Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 790 (2003) 365–372
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Preparative purification of soybean agglutinin by affinity chromatography and its immobilization for polysaccharide isolation

Laura Franco-Fraguas[a,*], Alicia Plá[a], Fernando Ferreira[b], Hugo Massaldi[b], Norma Suárez[b], Francisco Batista-Viera[a]

[a]*Cátedra de Bioquímica, Facultad de Química, CC 1157, Montevideo, Uruguay*
[b]*Laboratorio de Carbohidratos y Glicoconjugados, Departamento de Química Orgánica-Departamento de Desarrollo Biotecnológico, Facultades de Química y Medicina, Montevideo, Uruguay*

Abstract

Optimized procedures for the affinity purification of soybean agglutinin (SBA) from soybean flour, and its further immobilization, were developed. Lectin purification on galactosyl-Sepharose yielded 44.5±3.5 mg of pure SBA/50 g of flour. To prepare SBA adsorbents, the lectin was immobilized onto 1-cyano-4-(dimethylamino)pyridinium tetrafluoroborate (CDAP) activated Sepharose with high yields (77%). Feasibility of the use of this improved SBA adsorbent for affinity purification of *Streptococcus pneumoniae* capsular polysaccharides from strain 14 (CPS-14) at laboratory scale was demonstrated. Using SBA-Sepharose adsorbent (7.0 mg lectin per ml), amounts of 6.3 mg of pure CPS-14 per cycle were produced, the adsorbent being reused up to four times without loss of capacity.

Keywords: Preparative chromatography; Affinity adsorbents; *Streptococcus pneumoniae*; Soybean; Polysaccharides; Bicinchoninic acid; Cyan(dimethylamino)pyridinium tetrafluoroborate; Lectins; Proteins; Agglutinin

1. Introduction

Lectins constitute a remarkable group of proteins with the ability to interact reversibly with residues of specific sugars. Plant lectins have long been used as powerful tools in carbohydrate chemistry and at present, most of the typical plant lectin purification schemes include affinity adsorbents prepared by immobilization of simple specific sugars on to appropriate matrices [1]. Ligands such as mannose, galactose, glucose, etc., can easily be coupled onto agarose under alkaline pH using epichlorohydrin or divinylsulfone activation methods [2]. Besides, it is well known that the effectiveness and efficiency of an affinity adsorbent is strongly dependent on the ligand density which is, in turn, a function of the method and conditions chosen for the activation and immobilization procedure. In order to achieve satisfactory and reproducible results, it is very important to be able fully to characterize the prepared bioadsorbent, including the determination of its ligand density.

We are interested in finding a convenient method for the purification of *Streptococcus pneumoniae* capsular polysaccharides, due to their importance displayed in infectious diseases in our country. It is known that many pathogenic bacteria produce capsular polysaccharides (CPSs), which are essential for

*Corresponding author. Tel.: +598-2-924-1806; fax: +598-2-924-1906.
E-mail address: lfranco@fq.edu.uy (L. Franco-Fraguas).

1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(03)00086-2

virulence, and could also play an important role in the host immune response. The *Streptococcus pneumoniae* bacteria produces capsular polysaccharides which have been found to be important in infective processes such as pneumonia, meningitis, otitis media and other infectious diseases. More than 84 different serotypes have been identified, which differ in their capsular polysaccharides. In Uruguay the most predominant serotypes are 14, 5, 1, 6B, 3, 7F and 19A, in that order. A total of 80% of the resistant strains belong to serotype 14 [3,4]. Preparation of highly purified capsular polysaccharide from the most prevalent serotype is therefore of importance for immunological studies and vaccination. The *Streptococcus pneumoniae* capsular polysaccharide from strain 14 (CPS-14) is a regular high-molecular-mass polysaccharide composed by a tetrasaccharide repeating unit containing β-D-glucose, β-D-galactose and β-*N*-acetyl-D-glucosamine. Therefore, affinity adsorbents prepared with specific lectins could be valuable tools for the purification of the polysaccharide. Seeds from soya (*Glycine max*) beans contain several isolectins which exhibits very high affinity for *N*-acetylgalactosamine, its glycosides and oligosaccharides containing terminal *N*-acetylgalactosamine. They also react with galactose and its derivatives but with lower affinity [1]. In earlier work we demonstrated that a commercial soybean agglutinin (SBA)-agarose is a good adsorbent for the purification of CPS-14, through its D-galactose residues [5].

