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PROTEIN–CARBOHYDRATE INTERACTION

VI. ISOLATION OF CONCANAVALIN A BY SPECIFIC ADSORPTION ON CROSS-LINKED DEXTRAN GELS*,**

B. B. L. AGRAWAL*** AND I. J. GOLDSTEIN

Department of Biological Chemistry, University of Michigan, Ann Arbor, Mich. (U.S.A.)

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SUMMARY

1. A new method is described for the isolation and purification of concanavalin A based on its specific adsorption on cross-linked dextran gels and subsequent displacement with D-glucose.

2. The yield of concanavalin A by this method is approx. 2.0–2.4 g/100 g jack bean meal. The chromatographic recovery is of the order of 94 %.

3. Concanavalin A obtained by this procedure is approx. 98 % precipitable with dextran NRRL B-1355-S.

4. $(NH_4)_2SO_4$ fractionation of a saline extract of jack bean meal showed the maximum localization of concanavalin A activity to be in the fraction precipitated between 0.50 and 0.60 saturation of $(NH_4)_2SO_4$.

5. The nature and specificity of binding of concanavalin A to cross-linked dextran gels have been shown to be generally similar to that of concanavalin A–polysaccharide interaction by demonstrating the reversal of the binding and subsequent elution of the protein from Sephadex G-50 with the same low molecular weight carbohydrates which inhibit concanavalin A–polysaccharide interaction.

INTRODUCTION

Concanavalin A, a globulin from the jack bean (*Canavalia ensiformis*)[1] has been identified as a hemagglutinin[2,3]. Various workers[4–10] have also observed the ability of this protein to form a precipitate with glycogens, yeast mannans, amylopectins, dextrans, levans and animal sera. All the above polysaccharides are branched and contain terminal, non-reducing α-D-glucopyranosyl, α-D-mannopyranosyl or β-D-fructofuranosyl residues, the common configurational features being the disposition of the hydroxyl groups at the C-3, C-4, and C-6 positions of the α-D-hexopyranosyl and the β-D-fructofuranosyl rings, respectively. Carbohydrate–protein conjugates

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*** Present address: Department of Molecular Biology, Abbot Laboratories, Scientific Divisions, North Chicago, Ill. 60064 (U.S.A.).

have been synthesized and examined as precipitants of concanavalin A, thus confirming the stereochemical specificity of the protein's combining sites[11].

The fact that concanavalin A also gives rise to a precipitin-like curve in its reaction with specific polysaccharides and that polysaccharide–concanavalin A interaction may be inhibited by specific low-molecular-weight carbohydrates (hapten) suggested a close analogy with an antibody–antigen system[8,12,13]. It is largely for this reason that we have been engaged in the study of concanavalin A–polysaccharide interaction.

Inasmuch as concanavalin A has been shown to react with dextrans, a family of α-glucans containing predominantly α-D-(1 → 6)-glucosidic linkages, and since insoluble supports of cross-linked dextrans are commercially available (Sephadexes)[14,15] we investigated the feasibility of using these materials for the isolation and purification of concanavalin A by specific adsorption, in a manner analogous to the isolation of antibodies by the use of immunoadsorbents[16–20]. Indeed, we have observed such specific binding of concanavalin A to Sephadex G-50, -75, -100 and -200 (ref. 21).

Other recent attempts to isolate concanavalin A from jack bean meal include those of OLSON AND LIENER[22], and of NAKAMURA AND SUZUNO[23]. The approach of OLSON AND LIENER is similar to ours in that it is based on the adsorption of the protein to cross-linked dextran gels. However, these workers employed glycine–HCl buffer (pH about 2.0) for elution of the protein. The Japanese workers first removed canavalin, another jack bean protein, by adsorption on bentonite; concanavalin A then precipitated when the supernatant solution was dialyzed against water.

This communication describes in detail the manner in which we have employed Sephadex for the isolation of concanavalin A in a high state of purity. Succeeding communications will describe some physical and chemical properties of the protein. A preliminary report has already appeared[24].

EXPERIMENTAL PROCEDURE

Materials

Jack bean meal was purchased from General Biochemicals, Chagrin Falls, Ohio. Sephadexes were obtained in the fine-bead form from Pharmacia Fine Chemicals, Piscataway, N. J. The sugars used for hapten inhibition studies were obtained from the following sources:

Dawe's Laboratories, Chicago (D-fructose); Mann Research Laboratories, New York (D-galactose, D-glucose and cellobiose); Matheson, Coleman and Bell, East Rutherford, N.J. (D-arabinose); Merck and Co., Rahway, N.J. (sucrose); Paul Lewis Laboratories, Milwaukee, Wisc. (lactose); Pfanstiehl Laboratories, Waukegan, Ill. (D-mannose, methyl β-D-glucopyranoside and melibiose).

