APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2001, p. 969–971 Vol. 67, No. 2
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.2.969–971.2001


# Production of Capsular Polysaccharide of *Streptococcus pneumoniae* Type 14 and Its Purification by Affinity Chromatography

NORMA SUÁREZ,[1] LAURA FRANCO FRAGUAS,[2] ESTHER TEXEIRA,[3] HUGO MASSALDI,[3] FRANCISCO BATISTA-VIERA,[2] AND FERNANDO FERREIRA[1]*

*Laboratorio de Carbohidratos y Glicoconjugados, Departamento de Quimica Orgánica, Facultad de Química,[1] and Departamento de Desarrollo y Producción, Facultad de Medicina,[3] Instituto de Higiene, C.P. 11600, and Facultad de Química, Cátedra de Bioquímica, CC 1157,[2] Montevideo, Uruguay*

Received 23 December 1999/Accepted 20 October 2000

**We describe a rapid and efficient method for producing the capsular polysaccharide of *Streptococcus pneumoniae* by fermentation on tryptic soy broth and purification of this compound by using immobilized soybean lectin as an affinity adsorbent. In principle, the same strategy can be used to produce purified capsular polysaccharides from other streptococcal serotypes by selecting the appropriate lectin adsorbents.**

*Streptococcus pneumoniae* produces capsular polysaccharides that have been found to be important in infective processes, such as pneumonia, meningitis, otitis, and several other infectious diseases that cause considerable morbidity and mortality in children in most countries of the world (11). These polysaccharides are type specific and contain the main antigenic determinant of *S. pneumoniae*. The bacterium presents a multiplicity of serotypes, and serotype 14 is the predominant serotype in South American countries (2).

Chemical studies have shown that there is a relationship between capsular structure and immunological specificity, and both aspects of the serotype 14 capsular polysaccharide (CPS-14) have been extensively studied (3, 4). This polysaccharide is composed of a tetrasaccharide repeating unit containing D-glucose, *N*-acetyl-D-glucosamine, and D-galactose (6), as shown in Fig. 1. It is one of the few neutral capsular polysaccharides produced by pneumococcus strains, and several patents (1a, 3a) have been issued for its preparation and purification. Our purification procedure is based on the use of immobilized lectins. Soybean (*Glycine max*) lectin, which is specific for D-galactose and *N*-acetyl-D-galactosamine, can recognize the galactosyl residues present in the repeating unit of CPS-14 (8). Purification of CPS-14 by affinity chromatography as described here consists of a one-step procedure in which a commercial immobilized soybean lectin is used.

**Production and purification of CPS-14.** CPS-14 was prepared from *S. pneumoniae* strains obtained from The National Centre for Streptococcus (Alberta, Canada) as described by Lund and Henrichsen (7) in 5-liter flasks containing 2 liters of tryptic soy broth (Difco Laboratories, Detroit, Mich.) and incubated at 37°C for 18 to 24 h. Growth was stopped by adding formaldehyde to a final concentration of 0.2% (wt/vol); the cells were lysed with sodium deoxycholate (0.1%, wt/vol) and then centrifuged. The supernatant was concentrated by ultrafiltration (using a hollow fiber cartridge with a molecular mass cutoff of 10 kDa) to 1/10 the original volume, and it was extensively dialyzed against distilled water and lyophilized.

In a typical experiment, 165 mg of lyophilized powder was suspended in 5 ml of 50 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl (PBS buffer). The suspension was centrifuged at 4°C for 15 min at 4,000 × *g*. A 4-ml portion of the clear supernatant was applied to a column packed with 2 ml of commercial soybean lectin-agarose (Sigma Chemical Co., St. Louis, Mo.). According to the manufacturer, the ligand content was approximately 2 mg of lectin per ml of packed gel. Nonbound material was recycled three times, and the column was then washed with 16 ml of PBS buffer. Elution was performed with 0.1 M D-galactose in PBS buffer. Two 4-ml fractions were collected, and each fraction was dialyzed against distilled water or gel filtered by using prepacked PD-10 columns (Pharmacia Amersham, Uppsala, Sweden) with distilled water and finally lyophilized. For a small laboratory bench scale up of the procedure, a column was packed with 9.0 ml of soybean lectin-agarose and the protocol described above was used with appropriately scaled volumes.

**Specific latex reagent.** Rabbits were immunized with serotype 14 aldehyde-inactivated *S. pneumoniae* bacterial cells by using the immunization protocol of Lund and Henrichsen (7). Peripheral blood was collected, and the immunoglobulin fraction was purified from the serum by salt precipitation with 30% saturated ammonium sulfate. To avoid cross-reactions with the C polysaccharide produced by all serotypes, anti-C antibodies were eliminated by adsorption with a suspension of inactivated cells of a noncapsulated rugose C-mutant *S. pneumoniae* strain. The antisera were adsorbed onto latex reagent by the method of Battistoni (1). 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.