In order to reduce the cost of the affinity adsorbent and at the same time to improve its properties and performance, we herein describe an optimized protocol for the extraction and purification of SBA from soybean flour, and its further immobilization. We also report the application of this adsorbent to the isolation of CPS from the supernatant of a static culture of *Streptococcus pneumoniae* strain 14.

2. Experimental

Sepharose 4B and PD-10 columns (Sephadex G-25 pre-packed columns) were from Amersham Pharmacia Biotech (Uppsala, Sweden). The 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate (CDAP A reagent), epichlorohydrin (1-chloro-2,3-epoxypropane), α-D-galactose, soluble and immobilized soybean agglutinin were from Sigma (St. Louis, MO, USA). Triethylamine (TEA) was from Fluka (Buchs, Switzerland). All other reagents were of analytical grade.

2.1. Extraction of the lectin from soybean flour and its purification by affinity chromatography

2.1.1. Preparation of the soybean meal extract

This was done essentially according to Larsson et al. [6], slightly modified as follows: 50 g of soybean meal were defatted with *n*-heptane. The defatted meal (38 g) was extracted with 150 ml of 0.15 *M* NaCl with stirring for 2 h at room temperature and then centrifuged (2500 *g*, 1 h, +4 °C). The supernatant was centrifuged again (8500 *g*, 30 min, +4 °C). The precipitate was discarded, to the supernatant ammonium sulfate was added to 40% saturation and precipitation was allowed to take place for 4 h at +4 °C. The mixture was centrifuged as above (8500 *g*, 30 min, +4 °C). The precipitate was discarded and to the supernatant ammonium sulfate was added to 80% saturation, kept for 4 h at +4 °C and then centrifuged as above. The pellet was dissolved in 0.15 *M* NaCl solution and centrifuged (15 000 *g*, 30 min, +4 °C). The clear supernatant was gel filtered through PD-10 columns. The extract was ready to apply to the affinity column.

2.1.2. Preparation of the galactosyl-Sepharose affinity adsorbent

Sepharose 4B was washed with 200 ml distilled water on a glass filter and the residual water was removed by gentle suction. The drained gel (10 g) was suspended in 9 ml of 4 *M* sodium hydroxide in a 125 ml Erlenmeyer flask. A spatula tip of sodium borohydride and 5.5 ml of epichlorohydrin were added. The pH was confirmed to be 13–14 and the suspension was mixed for 90 min at room temperature. The gel was washed on a glass filter with 2 *M* sodium hydroxide and distilled water until neutral. The gel was divided into two portions: 4 g to be used for epoxy group analysis and the remaining 6 g of drained gel were washed with distilled water and then with 0.5 *M* sodium hydroxide. The drained gel was suspended in 6 ml of 10% galactose solution in 0.5 *M* sodium hydroxide. The suspension was gently

rotated end over end, overnight at room temperature. The gel was washed on the glass filter with 0.5 *M* sodium hydroxide and the remaining epoxy groups were blocked with β-mercaptoethanol (60 μl in 6 ml of 0.5 *M* sodium hydroxide) for 2 h at room temperature. The gel derivative was washed with water until neutral pH was attained, equilibrated with 0.15 *M* NaCl solution and kept at 4 °C until used.

2.1.3. Affinity chromatography for the isolation of SBA

A column was packed with galactosyl-Sepharose (5 ml of packed gel) and was equilibrated with 0.15 *M* NaCl. The extract of soybean meal prepared as described above was applied to the column (17 ml of clear extract) and re-circulated at least three times. The column was washed with 0.15 *M* NaCl and fractions of 5 ml were collected until the absorbance of the washings at 280 nm reached zero. Elution was performed with 0.1 *M* galactose solution in 0.15 *M* NaCl. The fractions containing hemagglutination (HAG) activity were pooled (10 ml) and this material was used for synthesis of SBA-Sepharose.

