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Note

Sephacryl S-300 — an affinity matrix which distinguishes concanavalin A from other D-mannose/D-glucose-specific lectins

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Sephadex G-50 is bead gel prepared by cross-linking dextran with epichlorohydrin[1]. It is commonly used as an affinity matrix for isolation of the D-mannose/D-glucose-specific lectins[2]. We have shown that a simple affinity matrix like cross-linked starch entrapped in agarose beads can be used as a substitute for Sephadex[3]. Sephacryl S-300 gel beads are prepared by cross-linking allyldextran with N,N'-methylenebisacrylamide to give a gel matrix with a controlled pore size[1]. The present report describes the use of Sephacryl S-300 as an affinity matrix for the isolation of concanavalin A (Con A).

MATERIALS AND METHODS

Reagents

Sephadex G-50 (Fine) and Sephacryl S-300 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), bovine serum albumin and rabbit liver glycogen from V. P. Chest Institute, New Delhi. All other chemicals were of analytical grade. Jack bean seeds (*Canavalia gladiata*, Abai seeds) were obtained from plants grown in the garden of the Botany Department, University of Poona, Pune, India. Fava beans (*Vicia faba*) were obtained from Sutton and Sons Pvt., Calcutta, India. Good quality pea (*Pisum sativum*) and lentil (*Lens culinaris*) seeds were obtained locally.

Protein concentrations were determined by absorbance measurements at 280 nm using bovine serum albumin as a standard ($A^{1\%}_{1\,cm}$ = 6.45). Haemagglutination assays were done at room temperature (26–28°C) in 0.02 *M* phosphate-buffered saline (pH 7.2, 0.1 *M* sodium chloride) at concentration of 0.01 mg/ml using a 4% suspension of papain-treated rabbit erythrocytes[3]. The turbidity of Con A–glycogen (1.0 mg/ml) in saline II solution (1.0 *M* sodium chloride) was monitored at 420 nm[4]. Centrifugation was carried out in a Sorwall SS-3, automatic centrifuge, using a rotor SS-34 (capacity 8 × 50 ml, 12080 *g*) at room temperature (26–28°C) and in a refrigerated centrifuge (capacity 2 × 180 ml, 2412 *g*) at +4°C. The saline solution I contained 0.145 *M* sodium chloride.

Seeds were soaked in water, the seed coats were peeled off and the softened cotyledons were homogenized with saline solution I (1:5, w/v) in a blender. The homogenate was mixed with 1-butanol (20 ml per 100 ml of homogenate), the mixture

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was stirred for 1 h at 0°C and the 1-butanol layer was removed by centrifugation at 12 080 *g*. The treatment was repeated three or four times to remove the lipids. An equal volume of cold acetone (−4°C) was added to the homogenate and the precipitate was collected by centrifugation at 12 080 *g*, dehydrated by repeated treatment with cold acetone (−4°C), air dried and stored in a deep freeze at −20°C.

The acetone-dried powder was extracted with saline solution I with stirring for 3–4 h at +4°C. The extract was centrifuged at 12 080 *g* and the residue was re-extracted with saline solution I as before. The supernatants were pooled and subjected to fractional precipitation with ammonium sulphate, essentially according to the procedure of Agrawal and Goldstein[5]. The protein fraction precipitated between 30 and 80% saturation by ammonium sulphate was isolated, dissolved in a minimum amount of water, extensively dialysed against water and finally against saline solution II. On dialysis the solution containing lectin was clarified by centrifugation at 12 080 *g* and then stored at −20°C. This solution was used to isolate lectin by affinity chromatography on Sephadex G-50 and Sephacryl S-300 columns (6.0 cm × 2.5 cm I.D.; capacity 28.0 ml), equilibrated with saline solution II. The protein was applied to the column at a flow-rate of 20 ml/h. Fractions of 5.0 ml were collected and monitored at 280 nm for the protein content, for lectin by the turbidity with glycogen at 420 nm[4] and by haemagglutination assay. The column was washed with saline solution II to remove the inert protein and the adsorbed Con A was eluted with D-glucose (0.1 *M* in saline solution II). The Con A-containing fractions were pooled and dialysed against saline solution II till free from D-glucose.

