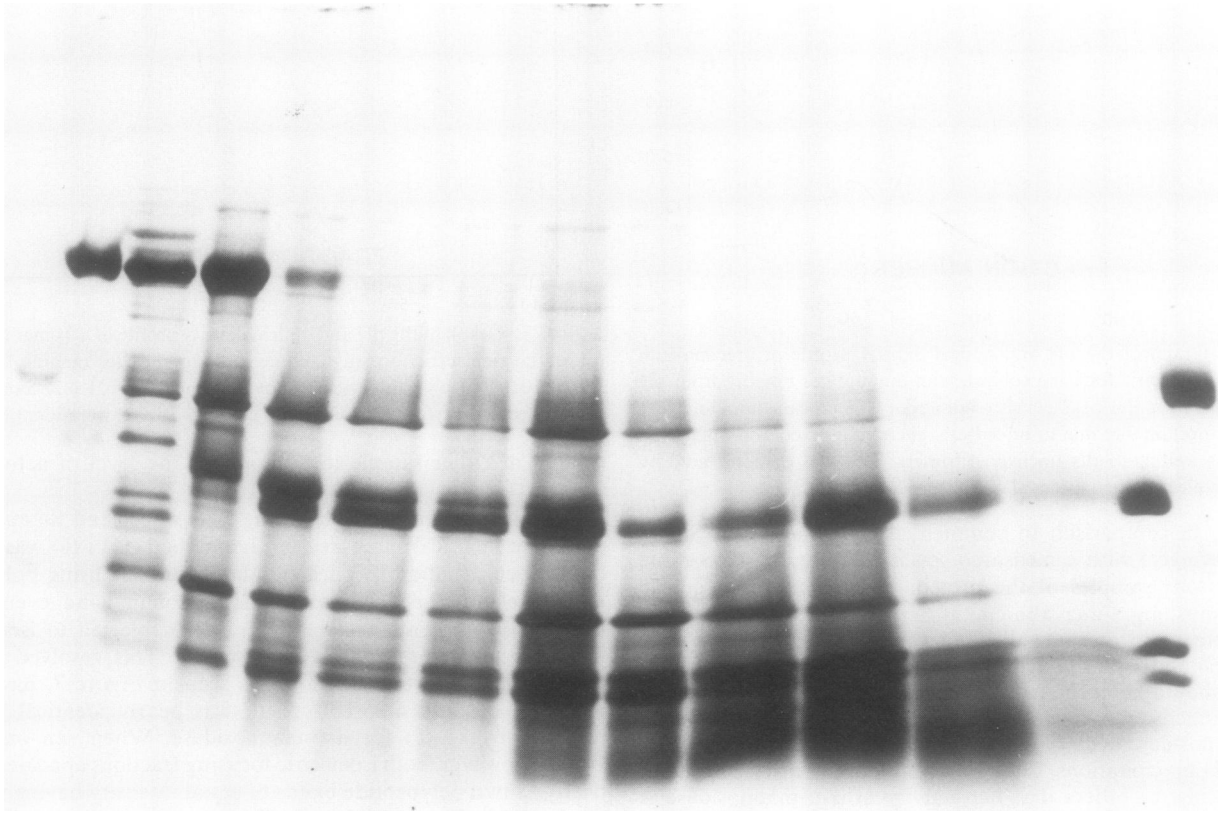


FIG. 8. SDS-PAGE showing the proteins extracted into 2% SDS from jack beans which have germinated over a period of 22 d, along with several marker proteins. From left: track (1), commercial concanavalin A; (2), purified precanavalin; (3), dry seeds; (4–13), imbibed seeds sacrificed at days 2, 4, 6, 8, 10, 12, 14, 16, 18, and 22; (14), canavalin; (15), concanavalin B. This gel demonstrates that precanavalin but not canavalin is present in the dry seeds and that it is almost entirely converted to canavalin by day 4 following imbibition. It further shows that canavalin and concanavalin A are virtually the only proteins still present in significant amounts in the cotyledons by the time they wither and fall from the growing plants.