2.2. Synthesis of SBA-Sepharose and its use for the isolation of CPS-14

2.2.1. Activation of Sepharose with CDAP

Sepharose 4B (6.0 g of drained gel) was activated as described by Giacomini et al. [7]. The gel was drained and mixed with acetone:water (6:4, v/v) pre-cooled to 4 °C. CDAP (150 mg), dissolved in 4 ml of cold acetone:water (6:4, v/v), was added to the gel suspension under vigorous stirring for 3 min at 4 °C; then 720 μl of 200 m*M* TEA solution was added dropwise over 1–2 min. After 3 min the entire reaction mixture was quickly added to 100 ml of ice-cold 50 m*M* HCl and after 3 min it was washed with 100 ml of ice-cold water and drained under vacuum. The activated gel (CDAP-activated Sepharose) was equilibrated with 0.1 *M* $NaHCO_3$ pH 8.3 (coupling buffer) and immediately used for coupling purposes.

2.2.2. Immobilization (coupling step) of the purified SBA on CDAP-activated Sepharose

The CDAP-activated Sepharose (6.0 g of drained gel) equilibrated in coupling buffer was incubated with 6 ml of solution containing 85 mg of purified SBA and mixed end over end at room temperature for 8 h. The SBA derivatives were washed on a glass filter with coupling buffer, distilled water and finally with 0.1 *M* NaCl solution. The immobilization yield was calculated as the percentage of the amount of immobilized lectin (determined by direct method, see under Section 2.3.1) related to the total amount of added lectin. We also determined that 1.0 g of drained gel corresponds to 1.55 ml of packed gel.

2.2.3. Preparation of the S. pneumoniae extract

The *S. pneumoniae* strains were obtained from the National Centre for Streptococcus (Alberta, Canada) and the extract was prepared as described by Suárez et al. [5]. The lyophilized powder was suspended in 0.15 *M* NaCl (12.4 mg of lyophilized powder/ml of solution) and used for affinity chromatography on SBA-Sepharose.

2.2.4. Affinity chromatography for the isolation of CPS-14

Commercial and synthesized SBA-Sepharose derivatives were packed in mini-columns (2.0 and 1.0 ml of packed gel, respectively) and equilibrated with 0.15 *M* NaCl. The *S. pneumoniae* extract was directly applied to each mini-column and thereafter, 0.15 *M* NaCl was applied to the column until the absorbance at 280 nm of the washings was less than 0.05. Elution of CPS-14 was performed with 6 ml of 0.1 *M* galactose dissolved in 0.15 *M* NaCl. The eluted fractions were dialyzed overnight against distilled water, lyophilized and used for analytical determinations.

2.3. Analyses

2.3.1. Determination of CPS-14 with specific latex reagent

Rabbits were immunized with serotype 14 from aldehyde inactivated *S. pneumoniae* bacterial cells. Peripheral blood was collected and the immunoglobulin fraction was purified from the serum by salt precipitation with 30% saturated ammonium sulfate. The antisera were adsorbed onto latex reagent [5]. Agglutination of the samples was quantified by a twofold serial dilution assay. The titer was defined as

the reciprocal of the highest dilution able to produce visible agglutination.

2.3.2. Determination of soluble and immobilized proteins

The concentration of soluble proteins was determined by the bicinchoninic acid (BCA) method [8] by incubation at 60 °C for 15 min. A calibration curve using bovine serum albumin (BSA) as standard was established by incubating 100 μl aliquots of appropriate dilutions (concentrations between 0.0 and 0.3 mg ml^{-1}) with 2 ml of BCA working reagent for 15 min at 60 °C. The absorbance of the solutions was measured at 562 nm against the reagent blank. Immobilized protein on Sepharose was determined with the BCA reagent using the protocol described by Giacomini et al. [7].

2.3.3. Hemagglutination activity

The lectin activity was determined by measuring hemagglutination (HAG) according to Novak et al. [9] using rabbit red cells and estimated by the two-fold serial dilution assay. Titer was defined as the reciprocal of the highest dilution giving visible agglutination of the rabbit erythrocytes after 30 min of incubation. Specific hemagglutination activity (S.A.) was defined as the ratio between the titer/ml and the mg/ml. The purification factor (P.F.) was defined as the ratio between the S.A. after, and the S.A. before, affinity chromatography.

2.3.4. Determination of gel-bound epoxy groups

This determination was performed as described by Axén et al. [10]. The degree of activation was expressed as μmoles of epoxy groups per ml of packed gel.