All reagents were analytical grade.

Dextran NRRL B-1355-S was a generous gift of Dr. A. JEANES, U.S. Department of Agriculture.

Methods

Distribution of concanavalin A in $(NH_4)_2SO_4$ fractions. Jack bean meal (200 g) was suspended in 0.15 M NaCl (1 l) and magnetically stirred overnight in the cold (about 5°). The suspension was strained through cheesecloth and the residue re-

extracted under the same conditions. The combined filtrates from the two extractions were centrifuged for 30 min at 9500 rev./min (14600 × *g*) in the GSA head of a Sorvall RC-2 refrigerated centrifuge and the residue was rejected. Protein in the supernatant solution was precipitated by the addition of $(NH_4)_2SO_4$ as the solid salt[25] in the following saturation ranges: 0.0–0.40, 0.40–0.50, 0.50–0.60, 0.60–0.70, 0.70–0.80 and 0.80–0.90. No further protein was precipitated above 0.90 saturation. These fractions were dialyzed against water and then 1.0 M NaCl. Concanavalin A activity in each fraction was determined by a rapid turbidimetric method. The assay medium contained the following components: protein in 1.0 M NaCl, 0.0–1.0 ml; 1.0 M NaCl, 1.0–0.0 ml; 4.0 M NaCl, 0.50 ml; 0.10 M phosphate buffer (pH 7.4), 0.50 ml; water, 0.50 ml; dextran NRRL B-1355-S (0.5 mg/ml), 0.50 ml; total volume, 3.0 ml.

The reaction was initiated by the addition of the dextran, the contents were stirred with a polyethylene rod and the tubes were incubated at 25°. The turbidity was read after exactly 12 min in a Spectronic 20 colorimeter at 420 mμ. 1 unit of concanavalin A activity was arbitrarily defined as the amount of protein (in mg) yielding an absorbance of 0.20 (linear range 0.05–0.40) at 420 mμ.

Procedure for the isolation of concanavalin A. A NaCl extract of jack bean meal (300 g) was prepared as described above. The extract was centrifuged (14600 × *g*, 30 min) and the residue was rejected. Solid $(NH_4)_2SO_4$ (176 g/l)[25] was added to the supernatant solution to make it 30% saturated with respect to the salt. The pH was adjusted to 7.0 with dilute NH_4OH and the suspension was allowed to stir for 2 h at room temperature (about 25°). The precipitate was removed by centrifugation at 9500 rev./min (14600 × *g*). Additional solid $(NH_4)_2SO_4$ (356 g/l) was added to make the supernatant solution 80% saturated, the pH adjusted to neutrality and the proteins allowed to precipitate at 25°. The precipitate was collected by centrifugation (14600 × *g*) and the supernatant solution discarded. The precipitate was suspended in water (500 ml) and dialyzed extensively against water and finally against 1.0 M NaCl. The contents of the dialysis sac were centrifuged (14600 × *g*) rejecting a small amount of insoluble material and the clear solution used for specific adsorption on a Sephadex G-50 (fine) column (4 cm × 50 cm) equilibrated with 1.0 M NaCl. The protein solution was passed through the column at a rate of approx. 30 ml/h. Fractions (20 ml) were collected and monitored at 280 mμ for protein content. After all the protein solution had been applied to the column, it was washed for 48 h with 1.0 M NaCl (1.5–2.0 l). A solution of D-glucose (0.10 M) in 1.0 M NaCl was used to displace concanavalin A from the Sephadex bed. Fractions with an absorbance (at 280 mμ) of 0.10 or greater were combined and dialyzed, with several changes (18–20), against large volumes of 1.0 M NaCl.

Assay of concanavalin A activity. At each stage of isolation, aliquots of the protein solutions were removed and dialyzed exhaustively against 1.0 M NaCl. The assay was conducted at constant protein concentration using varying amounts of dextran NRRL B-1355-S as the polysaccharide precipitant. The procedure of SO AND GOLDSTEIN[26] was employed for the quantitative determination of nitrogen in the precipitate. The assay system contained the following components: protein solution in 1.0 M NaCl, 0.10 ml; 4.1 M NaCl, 0.22 ml; 0.10 M phosphate buffer (pH 7.4), 0.18 ml; water, 0.0–0.50 ml; dextran (2 mg/ml), 0.0–0.50 ml; total volume, 1.00 ml.

