STROMA: AS AN AFFINITY ADSORBENT FOR NON-INHIBITABLE LECTINS

José-Luis OCHOA+ and Tore KRISTIANSEN
Institute of Biochemistry, Uppsala University, Box 576, 75123 Uppsala, Sweden

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1. Introduction

Lectins are carbohydrate-binding proteins characterized by their ability to agglutinate erythrocytes and other types of cells [1,2]. Some of them possess, in addition, other biological properties that are widely applied in various fields of biochemistry [1]. Recent techniques employed in their purification take advantage of their specific binding properties and are based on bioaffinity chromatography involving immobilization of inhibiting carbohydrates through covalent linkage to an inert matrix or support [2]. However, some lectins are inhibited only weakly, or not at all, by mono- or oligosaccharides found to be good inhibitors of other lectins. It has been concluded that the former type of lectins imposes very specific stereochemical restrictions on the oligosaccharide structures which are thought to be present only on the cell surface. For this reason the preparation of a biospecific adsorbent is difficult. The fact that these lectins possess a strong affinity for the cell membrane, as evidenced by their high agglutination titers, prompted us to attempt their purification by using physically entrapped glutaraldehyde-treated stroma as adsorbent [3–6]. This chromatographic system allows the biospecific separation of substances possessing affinity for cell membrane structures. As illustration we describe here the purification of two non-specific and non-inhibitable lectins (*Phaseolus vulgaris*, var. 'garbancillo' and *Phaseolus coccineus*, var. 'alubia') [7].

Abbreviations: Con-A, concanavalin-A lectin; Ph.a, phosphorylase *a*; BSA, bovine serum albumin; Ov, Ovalbumin

+ Permanent address: Instituto de Quimica, Universidad Nacional Autonoma de México, México DF.

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2. Materials and methods

All the reagents were of analytical grade purchased from commercial sources. Sephadex G-25 superfine was obtained from Pharmacia Fine Chemicals (Uppsala). The seeds (*Phaseolus vulgaris*, var. 'garbancillo' and *Phaseolus coccineus*, var. 'alubia') were provided by Dr Felix Cordoba (Faculty of Medicine, National University of Mexico) and classified at the Agriculture School of Chapingo, Mexico.

2.1. *Extraction of the lectins*

Seeds, 100 g, were finely ground and the lectins extracted with saline phosphate buffer, PBS (0.02 M K_2HPO_4/KH_2PO_4, pH 7.4 containing 0.9% NaCl and 0.02% NaN_3), in proportion 1 : 10, w/v, over 2 h at 4°C. After centrifugation at 2500 × *g*, 15 min, the supernatant was adjusted with glacial acetic acid to pH 4 and stirred 2 h before letting it settle overnight. This new suspension was centrifugated at 25 000 × *g*, 15 min and the corresponding supernatant filtered through a glass filter paper. The clear solution was fractionated by salting-out with solid ammonium sulphate in the range 40–80% of saturation. Finally, the latter fraction was dialyzed extensively against PBS and, after removal of insoluble materials by centrifugation, the solution was kept at 4°C.

2.2. *Stroma preparation*

The erythrocyte membrane residues, or stroma,

were obtained by hemolysis of 250 ml pig red cells, washed 3 times with PBS as in [8]. After freeze-drying, the stroma was polymerized with glutaraldehyde in order to preserve the integrity of its protein composition. This was done following the technique in [9,10]; 52 mg freeze-dried stroma in 23 ml PBS were mixed with 2.5 ml 25% glutaraldehyde solution adjusting to pH 7.2 with 2 M KOH solution. After 16 h incubation at 20°C, the polymerized material was extensively washed with PBS and incubated overnight with 25 ml 1 M glycine in PBS in order to neutralize the free-reactive glutaraldehyde groups on the stroma. The particulate glutaraldehyde-treated stroma was prepared with the aid of a homogenizer and then mixed with swelled Sephadex G-25 (superfine) in proportion 1 : 10, w/v, before pouring over the column. To avoid contamination of the sample by materials arising from the stroma during desorption, the column was washed with 4–5 times the bed volume of the different eluant systems (distilled water, 0.2 M glycine, pH 2.5) and finally equilibrated with PBS. The hemagglutinating activity was assayed with 2% saline suspension of 3 times washed erythrocytes, using the doubling dilution procedure with a lectin solution. The protein concentration was found by measuring the A_{280} or by the method in [10]. The hemagglutination titer was interpreted visually after 1 h and expressed as the higher dilution showing detectable agglutination.