2.3.5. Determination of gel-bound galactosyl groups

The amount of galactose immobilized on Sepharose was determined using the BCA reagent as follows. An amount of 0.10 g of drained Galactosyl-Sepharose was mixed with 2.0 ml of BCA working reagent and incubated for 30 min at 60 °C in a water bath provided with a shaker. The gel suspensions were cooled to room temperature and the absorbance of the supernatants was measured at 562 nm. Sepharose 4B was treated exactly as each of the samples containing galactose, and used as a blank. The absorbance of blanks and each of the derivative samples was determined at least in triplicate. A calibration curve using galactose as standard was established by incubating 100 μl aliquots of appropriate dilutions (concentrations between 0 and 10 m*M*) with 2 ml of BCA working reagent for 30 min at 60 °C. The standard curve was prepared by plotting the absorbance at 562 nm versus the amount of galactose per standard assay (in μmol).

2.3.6. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis

Fractions from the affinity chromatography of SBA on Galactosyl-Sepharose column were analyzed by SDS–PAGE, using a combination of PhastSystem equipment (Pharmacia, Uppsala, Sweden) with preformed PhastGel Gradient 8-25 gels according to the manufacturer's instructions. A low M_r electrophoresis calibration kit (Pharmacia) was used for the markers. Proteins were stained with Coomassie Brilliant Blue.

3. Results and discussion

3.1. Preparation of galactosyl-Sepharose adsorbent

In order to carry out the purification of SBA from a soybean extract by affinity chromatography, the galactosyl-Sepharose adsorbent was synthesized, via activation of the matrix with epichlorohydrin in alkaline media. This activation technique has been reported by different authors [11–14] and the protocols show only slight differences in the experimental conditions for the activation. Scoble and Scopes [15] have also discussed in detail the conditions for epichlorohydrin activation of Sepharose 4B. These authors report amounts up to 31 μmol ml^{-1} active groups, when using 0.2 ml of epichlorohydrin per gram of drained gel. Higher levels of reagent did not cause much increase in the activation, with only 34 μmol ml^{-1} active groups obtained from 0.4 ml of epichlorohydrin per gram of Sepharose. We have presently used 0.55 ml of epichlorohydrin per gram of drained Sepharose 4B and we have shortened the incubation time to 1.5 h at room temperature. Under these experimental conditions we were able to reach

levels of 44.6±1.3 μmol of oxirane groups per ml of packed gel, which is equivalent to 75% of the oxirane groups present in the gel after 19 h of incubation at room temperature (data not shown). The oxirane groups introduced into the matrix were determined by reaction with sodium thiosulfate [10]. The reaction between the oxirane groups and sodium thiosulfate proceeds with the release of hydroxide groups that can be used to determine the amount of active epoxy structures in the gel by titration with HCl. The galactose was then coupled to this highly epoxy-activated-Sepharose at alkaline pH by ether formation (Fig. 1), followed by blocking of remaining active oxirane groups with β-mercaptoethanol.

3.1.1. Direct determination of immobilized galactose with BCA reagent

The determination of the amount of immobilized galactose was performed using the BCA reagent with a standard curve prepared with soluble galactose. The BCA reagent has been mainly used to determine soluble proteins, but it has been also suggested to determine soluble reducing sugars [16]. Reducing groups in proteins and certain carbohydrates react with alkaline Cu^{2+} to produce Cu^{+}. Bicinchoninic acid, the key component in the BCA Protein Assay Reagent, forms alkali metals salts which are soluble in water; in this case it reacts with Cu^{+} to give an intense purple color which can be measured by absorbance at 562 nm. The Cu^{2+} and Cu^{+} ions as well as the BCA and the metal complex are free to move in and out of the gel particles. We have used the BCA reagent, as far as we know for the first time, to determine the number of galactose ligands in the agarose derivative. By using the calibration curve ($y = 0.5175x + 0.0166$, $R^2 = 0.9918$) we determined that the galactosyl-Sepharose derivative contained 5.2 μmol of galactose groups/ml of packed gel. Thus, only 12% of the total epoxy groups of the gel were used during the sugar coupling. This low percentage is a common feature observed by other authors [11,12]. The method used to determine the amount of gel-bound galactose needs free anomeric carbon, this may mean that this group was not involved in the immobilization of the galactosyl residue; most probably, the immobilization could take place via the primary hydroxyl group in position 6 of the galactose.