The precipitation was carried out in 3-ml glass centrifuge tubes specially calibrated for these studies (Bellco Glass, Inc., Vineland, N.J.). The amount of nitrogen

in the specific precipitates was determined by ninhydrin analysis following the digestion of the precipitate with 3.5 M H_2SO_4 and subsequent treatment with H_2O_2 (30 %, v/v)[27]. The analysis of concanavalin A was expressed in terms of the percentage of the total nitrogen precipitated.

Concanavalin A–Sephadex interaction. Various substances were evaluated for their ability to displace concanavalin A bound to Sephadex G-50.

(a) Hapten displacement. Concanavalin A was adsorbed on a Sephadex G-50 column (2.4 cm × 24 cm) by passing through a solution of an $(NH_4)_2SO_4$ fraction precipitated between 0.30 and 0.80 saturation. A 0.01 M solution of non-inhibitor sugar[28] (D-galactose) was added to the column and the effluent was assayed for protein by the absorbance at 280 mμ and carbohydrate by the phenol–H_2SO_4 method[29]. After washing the column free of D-galactose with 1.0 M NaCl solution, a 0.01 M solution of D-fructose (a known inhibitor of concanavalin A–polysaccharide interaction) was added and the effluent again monitored for protein and carbohydrate.

In a similar fashion D-arabinose, cellobiose, lactose, melibiose, methyl β-D-glucopyranoside, D-mannose and sucrose were tested for their capacity to displace concanavalin A.

(b) Displacement by alterations in physical environment. Solutions of 1.0 M acetic acid (pH 2.4), 0.20 M sodium phosphate (pH 8.5–9.0), 3.0 M $MgCl_2$, and 8.0 M urea were also individually evaluated for their ability to elute concanavalin A.

Prior to testing for the ability of concanavalin A to precipitate specific polysaccharides, the solutions were freed of eluting agent by dialysis against 1.0 M NaCl. Acetic acid was removed by passage through a Sephadex G-25 column equilibrated with 1.0 M NaCl. The agar gel diffusion technique[9] was used for evaluating activity against various polysaccharides.

RESULTS

Distribution of concanavalin A activity

The results summarized in Table I show that approx. two-thirds of the total concanavalin A activity resided in the protein fraction which was precipitated between 0.50 and 0.60 saturation of $(NH_4)_2SO_4$. The remaining activity was distributed in

TABLE I

DISTRIBUTION OF CONCANAVALIN A IN $(NH_4)_2SO_4$ FRACTIONS

Serial No.	*$(NH_4)_2SO_4$ fraction**	*Volume (ml) (X)*	*Protein concentration (mg/ml) (Y)*	*Specific activity (units/mg) (Z)*	*Total units X × Y × Z*	*Percentage of the total*
1	0.00–0.40	72	18.00	0.59	765	7.8
2	0.40–0.50	53	29.00	0.83	1277	13.0
3	0.50–0.60	125	36.50	1.25	5694	58.1
4	0.60–0.70	65	26.25	0.67	1126	11.5
5	0.70–0.80	100	35.50	0.26	923	9.4
6	0.80–0.90	40	33.25	--	--	-

* The figures indicate the saturation range in which the protein fractions were precipitated with $(NH_4)_2SO_4$.

various fractions ranging from 0.30 to 0.80 saturation. Concanavalin A activity could not be detected in the fraction precipitated above 0.80 $(NH_4)_2SO_4$ saturation.

Sephadex adsorption method for the isolation of concanavalin A

An effective separation of concanavalin A from inert proteins was achieved by specific adsorption on Sephadex G-50 as illustrated in the elution profile (Fig. 1), and the agar gel diffusion patterns (Fig. 2). Only the protein fraction eluted with 0.10 M D-glucose (referred to as Peak No. 2) formed a precipitation band with specific polysaccharides.