3. Results and discussion

Physically entrapped stroma has been already applied in the purification of some carbohydrate-specific, or inhibitable, lectins in a number of cases [3–6]. The purification process is attributed to the ability of lectins to recognize carbohydrate structures located on the cell membrane residues (stroma). Thus, the necessity of synthesizing a biospecific adsorbent by coupling of diverse carbohydrate inhibitors to an innert matrix or support is avoided [2]. The widespread specificity of adsorption permits stroma to be used for the purification of different carbohydrate-specific lectins, and of other cell membrane-binding proteins of interest. Selective desorption is then possible by the action of the corresponding competitive inhibitor which, in the case of chemo-specific lectins, consists of carbohydrate solutions. However, it has been observed that in some cases oligosaccharide solutions are not efficient in displacing all the adsorbed material. An improvement in the total recovery has been reported [6] by use of low pH buffer. We have further improved this method by entrapment of stroma in columns. In this way, a chromatographic system, in contrast to the batch procedures employed before [3–6], allows us to follow the purification spectrophotometrically. The method has been successfully applied in the purification of non-chemo-specific and non-inhibitable lectins (a report about the characterization of these lectins will appear soon) adding this possibility to those mentioned above.

The patterns obtained after chromatography of an extract of *Phaseolus coccineus*, var. 'alubia' and *Phaseolus vulgaris*, var. 'garbancillo', as in section 2, are shown in fig.1a and 1b, respectively, and in table 1.

The absence of hemagglutinating activity in the substantial fraction of starting material passing through the columns (peaks A), means that a selective adsorption of lectin to stroma does indeed occur. Since specific desorption of lectin allegedly bound to carbohydrate receptors on the stroma was not possible with soluble sugars, non-specific desorption had to be used. The fact that 43% of the applied agglutination activity from 'alubia' and 31% from 'garbancillo' could be eluted with distilled water, suggests that hydrophobic adsorption may participate in the interaction between lectin and stroma. There are additional evidences in favour of different mechanisms of binding lectins to stroma: in both cases, if the sequence of elution with water and low pH is inverted, peaks B and C exchange positions but retain their size. In other words, fraction B is eluted by a decrease in ionic strength and fraction C only by lowering the pH, and this applies to both the 'alubia' and 'garbancillo' lectins. Moreover, since the electrophoretic patterns of B and C (fig.2) are quite similar in both cases, this observation suggests that these proteins can adsorb to stroma by different mechanisms. The large stability of stroma has also permitted us the weekly use of the systems over 6 months without any appreciable change either in binding capacity, or selectivity. Even if the adsorption of lectin to stroma may not be entirely similar to the way it

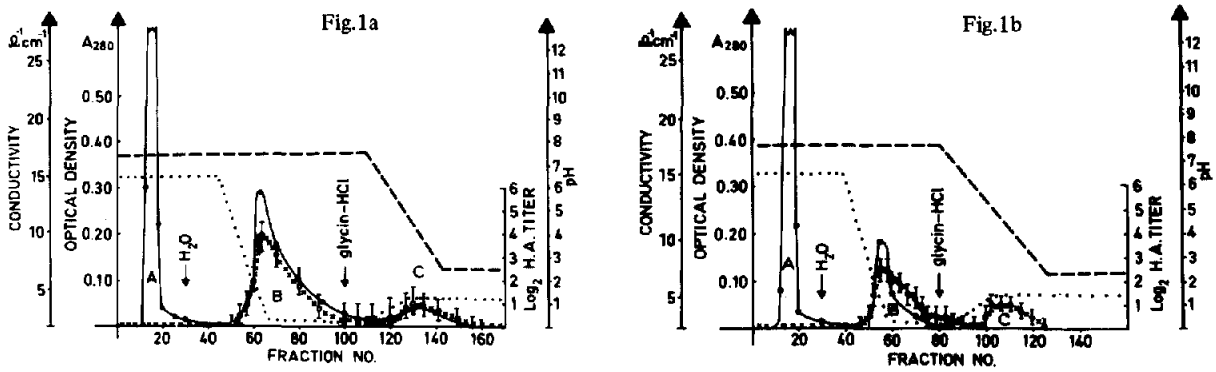

Fig.1a,b. Chromatography of 'alubia' and 'garbancillo' lectins on physically immobilized stroma.