RESULTS AND DISCUSSION

The chromatographic data given in Table I show that Sephacryl S-300 has an high adsorption capacity for Con A in contrast with Sephadex G-50 under identical conditions. The adsorbed Con A is completely eluted with D-glucose in the case of Sephacryl S-300, but only 87% is eluted in the case of Sephadex G-50.

The Con A isolated by both procedures was found to be identical as judged by the electrophoretic patterns (Fig. 1), haemagglutination assay and turbidimetric

TABLE I

CHROMATOGRAPHIC ANALYSIS DATA

Total carbohydrate content: Sephadex G-50, 2.0 g; Sephacryl S-300, 0.93 g.

Affinity medium	*Protein loaded (mg)*	*Protein retained (mg)*	*Protein eluted (mg)*	*Elution (%)*	*Adsorption efficiency in mg Con A adsorbed per g carbohydrate*
Sephadex G-50 (0.75)*	450**	217	187	87	108.5
Sephacryl S-300 (0.23)*	450 (600)**	280 (300)**	280	100	322.6

* Carbohydrate content (mg of dry powder) determined by phenol-sulphuric acid method[21].

** Saturation level of adsorbed protein.

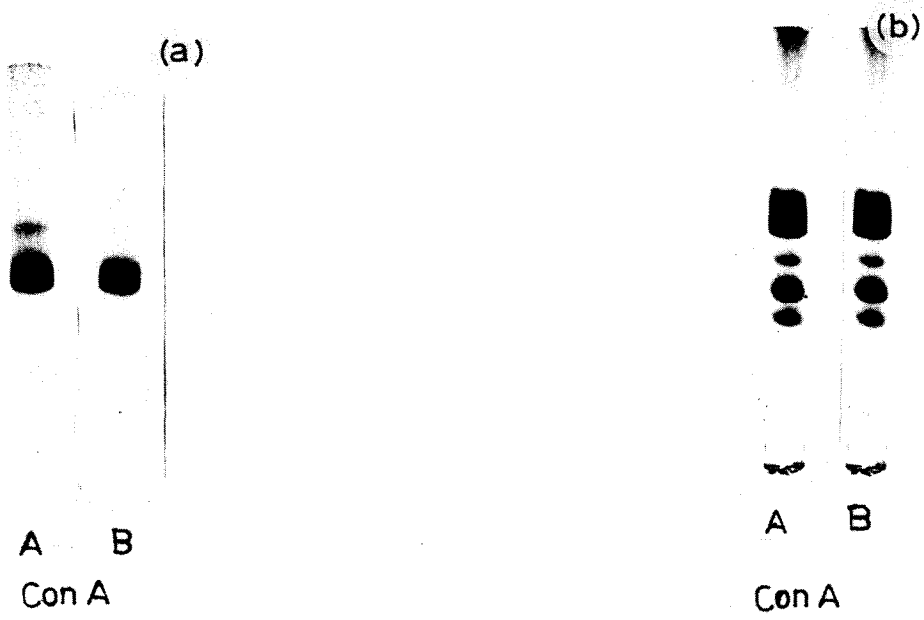

Fig. 1. Gel electrophoresis patterns of Con A: (a) PAGE, pH 4.5[22]; (b) SDS-PAGE, pH 7.2[23]. A, Sephadex G-50; B, Sephacryl S-300.

assay using rabbit liver glycogen. Polyacrylamide gel electrophoresis (PAGE) at pH 4.5 gave one major and one minor band. At pH 4.5, Con A exists essentially as a cationic dimer and in PAGE the slow-moving band is probably due to aggregation of Con A promoters.