**Protein content.** The protein content was determined both spectrophotometrically by using the relationship between ab-

* Corresponding author. Mailing address: Laboratorio de Carbohidratos y Glicoconjugados, Departamento de Desarrollo y Producción, Instituto de Higiene, C.P. 11600, Av. A. Navarro 3051, Montevideo, Uruguay. Phone: (598) 2 4871288. Fax: (598) 2 4873073. E-mail: fferreir@bilbo.edu.uy.

→4)-β-D-Glc*p*-(1→6)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→
4
↑
1
β-D-Gal*p*

FIG. 1. Structure of the tetrasaccharide repeating unit of *S. pneumoniae* type 14 capsular polysaccharide (CPS-16) (6).

sorbance at 280 nm and absorbance at 260 nm (5) and by the bicinchoninic acid (Pierce, Rockford, Ill.) method (10).

**$^1$H-NMR analysis.** The $^1$H nuclear magnetic resonance ($^1$H-NMR) spectrum of a sample dissolved in $D_2O$ was determined with a Brucker DPX-400 spectrometer by using standard pulse sequences. Sodium 3-trimethylsilylpropionate-$d_4$ ($\delta_H$ 0.00) was used as an internal reference.

**Sugar analysis.** Purified polysaccharide (0.5 mg) was hydrolyzed with 2 M trifluoroacetic acid at 120°C for 1 h. The resulting monosaccharides were analyzed as their alditol acetates by gas-liquid chromatography on an SE-54 fused-silica capillary column by using the following temperature program: 140°C for 3 min, followed by an increase to 240°C at a rate of 3°C min$^{-1}$. Gas chromatography-mass spectrometry was performed by using the column and conditions mentioned above with a Shimadzu QP-5050 gas chromatograph-mass spectrometer.

TABLE 1. Affinity chromatography of CPS-14 on soybean lectin-agarose[a]

| Fraction | Vol (ml) | $A_{280}$ (μ/ml) | $A_{260}$ (μ/ml) | Latex titer[b] | Dry wt (mg)[c] |
|---|---|---|---|---|---|
| Applied material | 4.0 | 26.50 | 33.00 | 16,384 | 132 |
| Column breakthrough | 4.0 | 10.25 | 13.00 | 512 | |
| First fraction eluted | 4.0 | 0.04 | 0.08 | 16,384 | 3.1 |

[a] The data are averages based on at least three independent experiments.
[b] Determined by agglutination by using the two-fold dilution system (see text).
[c] Sample weight after lyophilization.

**Production and affinity purification of CPS-14.** The latex reagent was calibrated with a pure sample of *S. pneumoniae* CPS-14, and the detection limit was found to be 60 ng/ml. This value was used to estimate the amounts of CPS-14 in the culture medium and the culture supernatant after cell lysis, and the data indicated that almost all of the CPS-14 was released into the medium.

Table 1 summarizes the data regarding affinity purification of CPS-14 from culture medium supernatant. Although the estimated amount of CPS-14 in the starting material was very small (3.9 mg), our one-step procedure resulted in a polysaccharide preparation that was virtually free of protein (Table 1). The soluble protein content represented 50% of the starting dry weight. In the purified polysaccharide, the protein content