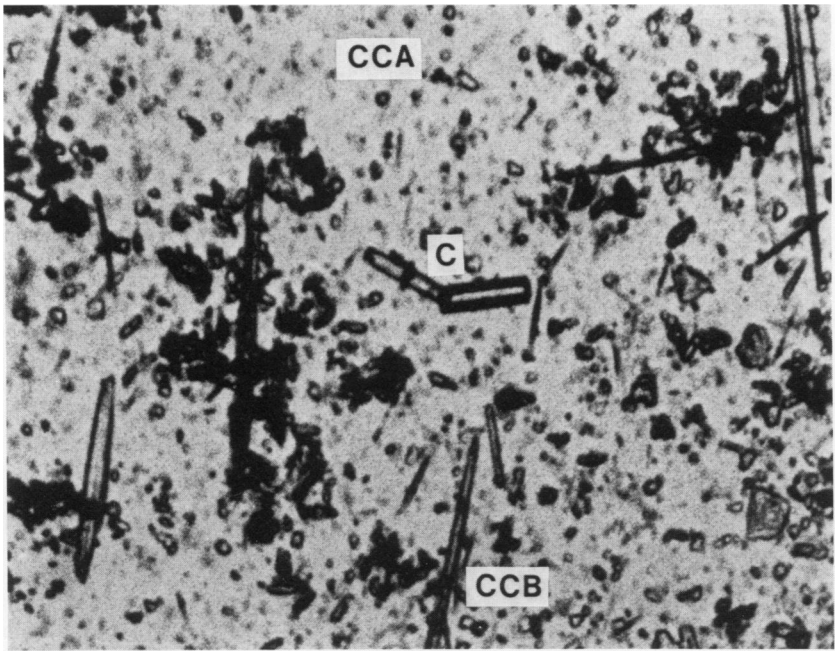


FIG. 9. The crystalline precipitate, seen under a low power light microscope, obtained by extracting 9-d-old jack bean seedlings and dialyzing this extract against physiological saline. Recognizable in the precipitate are crystals of canavalin (C) and concanavalin B (CCB), and a grainy background of small concanavalin A crystals (CCA). By day 9 of germination, these are essentially the only intact proteins still present in the cotyledons; all crystallize spontaneously under these conditions.

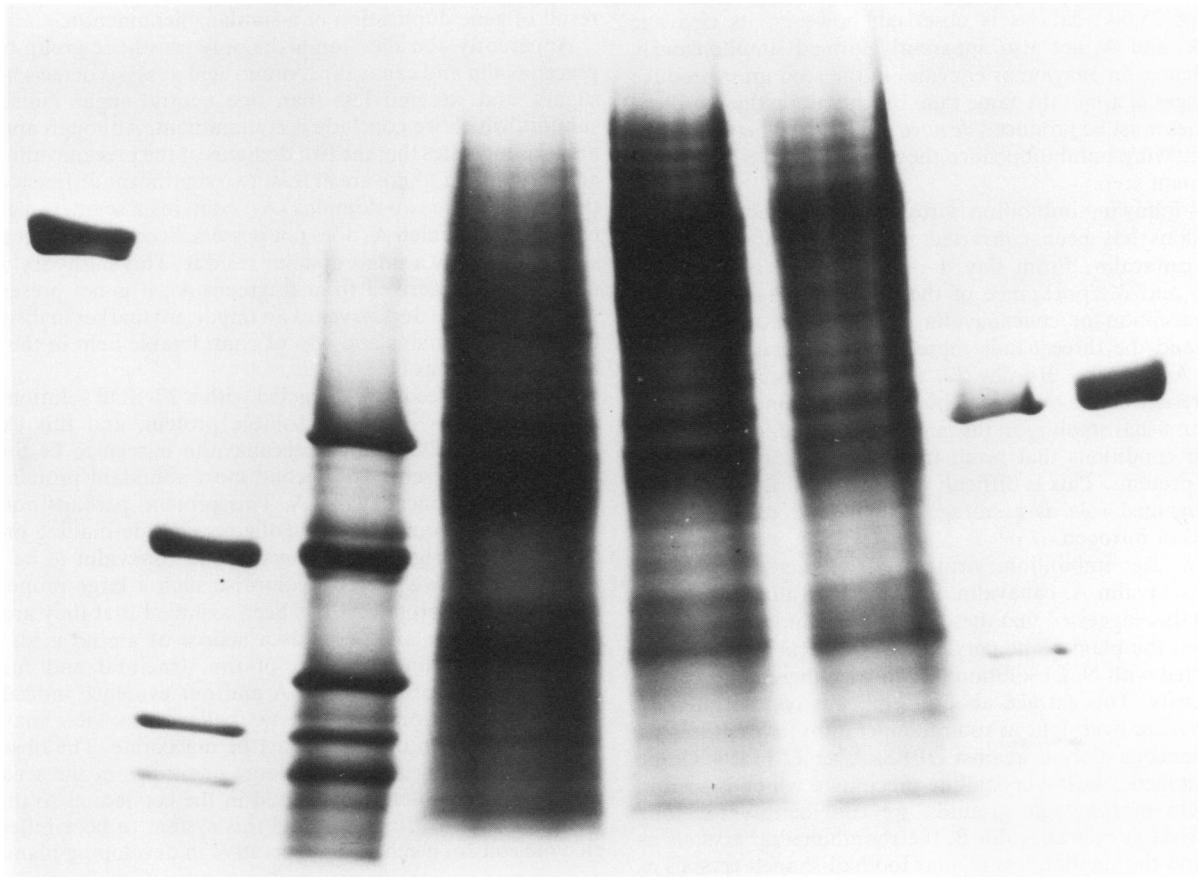
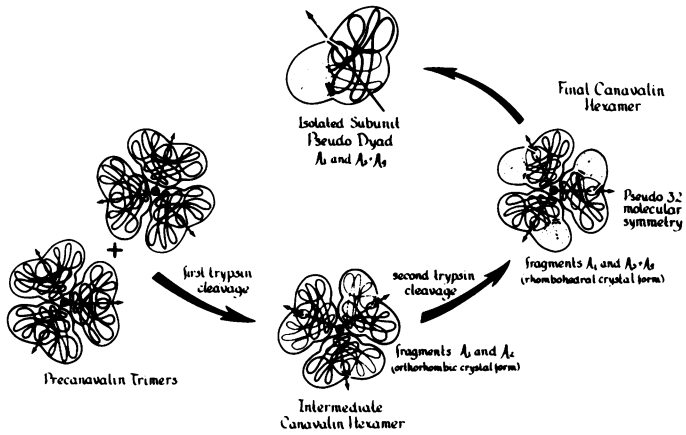