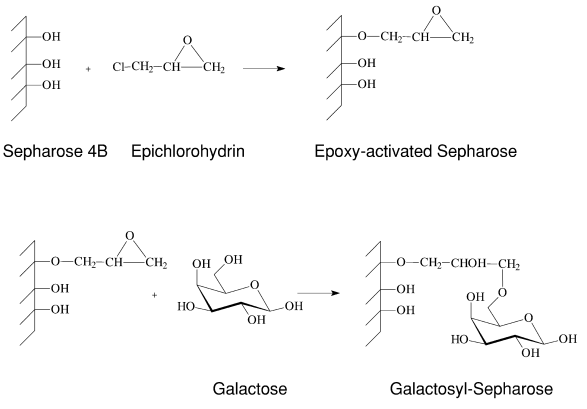

Fig. 1. Scheme for the synthesis of galactosyl-Sepharose.

3.2. Purification of SBA

The galactosyl-Sepharose adsorbent was used for the purification of the soybean lectin from soybean flour extract. Data of the affinity chromatography of the lectin are summarized in Table 1; a purification factor of 15 was achieved. The purity of the lectin was checked by SDS–PAGE (Fig. 2). The HAG activity of the lectin was estimated with the two-fold serial dilution and almost all the activity of the extract was recovered during the affinity chromatography procedure. The described protocol yielded 44.5±3.5 mg of pure SBA from 50 g of soybean flour. The affinity column was reused up to six times without loosing capacity. When the process was scaled up for 300 g of soybean flour it yielded 324 mg of pure lectin, using a column packed with 20.0 ml of galactosyl-Sepharose (5.8 μmol of galactose

Table 1
Purification of SBA from soybean meal extract by affinity chromatography on galactosyl-Sepharose

Fraction	Volume (ml)	Protein (mg/ml)	Lectin activity (titer/ml)	S.A.[a]	P.F.[b]
Extract	17	35.5	2560	72	–
Eluate	10	4.6	5120	1113	15

[a] S.A.: Specific hemagglutination activity (titer/ml:mg/ml).
[b] P.F.: Purification factor (S.A. after: S.A. before affinity chromatography).

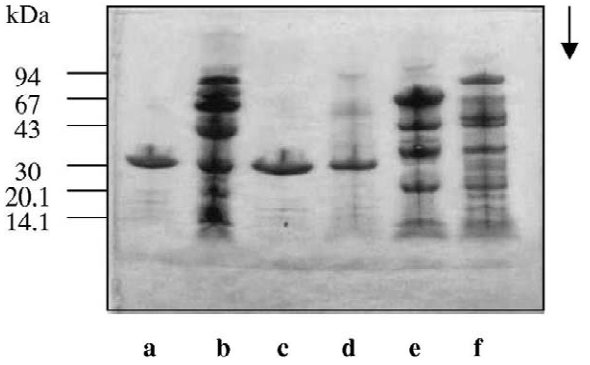

Fig. 2. SDS–PAGE of fractions from the affinity chromatography of soybean meal extract on galactosyl-Sepharose. The PhastGel 8-25 gradient gels were stained with Coomassie Brilliant Blue. Lanes: a and c = eluted lectin; b = low M_r markers; d = commercial soybean lectin; e = column flow-through; f = applied soybean extract. kDa = kilodaltons.

per ml of packed gel) prepared as described above. It has also to be mentioned that a content of between 4.0 and 6.0 μmol of galactosyl groups per ml of packed gel was found to be optimal for an efficient lectin purification. Amounts of immobilized galactose higher than 6.0 μmol ml^{-1} led to very strong lectin adsorption which in turn, made very difficult the elution of the lectin and decreased the recovery yields (data not shown). We also found that in order to prepare galactosyl-adsorbents containing between 4.0 and 6.0 μmol ml^{-1} of packed gel it was necessary to begin with at least 40 μmol of oxirane groups per ml of Sepharose (data not shown).