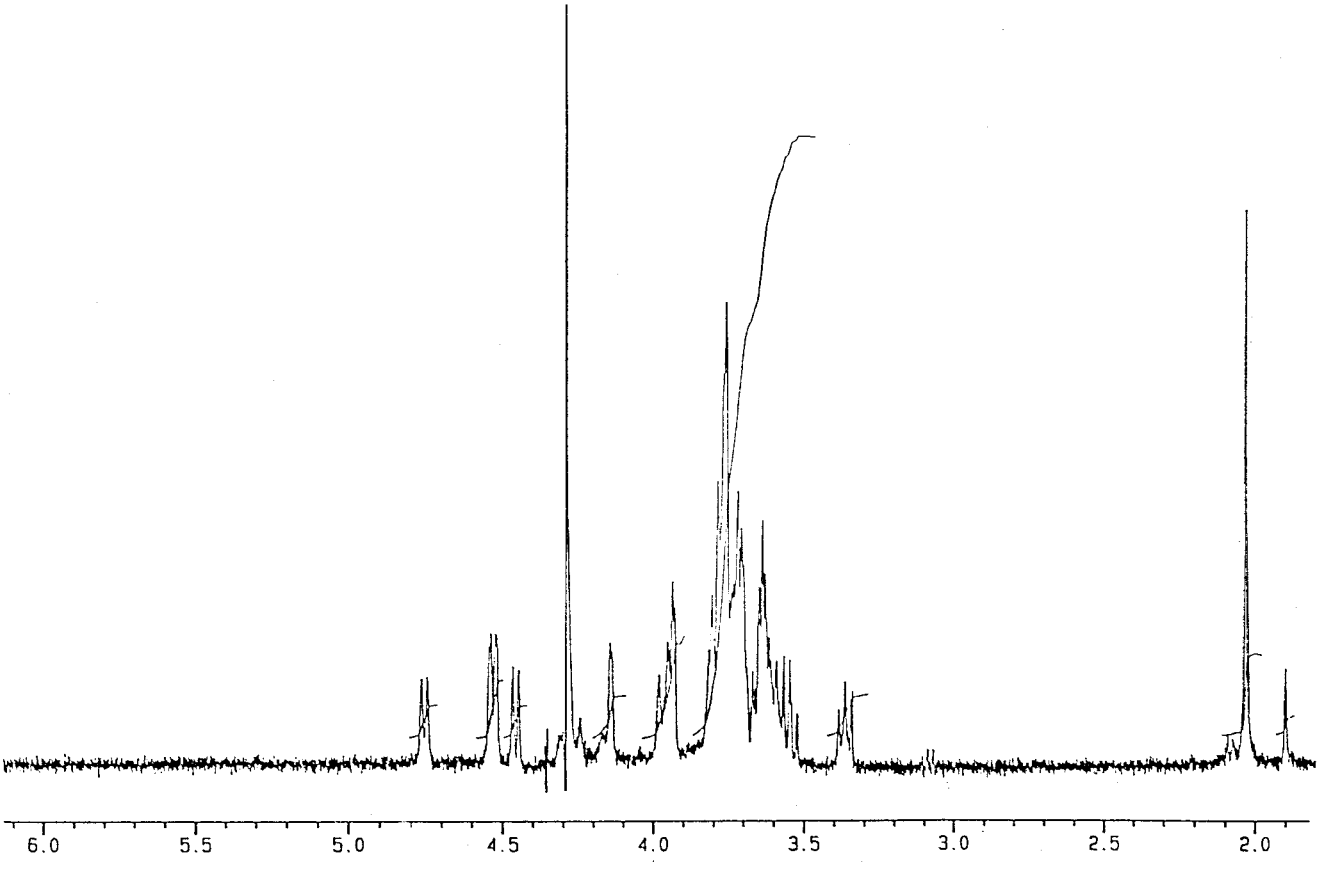


FIG. 2. $^1$H-NMR spectrum of CPS-14 obtained by affinity chromatography.

was 0.15% (wt/wt) (spectrophotometric method) or zero (bicinchoninic acid method). The results for the eluted material in Table 1 are the results for the first elution fraction. The polysaccharide content of the second elution fraction was very low, as indicated by the latex technique (titer, 64). Furthermore, the protein content was 4.5%, and so the second elution fraction was discarded. The maximum capacity of the column was found to be approximately 1.5 mg of polysaccharide per ml of soybean lectin-agarose-packed gel (Table 1).

The process was scaled up to a 9.0-ml packed gel column. In this case the capacity of the column was 1.2 mg of polysaccharide per ml of packed gel, and after three cycles the capacity was 1.1 mg of polysaccharide per ml of packed gel.

**Identity and quality of the product.** The polysaccharide obtained reacted positively with CPS-14-specific latex reagent, giving a high titer, as indicated in Table 1. Sugar analysis of the product revealed the presence of D-glucose, *N*-acetyl-D-glucosamine, and D-galactose at relative proportions of 1:1:2, which corresponded to the reported composition of the polysaccharide (6). The $^1$H-NMR spectra (Fig. 2) showed the tetrasaccharide repetitive unit having all of the monosaccharides in the anomeric β-configuration and the presence of an *N*-acetyl group, as expected. No leakage of lectin from the column was observed, as indicated by negative hemagglutination (9) performed with the eluted material.

The one-step purification procedure described here is easy to perform and faster than traditional multistep methods that include fractional precipitation, protease and DNase treatments, and/or ion-exchange chromatography, among other procedures (1a, 3a). In contrast, a good yield of high-purity CPS-14 is produced by our one-step method, and our procedure may be easily scaled up.

We are indebted to J. Battistoni for his valuable advice.
We are grateful for financial support from the Program for Development of Basic Sciences, the Pan American Health Organisation, the International Program in the Chemical Sciences (project URU:02), the International Foundation for Science (project F-2834-1), and the Organisation for the Prohibition of Chemical Weapons.

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