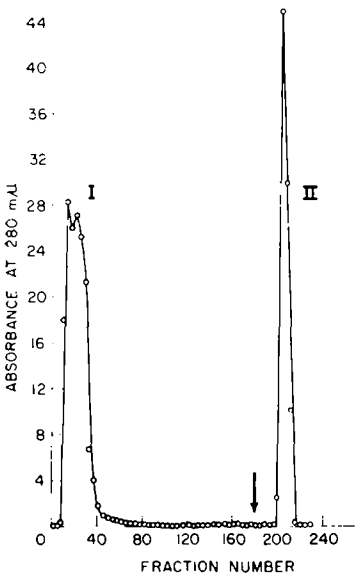

Fig. 1. Chromatography of the jack bean protein fraction precipitated between 0.30 and 0.80 saturation of $(NH_4)_2SO_4$ on a Sephadex G-50 column (4 cm × 50 cm). Arrow indicates the addition of 0.10 M glucose. Peak No. 1 was inert towards dextran. All the polysaccharide-precipitating activity was recovered in Peak No. 2. Chromatographic recovery about 94 %.

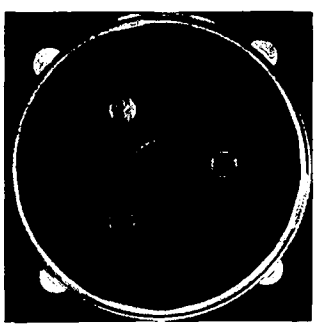

Fig. 2. Agar gel diffusion pattern of the fractions isolated from Sephadex G-50 chromatography. Center well contained rabbit-liver glycogen. Peripheral wells No. 1, 3, and 5 contained the $(NH_4)_2SO_4$ fraction (precipitated between 0.30 and 0.80 saturation), Peak No. 1 and Peak No. 2, respectively.

The purification and recovery data of concanavalin A are summarized in Table II. The nitrogen values in each fraction represent non-dialyzable protein nitrogen. The ultraviolet absorption spectra of these fractions are characteristic of proteins with an $A_{280\,m\mu}/A_{260\,m\mu}$ ratio of 1.80 indicating the absence of nucleotides. Concanavalin A isolated by Sephadex adsorption is in a highly purified state; 97.6 % of the total protein can be precipitated by the specific assay polysaccharide, dextran NRRL B-1355-S. The chromatographic recovery is of the order of 94 % and the total yield of concanavalin A is usually 2.0–2.4 g/100 g meal.

Hapten displacement

The results clearly demonstrate that 0.01 M D-galactose, a non-inhibitor of

TABLE II

PURIFICATION AND RECOVERY DATA OF CONCANAVALIN A

Isolation stage	*Volume (ml)*	*mg N/ml*	*Total N (mg)*	*Recovery of N (percentage of the total)*	*Percentage purification*
0.15 M NaCl	2766	1.941	5357	100	23.8
Supern. 30*	3000	1.350	4860	97.2	22.9
$(NH_4)_2SO_4$ fraction 0.30–0.80	530	8.125	4316	80.6	26.8
Peak No. 1	1500	1.973	2961	55.2	
Peak No. 2	450	2.436	1096	20.4	67.6

* Denotes the supernatant solution remaining after the precipitation of proteins at 0.30 saturation of $(NH_4)_2SO_4$.

concanavalin A–polysaccharide interaction, did not elute concanavalin A specifically bound to Sephadex whereas D-fructose (0.01 M) readily displaced the protein from the Sephadex bed (Fig. 3).

The various sugars tested as eluants can be classified into two groups based on their ability to displace concanavalin A from Sephadex (Table III). Sugars in

ELUTION PROFILE SHOWING THE SPECIFICITY OF ELUTING SUGAR.

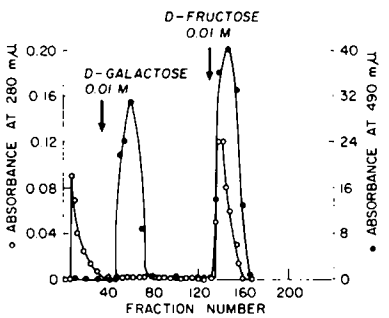

Fig. 3. Elution of concanavalin A from Sephadex G-50 by various sugars. 0.01 M D-galactose is a non-inhibitor. Left ordinate (○—○) represents absorbance of the effluent at 280 mμ. Right ordinate (●—●) shows the phenol–H_2SO_4 color values at 490 mμ.

TABLE III

CLASSIFICATION OF SACCHARIDES

All sugars tested as 0.01 M solution.