3.3. Immobilization of SBA

The immobilization of the SBA can be performed in several different ways, using different groups in the amino acid side chains involved in coupling it to the matrix. The most common groups involved in the immobilization of a protein to a solid support are in decreasing order: amino (lysine), sulfydryl (cysteine), hydroxyl (serine), and imidazole (histidine) groups [2]. Gel activation based on the CNBr method leads to the introduction of cyanate ester and imidocarbonate groups into the matrix; the bonds established between the protein ligand and the activated matrix are of isourea- and N-substituted imidocarbonate-types, respectively. N-substituted carbamates also occur when the ligand reacts with cyclic carbonates, which means that the immobilization of amino-containing ligands is a heterogeneous process. In spite of being one of the most widely used activation techniques, the CNBr method has the additional serious disadvantage of posing health risks for workers. Because of this and its high vapor pressure, all work with CNBr must be done in strictly controlled and ventilated environments.

Several cyano transfer complexes have been described as coupling reagents [17] and one of the most useful is CDAP (Fig. 3). This cyanylating agent is a slightly hygroscopic, non-volatile solid, that can be stored at room temperature for extended periods and, most importantly, can be easily handled in laboratories without undue health risks. Furthermore, the reagent introduces cyanate ester structures into the matrix and, as a result, immobilization is a more homogeneous process since virtually all the linkages are of the isourea type (Fig. 3). In a previous work we have optimized a protocol to prepare CDAP-activated Sepharose [18]. We have used here this optimized protocol and we performed the coupling of SBA by its amino groups. The immobilization yield was 77% and the adsorbent contained 7.0 mg of lectin per ml of packed gel. Using the described

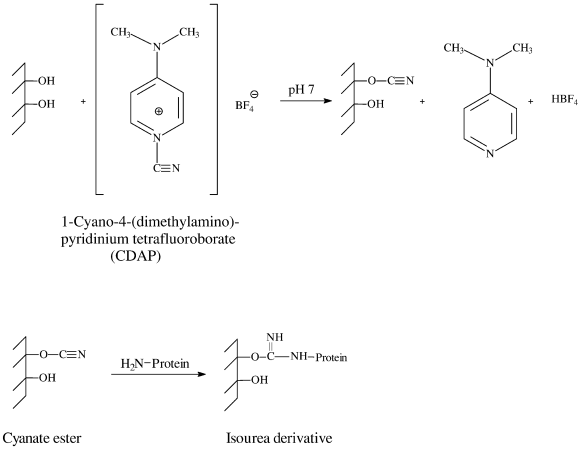

Fig. 3. Scheme for the synthesis of SBA-Sepharose.

protocol we were able to produce derivatives that contain three-times higher amounts of immobilized lectin than the commercial gel.

3.4. Purification of the CPS-14

Finally, the performance of the synthesized SBA-Sepharose adsorbent was compared with that of a commercial one, for the isolation of CPS-14 by affinity chromatography. The presence of CPS-14 in the samples was detected as previously reported [5] using specific latex reagent, which was calibrated using a pure sample of CPS-14. The detection limit for the highest dilution able to produce visible agglutination was found to be 60 ng ml^{-1}. This value was used to estimate the amount of CPS-14 in the *S. pneumoniae* extract (3.9 mg of CPS-14 in 4 ml of extract prepared as described under Section 2.2.3.). Results are indicated in Table 2. Two SBA-Sepharose derivatives containing different ligand densities (4.9 and 7.0 mg of lectin per ml of packed gel) were synthesized and their performances compared with that of a commercial SBA-Agarose derivative (2.0 mg of lectin per ml of packed gel). The recovery of pure CPS-14 per cycle were 3.4, 6.3 and 1.6 mg of polysaccharide per ml of packed adsorbent, respectively. The capacity of the adsorbents, expressed in terms of mg of CPS-14 per mg of immobilized lectin, were similar: 0.8, 0.7 and 0.9, respectively, for the commercial and the two synthesized adsorbents. The recovery yields when working until saturation were 79.5 and 81.0% for the commercial and the synthesized adsorbent with the highest lectin content (7 mg lectin/ml packed gel), respectively. This derivative was reused up to four cycles without decrease in its capacity.

4. Conclusions

A simple, easy and cheap protocol for the extraction and purification of the soybean lectin from soybean flour was achieved. The purified lectin was successfully immobilized on to Sepharose and used to purify *S. pneumoniae* strain 14 polysaccharide. The synthesized adsorbent had improved properties with respect to the commercial gel. The yield of pure polysaccharide obtained was doubled, by increasing three times the amount of lectin immobilized onto the adsorbent. The preparation of soybean lectin affinity adsorbent and the possibility of its reuse resulted in overall economy of the process, and improves the feasibility of the purification of CPS-14 at the laboratory bench scale.