FIG. 10. Proteins extracted from various tissues of jack bean seedlings 9 d after imbibition. From left: track (1), purified precanavalin; (2), crystalline canavalin; (3), cotyledon extract; (4), root extract, concentrated 8×; (5), stem extract, concentrated 8×; (6), leaf extract, concentrated 5×; (7), commercial concanavalin A; (8), concanavalin B.

proportion of the concanavalin A is present as its proteolytic fragments, suggesting that some nicking of the concanavalin A polypeptide occurs when the protein is stored in or is extracted from the seed.



Conversion of Precanavalin to Canavalin

FIG. 11. The conversion of precanavalin to its proteolytic product canavalin. Although the molecular symmetry and aggregation state of canavalin has been proven by detailed x-ray diffraction analysis, the symmetry and aggregation state shown for precanavalin is deduced from biochemical evidence and has not yet been demonstrated by x-ray diffraction analysis.

At day 2, precanavalin is clearly being cleaved and the three fragments of canavalin at 24,000 (A_1), 12,000 (A_3), and 13,000 (A_4) D are beginning to appear in the germinating seeds. It is interesting to note that in the cotyledons of the seedlings, the intermediate chain A_2 of 25,000 daltons is observed; however, its cleavage products A_3 and A_4 are also apparently formed simultaneously with A_1. Hence, an enzyme or enzymes in the seed must produce both cleavages at about the same rate. Furthermore, these proteolytic enzymes must be produced *de novo* or activated between days 0 and 2 following imbibition since they are not active in extracts of the dormant seed.

By day 4 following imbibition, virtually all the precanavalin in the cotyledons has been converted into the three polypeptide chains of canavalin. From day 4 onward, there is a general breakdown and disappearance of the total protein in the seed, with the exception of concanavalin A and its two proteolytic fragments and the three bands representing the canavalin fragments A_1, A_3, and A_4. By day 22, these are virtually the only proteins present in the extracts. Thus, these proteins apppear to exhibit an unusual stability in the germinating seed, as they do *in vitro*, under conditions that result in the degradation of all the other seed proteins. This is difficult to understand in light of the protein's assumed role as a storage protein utilized to provide amino acids or nitrogen.