Non-inhibitor	*Inhibitor*
D-Arabinose	D-Glucose
D-Galactose	D-Fructose
Cellobiose	D-Mannose
Melibiose	Sucrose
Lactose	Methyl β-D-glucopyranoside*

* A poor inhibitor; the protein emerged in a broad peak.

the non-inhibitor class did not elute concanavalin A from the Sephadex column whereas all inhibitor sugars caused elution of the protein.

Elution by various agents

Whereas 1.0 M acetic acid successfully released concanavalin A, elution with 0.20 M sodium phosphate (pH 8.5) was incomplete. Concanavalin A could also be eluted with 3.0 M $MgCl_2$, and 8.0 M urea.

Removal of haptens and other eluting agents

All agents used for the elution of concanavalin A were removed most readily by dialysis against 1.0 M NaCl. Dialysis against water followed by 1.0 M NaCl often lead to aggregation of the protein. Acetic acid was removed by gel filtration on Sephadex G-25, as the dialysis of concanavalin A solution at low pH values resulted in loss of its activity. Following removal of the eluting agents, concanavalin A was shown to be active as illustrated in Fig. 4 by the gel diffusion patterns with rabbit-liver glycogen.

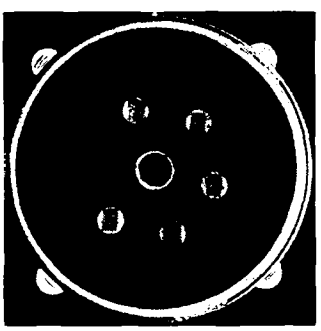

Fig. 4. Agar gel diffusion patterns of concanavalin A isolated from Sephadex G-50 by elution with various agents. Center well contained rabbit-liver glycogen as the precipitating polysaccharide. Peripheral wells No. 1, and 5 contain the protein eluted with 3.0 M $MgCl_2$, wells No. 2, 3, and 4 contained concanavalin A eluted with 0.1 M glucose, 1.0 M acetic acid, and 8.0 M urea, respectively.

DISCUSSION

Our studies indicated that approx. two-thirds of the total concanavalin A present in the jack bean was precipitated at about 0.60 saturation of $(NH_4)_2SO_4$ in agreement with earlier work[30]. However, a satisfactory separation of concanavalin A from other proteins was not achieved by $(NH_4)_2SO_4$ fractionation confirming SUMNER's observations[1]. Since our present method of isolation depends solely on the specific adsorption of concanavalin A to Sephadex we employed a rather wide protein fraction which is precipitated between 0.30 and 0.80 saturation of $(NH_4)_2SO_4$. (The fraction precipitated in the 0-0.30 $(NH_4)_2SO_4$ saturation range removed inert materials which often interfered with subsequent processing.) This fraction (0.30-0.80) contains virtually all the concanavalin A activity and, in addition, most of the jack bean proteins which are extractable by salt. Upon chromatography of this $(NH_4)_2SO_4$ fraction on Sephadex G-50 all of the inert proteins emerged with the void volume (Peak No. 1). The remainder was eluted by a solution of an inhibitor (0.10 M glucose).

Our method for the isolation of concanavalin A depends upon the specific binding of the protein to cross-linked dextran gels[21]. The isolation and fractionation of human antidextran antibodies by KABAT and co-workers[31,32] using cross-linked dextran gels is based upon the same principle. The present method for the isolation of concanavalin A is analogous to the isolation of antibodies by specific adsorption on immunoadsorbants. The latter consist of soluble antigens or haptens which are chemically bound to insoluble polymer supports, for example the coupling of proteins or haptens to cellulose and polystyrene frameworks[16–18]. Use of these immunoadsorbants involves the specific adsorption of the antibodies from the antiserum, removal of extraneous serum components by washing with buffered saline followed by displacement of antibodies from the support by lowering the pH (about 2–3), or in the case of antihapten antibodies by solubilization with hapten. Other applications of this very versatile approach involve purification of liver flavokinase by chromatography on flavin–cellulose compounds[33], of nucleotides by passage over thymidylate–cellulose[34], and of α-amylase by complexation with glycogen[35]. The use of water-insoluble derivatives of enzymes, antigens and antibodies was reviewed recently[36].

Concanavalin A binds readily to Sephadex G-50, -75, -100 and -200 but emerged with the void volume from Sephadex G-10 and G-25. It is believed that the latter two Sephadexes are so extensively cross-linked as to have eliminated the binding sites required for interaction with concanavalin A.