One major and three minor bands were observed in sodium dodecyl sulphate (SDS)-PAGE of Con A isolated by both procedures under reducing as well as non-reducing conditions. The major band corresponds to the intact subunit (MW 27000) and the three bands are due to fragments of this subunit. The fragments with MW 13300 and 11200 are derived from the intact subunit by hydrolysis of the peptide bond between Asn-118 and Ser-119[6]. No explanation has been given for the fragment with MW 17500.

The D-glucose gradient elution of Con A adsorbed on Sephacryl S-300 gave two fractions, one eluted by 0.019 *M* D-glucose and containing a mixture of intact subunits and nicked subunits, the other by 0.1 *M* D-glucose contained intact subunits only (Fig. 2).

Sephacryl S-300 was also tested as an affinity matrix for the isolation of other D-mannose/D-glucose-specific lectine such as those of the pea, lentil and favin. However, these lectins are not retained by the column.

The present investigation has shown that the adsorption capacity of Sephacryl S-300 for Con A is greater than that of Sephadex G-50 (Fine), though the polysaccharide content of Sephacryl S-300 is about 1/3 that of Sephadex G-50. Dextran is a homopolymer of D-glucose in which the D-glucose units are chiefly linked through α-(1 $\rightarrow$ 6) bonds and to a small extent through α-(1 $\rightarrow$ 3) bonds. The cross-linking

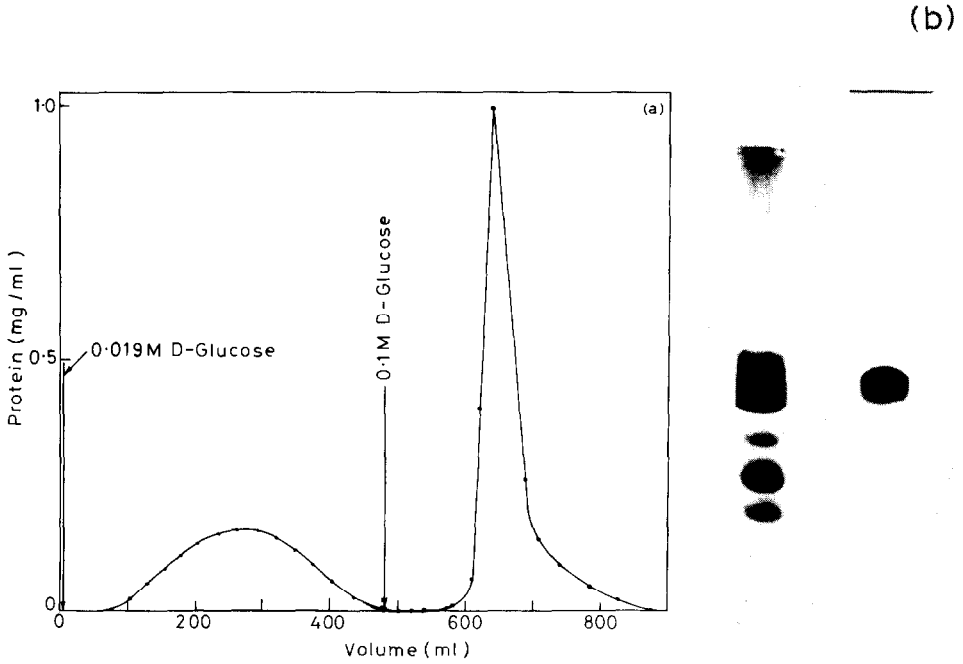

Fig. 2. Isolation of the intact subunit of Con A. (a) Elution profile of Con A from Sephacryl S-300 in 0.02 *M* Tris–HCl buffer (pH 7.4) using 0.019 *M* D-glucose followed by 0.1 *M* D-glucose[24]. (b) SDS-PAGE patterns of the respective protein fractions.

of dextran chains with epichlorohydrin introduces cross-bridges ($-O-CH_2-CHOH-CH_2-O-$) across certain hydroxyl groups (2, 3 and 4) of the D-glucose units. The interaction of Con A with cross-linked dextran (Sephadex G-50) and cross-linked allyldextran (Sephacryl S-300) takes place through hydrogen bonding of free –OH groups of the D-glucose units. It appears that the D-glucose units in the allyldextran chains of Sephacryl S-300 are oriented in such a way that they can interact with Con A to give a maximum retention. The adsorbed Con A is completely eluted with D-glucose, indicating the absence of non-specific adsorption on the Sephacryl S-300 gel matrix.