Fig.1a. 102 mg of a sample from 'alubia' seeds, prepared as in section 2, in 2 ml equilibrium buffer (PBS), were applied on a 'stroma-column' (40 × 2.5 cm) of a total adsorbing capacity of 25 × 10^3 HA units. The column was run at a flow rate of 15 ml/h at room temperature and samples of 6.6 ml were collected. Different eluant systems were applied as indicated in the figure pooling the various fractions corresponding to: (A) the material that is not adsorbed; (B) fraction eluted by distilled water; (C) protein eluted by 0.2 M glycine–HCl, pH 2.5. The chromatography was followed by determining the A_{280} (•–•–•) and the hemagglutinating capacity (×–×–×) of each sample. The ionic strength (. . . .) and pH (- - - -) gradients were also determined.

Fig.1b. 60 mg 'garbancillo' extracted lectins in 2 ml PBS were applied in a way similar to the one indicated for 'alubia' lectins in fig.1a. The total adsorbing capacity of the 'stroma-column' for this lectin was of 40 × 10^3 HA units. Chromatography and sample collection was performed as in the case of 'alubia' lectins.

binds to intact erythrocytes surfaces, the ability of distilled water to release part of the lectin activity from the immobilized stroma points to a possible importance of hydrophobicity as a contributing factor to hemagglutination by some lectins. This applies not only to panagglutinating lectins which are inhibited only weakly, or not at all, by simple sugars, but also to lectins that are inhibited in a more or less specific way by carbohydrates [12,13]. For example, it was shown that Con-A agglutinates liposomes which are devoid of carbohydrate and this observation was interpreted [12] as possibly being due to a hydrophobic effect.

Preliminary studies in our laboratory involving

Table 1
The purification process of 'alubia' and 'garbancillo' lectins on physically immobilized stroma

Fraction	Weight (mg)	Spec. act. (HA units/mg protein)	% weight	% initial activity	Purification factor
A1-F_{40}^{80}	102	196	100	100	–
A1-A	58	0	57	0	–
A1-B	14	615	14	43	3.1
A1-C	4.8	1600	5	40	8.2
G-F_{40}^{80}	60	170	100	100	–
G-A	37	0	62	0	–
G-B	3.6	800	7	31	4.7
G-C	1.4	2000	2	27	11.8

Fractions F_{40}^{80} correspond to the precipitating proteins in the range of 40–80% saturation with ammonium sulphate. Both samples were dialyzed against the equilibrium buffer before being applied onto the column. The other A, B, C fractions correspond to those obtained from the chromatography as indicated in fig.1a,b. For more details see section 2

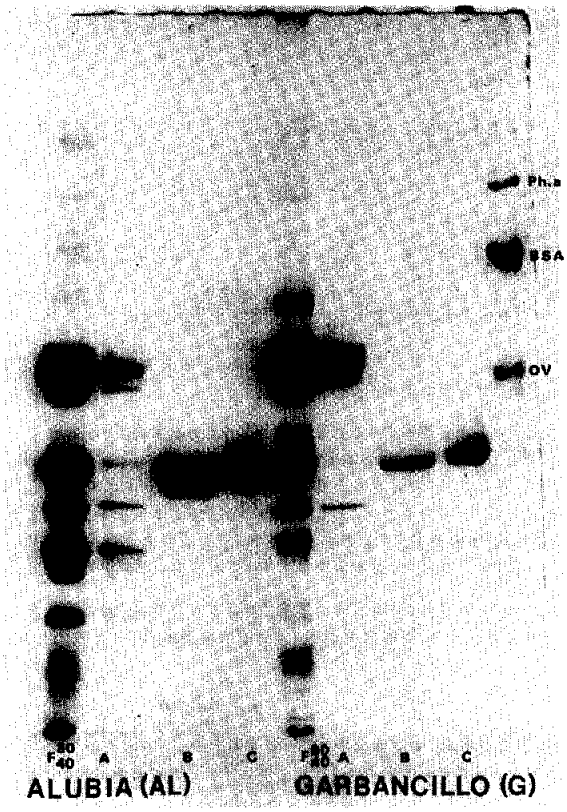

Fig.2. SDS gel electrophoresis of 'alubia' and 'garbancillo' lectins. Fractions A, B, C correspond to those obtained from chromatography on physically immobilized stroma. 10–50 μg protein sample were applied to a 10–20% polyacrylamide gradient gel prepared according to the technique in [15]. For more details see section 2 and fig.1a,b. Protein used as markers were (mol. wt); Ph.a (94 000); BSA (68 000); Ov. (43 000).

variation of temperature and polarity of the eluant indicate that the binding of lectin to stroma is compatible with accepted criteria of hydrophobic interaction [14]. A study is under way aimed at assessing the generality of hydrophobic interaction both in specific and non-specific hemagglutination by lectins.

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