By day 8 after imbibition, virtually all of the seed proteins except concanavalin A, canavalin, and concanavalin B were degraded and this suggested that these proteins might be crystallized directly from the plants. The cotyledons of several 9-d seedlings were extracted with NaCl solution, which was filtered and centrifuged to clarity. This extract, about 25 ml, was concentrated by reduced pressure overnight at room temperature to 5 ml volume with simultaneous dialysis against DPBS. After 12 h at 4°C, the extract contained a heavy crystalline precipitate which is seen in Figure 9. On morphologic grounds, we can identify the long hexagonal rods as concanavalin B, the rhombohedral crystals as canavalin and the smaller, less regular football-shaped crystals as concanavalin A. This simple procedure demonstrates that the proteins prepared and crystallized *in vitro* are the same as those *in vivo* proteins visualized on SDS-polyacrylamide gels.

We prepared and concentrated SDS extracts of the leaves, stems, and roots of 10-d seedlings and examined these extracts on polyacrylamide gels (Fig. 10). In none of these tissue extracts, even when concentrated, was either canavalin or precanavalin observed. Although they might be present at levels too low to be detected using this technique, they do not appear in amounts approaching those in the cotyledons.

DISCUSSION

The jack bean protein known as canavalin is derived as in Figure 11 by proteolytic cleavage of a precursor protein referred to here as precanavalin. Our evidence indicates that precanavalin possesses α-D-mannosidase activity. Intact precanavalin appears to be a trimer of identical 49,000 D subunits with one Zn^{2+} ion and very probably triad (C_3) point group symmetry. These subunits can be cleaved by trypsin or chymotrypsin to yield two nondissociated fragments of about 24,000 and 25,000 D (denoted A_1 and A_2) the heavier of which can be further cleaved to yield fragments of 12,000 (A_3) and 13,000 D (A_4). Thus, the final proteolytic product of precanavalin is a single subunit containing tightly associated fragments of 24,000 and 12,000 plus 13,000 D (A_1 and $A_3 + A_4$). Three of these subunits are aggregated to form the canavalin molecule which has an overall mol wt of about 147,000 D.

We find that, with a few small but significant exceptions, the chain A_1 has very nearly the same amino acid composition as the two chains $A_3 + A_4$, implying that primary structural homologies may exist. The preliminary crystallographic results (13, 14), indicating that the two halves of the canavalin subunit have similar tertiary structures and are related by pseudo 2-fold axes have been amply confirmed by the full three-dimensional structural analysis (15). Thus, it seems likely that the structure of the protein is a result of gene duplication or a similar phenomenon.

Apparently, the Zn^{2+} ion is the only prosthetic group bound to precanavalin and canavalin. Amino acid analysis detects no amino sugars, and we find less than one neutral sugar molecule per subunit, which we conclude is contaminant. Although amino acid analysis indicates that the two domains of the precanavalin subunit are very similar, there are at least two significant differences. First, the larger of the two domains (A_2) contains a segment vulnerable to proteolysis which A_1 does not possess. Second, the precanavalin subunit has only a single cysteine residue. This sulfhydryl is found in the domain derived from fragment A_1; it is not present in $A_3 + A_4$. This sulfhydryl serves as an important marker in distinguishing the two domains and was of considerable help in the crystallographic analysis (15).

If jack bean seeds are extracted with a 2% SDS solution so as to retrive virtually all of the soluble protein, and this extract is examined by SDS-PAGE, precanavalin is seen to be the major seed protein present. The second most abundant protein in jack bean seeds is concanavalin A. This protein, perhaps not coincidentally, is a mannose-specific oligosaccharide-binding protein as our evidence suggests precanavalin and canavalin to be as well. Because these two proteins comprise such a large proportion of the seed's total protein, it has been assumed that they are simply nutrient proteins and serve as a source of amino acids for the developing seedling. In light of the structural and functional complexities of concanavalin A and our evidence indicating the presence of enzymic activity, we believe this view may be an oversimplification or may in fact be inaccurate. The observation that precanavalin is stored in large quantities in the seed and is rapidly and completely converted in the cotyledons to the stable form canavalin further suggests this system to be a reflection of enzyme control mechanisms operative in developing plant tissues.

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