On the basis of haemagglutination inhibition studies as well as binding studies with various mono- and disaccharides, lectins from *C. ensiformis* (Con A), lentil, pea and *V. Faba* (favin) are classified as D-mannose/D-glucose-specific lectins[2]. Lentil lectin and pea lectin consist of a mixture of two isolectins which can be separated by ion-exchange chromatography[2]. Con A is a single-chain lectin whereas pea, lentil and favin lectins consist of two non-identical chains (A and B). A profound homology[7,8] exists between the amino acid sequences of these lectins. In addition there is a striking conservation of the amino acid residues which are involved in binding to metals, sugars and the hydrophobic cleft[9]. These lectins are mitogenic and selectively agglutinate transformed and protease-treated cell lines. They have been used for the isolation and structural elucidation of glycopeptides and complex carbohydrates. The ability of lectins to interact specifically with cell surface glyco-conjugates

makes them a useful tool in the field of cell biology. All these lectins are metalloproteins containing Ca^{2+} and Mn^{2+} in the native state. Recently Bhattacharya *et al.*[10] reported the preparation and properties of metal ion derivatives of lentil and pea lectin. Their results indicate that the removal of metal ions and remetallization of these lectins are very different from that of Con A . Con A exists as a dimer or tetramer depending upon the pH, but lentil and pea lectins exist as dimers (MW = 47000)[11,12]. All these lectins are usually isolated by affinity chromatography on Sephadex G-50, G-100. Recently it was reported that the precursor of Con A is a glycoprotein, with no lectin activity, and undergoes a unique post-translational peptide cleavage and ligation to produce mature Con A with lectin activity[13,14].

Since Con A was adsorbed on Sephacryl S-300 it was expected that other D-mannose/D-glucose-specific lectins, being structurally similar, would also be adsorbed. However, pea, lentil and favin lectins were not retained. When a mixture of pure Con A and pure pea lectin was loaded on a Sephacryl S-300 column only Con A was adsorbed and the pea lectin appeared in the effluent.

Our present knowledge about the binding specificity of most lectins is limited to the inhibitory activities of simple saccharides against haemagglutination. It is now well established that lectins bind to terminal as well as interior sugar residues of the oligosaccharide chains[15]. Ohyama *et al.*[16] recently determined the dissociation constants of ten asparagine-linked oligosaccharides derived from ovalbumin, by studying their interaction with Con A immobilized on Sepharose 4B, by the method of frontal affinity chromatography. A comparison of K_d values suggests that the binding of Con A varies considerably with slight structural differences in the glycosyl chain. The K_d values for the binding of *p*-nitrophenyl-α-D-mannoside to Con A and Con A-Sepharose were essentially the same. In the case of Con A and lentil lectin it is observed that, besides α-mannosyl groups, these lectins probably interact with multiple sugar residues in the oligosaccharide chains of the glycopeptides to promote tight binding[17,18]. According to Kornfeld *et al.*[19], fucose is an important determinant in the tight binding of glycopeptides to pea- and lentil-Sepharose, but not Con A-Sepharose. These four lectins, though considered to be identical in terms of sugar specificity, possess the ability to recognize fine structural differences in more complex oligosaccharide chains[20]. Thus it appears that the two-chain lectins like those of the pea, lentil and favin are related more closely to each other than to the one-chain lectin Con A, confirming the immunological and taxonomical diversification.

Our results provide support for these observations that the interaction of the sugar binding site of the lectins with sugar residues in the oligosaccharide chain is influenced by the neighbouring sugar residues. Sephacryl S-300 is able to distinguish between these fine structural requirements of D-mannose/D-glucose-specific lectins, the two-chain lectins of the pea, lentil and fava beans and the one-chain lectin Con A.

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