The specificity of the interaction of concanavalin A with cross-linked dextran gels is similar to that observed with several other polysaccharides (*e.g.* glycogens and yeast mannans), as only those sugars shown to be inhibitors of concanavalin A–polysaccharide interaction[28] were effective in displacing concanavalin A from Sephadex.

As is well known, the biological activity of proteins is markedly affected by the pH and ionic strength of the medium, and the presence of specific ions and denaturing agents. These various factors influence the distribution of charges and orientation of side-chain groups. Since most proteins display maximum biological activity over a limited pH range, a shift in pH away from this optimum usually leads to a decrease and eventual loss of activity. Inasmuch as the optimum range for concanavalin A–polysaccharide interaction lies between pH 6.5 and 8.0 one would anticipate the possibility of displacing concanavalin A bound to the cross-linked gels by altering the pH of the eluant. This is indeed the case as 1.0 M acetic acid (pH 2.4) can elute concanavalin A. The resulting proteins can be neutralized to pH 7.0 (NaOH or sodium phosphate) or the acetic acid may be removed by gel filtration by passage through Sephadex G-25. Dialysis in the presence of acid resulted in the loss of Mn^{2+} which is essential for the polysaccharide-precipitating activity of concanavalin A. The failure of OLSON AND LIENER[22] to observe loss in activity of acidified solutions of concanavalin A upon dialysis could be attributed to the brief duration of the dialysis or to their method of assay (hemagglutination) or both. Since only trace amounts of bivalent cations, *e.g.* Mn^{2+}, Ca^{2+}, Mg^{2+}, *etc.* are required for activity it is possible that such cations are present on the surface of the rabbit erythrocytes which these workers employed in their hemagglutination assay.

Elution of the protein from Sephadex G-50 by raising the pH to 8.5–9.0 with phosphate buffer is unsatisfactory as the recoveries are of the order of 60 %, or less. This difficulty is due mainly to the tendency of concanavalin A to aggregate at

alkaline pH, resulting in incomplete recovery. Denaturation is also probably initiated at this pH.

The presence of Mn^{2+} in crystalline concanavalin A was reported by SUMNER AND HOWELL[37]. We have confirmed its presence, by neutron activation analysis. Bivalent cations are necessary for the interaction of concanavalin A with polysaccharides, the most effective cation being Mn^{2+}. However, excess of bivalent cations inhibited the activation of the metal-free concanavalin A as well as concanavalin A–polysaccharide interaction. For example, precipitation of concanavalin A with polysaccharides was not observed in 3.0 M $MgCl_2$, which salt solution also acted to effectively displace concanavalin A from dextran gels. The elution of concanavalin A from Sephadex gels by 8.0 M urea can be rationalized on the basis of its action as a denaturing agent.

Of the four procedures examined for the displacement of concanavalin A from Sephadex G-50 the most effective is elution by haptens *e.g.* 0.10 M glucose. This treatment is very mild, the only disadvantage being the time required for removal of the displacing sugar. The effectiveness of the hapten elution method arises from the fact that the hapten possesses the same structural features required for the interaction of concanavalin A with polysaccharides. Because of its low molecular weight the hapten can readily diffuse into the gel matrix and compete with Sephadex for the combining sites on the protein. The remaining three methods for displacing concanavalin A from Sephadex are based on alterations in the medium with the consequent changes in the conformation of the active protein.

Removal of D-glucose from concanavalin A by dialysis against water followed by 1.0 M NaCl solution frequently leads to aggregation of the protein. This may be due to the sudden changes in the ionic environment of the medium. The removal of glucose by gel filtration on Sephadex G-25 was not complete and resulted in unnecessary dilution with each cycle. However, preliminary experiments using Biogel have been encouraging. Poor separation of glucose from the protein and glucose on Sephadex G-25 may be due to the mutual affinity of glucose, concanavalin A, and dextran support, whereas Biogels[38] are composed of non-carbohydrate material.

Concanavalin A obtained by the Sephadex adsorption method is approx. 98 % active as measured by its ability to precipitate a specific dextran. This compares favorably with the purity (about 90 %) of antibodies obtained by use of immunoadsorbants. The yield of concanavalin A by our method is about 2.0–2.4 g/100 g meal. Variations in yield are likely to occur depending on the source and time of harvest of the beans, and finally on the method of isolation. Such variations of urease content have been reported[39,40].

The simplicity of the Sephadex method for the isolation and purification of concanavalin A makes it possible to prepare relatively large quantities of this protein for investigational purposes.

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