Acknowledgements

We are grateful for financial support from the International Foundation for Science (IFS, project F-2834-2), the Program for Development of Basic Sciences (PEDECIBA) Uruguay and the International Program in the Chemical Sciences (IPICS, project URU:02), Sweden. We also thank Dr Valerie Dee for linguistic revision.

Table 2
Isolation of CPS-14 using commercial (A) and synthesized (B) SBA-adsorbents

Affinity adsorbent	Immobilized lectin (mg/ml)	Bed volume (ml)	Extract[a] (ml)	CPS-14[b] (mg)	Capacity[c]
SBA-Agarose (A)	2.0	2.0	4.0	3.1	0.8
SBA-Sepharose (B)	4.9	1.0	4.0	3.4	0.7
	7.0	1.0	8.0	6.3	0.9

[a] Volume of *S. pneumoniae* extract (prepared as described under Section 2.2.3) applied to the column.
[b] Amount of CPS-14, as mg of material eluted from each column, dialyzed and lyophilized.
[c] Binding capacity of the adsorbents, expressed in terms of mg of pure CPS-14 per mg of immobilized lectin.

References

[1] E.J.M. Van Damme, W.J. Peumans, A. Pusztai, S. Bardocz, in: Handbook of Plant Lectins: Properties and Biomedical Applications, Wiley and Sons Inc, New York, 1998, p. 7.
[2] J. Carlsson, J.-C. Janson, M. Sparrman, in: J.-C. Janson, L. Rydén (Eds.), Protein Purification, Principles, High Resolution and Applications, VCH, New York, 1998, p. 375.
[3] M. Hortal, G. Algorta, I. Bianchi, G. Borthagaray, I. Cestau, T. Camou, M. Castro, M. De Los Santos, R. Diez, L. Dell' Acqua, A. Galiana, A. Giordano, P. Giordano, G. Lopez-Ghemi, N. Milanese, C. Mogdasy, R. Palacio, W. Pedreira, A. Pisano, L. Pivel, Microb. Drug Resist. 3 (1997) 159.
[4] M.C. Pírez, O. Martínez, A.M. Ferrari, A. Nairac, A. Montano, I. Rubio, M.J. Saráchaga, S. Brea, T. Picón, M.C. Pinchack, P. Torello, G. Algorta, M.C. Mogdasy, Pediatr. Infect. Dis. J. 20 (2001) 283.
[5] N. Suárez, L. Franco Fraguas, E. Texeira, H. Massaldi, F. Batista-Viera, F. Ferreira, Appl. Environ. Microbiol. 67 (2001) 969.
[6] E.L. Larsson, B. Mattiasson, J. Biotechnol. 49 (1996) 189.
[7] C. Giacomini, A. Villarino, L. Franco Fraguas, F. Batista, J. Mol. Catal. B Enzym. 4 (1998) 313.
[8] P.K. Smith, R.I. Khron, G.F. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klent, Anal. Biochem. 150 (1985) 76.
[9] T.P. Nowak, P.L. Haywood, S.H. Barondes, Biochem. Biophys. Res. Commun. 68 (1976) 650.
[10] R. Axén, H. Drevin, J. Carlsson, Acta Chem. Scand. B 29 (1975) 471.
[11] J. Porath, N. Fornstedt, J. Chromatogr. 51 (1970) 479.
[12] L. Sundberg, J. Porath, J. Chromatogr. 90 (1974) 87.
[13] J. Porath, S. Oscarsson, Makromol.Chem. Macromol. Symp. 17 (1988) 359.
[14] A. Schwarz, F. Kohen, M. Wilchek, J. Chromatogr. B 664 (1995) 83.
[15] J. Scoble, R. Scopes, J. Chromatogr. A 752 (1996) 67.
[16] S. Waffenschmidt, L. Jaenicke, Anal. Biochem. 165 (1987) 337.
[17] J. Kohn, M. Wilchek, Appl. Biochem. Biotechnol. 9 (1984) 285.
[18] L. Franco Fraguas, J. Carlsson, F. Batista-Viera, Int. J. Bio-Chromatogr. 5 (2000) 255.