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Composition of the coagulant polysaccharide fraction from *Strychnos potatorum* seeds

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Abstract

The composition of the coagulant polysaccharide fraction from *Strychnos potatorum* seeds is described. This fraction comprises a 1:1.7 mixture of a galactomannan and a galactan. The structure of these polysaccharides is also discussed. In addition, the coagulant properties of the polysaccharide fractions of two other *Strychnos* species, *innocua* and *nux-vomica*, have been assayed.

Keywords: *Strychnos potatorum*; Polysaccharides; Galactomannan; Galactan; Coagulant properties

1. Introduction

The seeds of *Strychnos potatorum* L. (Loganiaceae) are used as a coagulant in water treatment in Third World countries. Previous investigations [1] of the polysaccharide content of the seeds described the structure of a mannogalactan, which is made up of a backbone of β-(1 → 4)-linked D-galactopyranose residues that bear side chains with mannopyranosyl nonreducing ends. No coagulant activity of this polysaccharide was reported. We have now extracted from *Strychnos*

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potatorum the fraction responsible for the coagulant activity, namely a 1:1.7 mixture of two polysaccharides, a galactomannan and a galactan.

2. Experimental

Extraction and purification of the polysaccharide fraction from Strychnos potatorum.—Seed powder (1 g) obtained by filing the seed kernel was added to 50 mL of distilled water, stirred for 15 min, and centrifuged at 10000 rpm for 10 min. The supernatant solution was filtered through Whatman GFC glass fiber discs (0.45 μm) followed by Whatman cellulose acetate filters. The filtrate was added to an equal volume of a 1:1 mixture of 10 mM pH 7.5 Tris-saturated phenol and $CHCl_3$, and the emulsion was stirred at room temperature for 30 min followed by centrifugation at 10000 rpm for 10 min. The aqueous layer was removed, shaken with an equal volume of $CHCl_3$, centrifuged as above, separated from the organic phase, and mixed with 2.5 volumes of EtOH. The polysaccharide fraction was precipitated overnight at −20°C, washed in EtOH, and dried in a vacuum desiccator.

The sample (100 mg), denoted PS, was chromatographed on Bio-Gel P-100 (Bio-Rad), using 50 mM (pH 5.2) NaOAc as eluent. The chromatographic profile, revealed by the phenol test [2], showed a broad peak with two maxima (Fig. 1). The peak fractions were pooled as fractions A and B; $[\alpha]_D + 36°$ (c 0.1, H_2O) was measured for both fractions. Fractions A and B were separately rechromatographed on the above column. In both cases, a uniform peak, eluted with similar volumes of eluent, was obtained. Analysis of the acid hydrolysate of representative fractions of either peak revealed, in all cases, a galactose–mannose ratio in the range 2.5–2.7:1. In addition, coagulant activity was measured for fractions A and B (see below and Table 1).

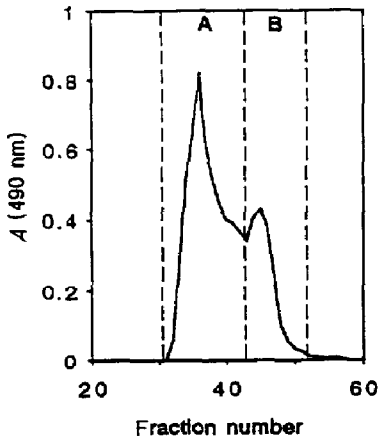


Fig. 1. Bio-Gel P-100 gel-filtration chromatography of fraction PS (column, 1.0×48 cm; flow rate, 3.6 mL/h; fraction volume, 0.5 mL).

Table 1
Coagulant test evaluated on a suspension of kaolin clay in distilled water, at room temperature [a]

Sample	Dose (mg/L)	Residual turbidity (NTU)	Comments
	Control	230	
Fraction A	0.25	47	Floc size ca. 1 mm
	0.50	38	diameter, slow growing.
	1.00	34	Final floc, fast settling.
	2.00	37	
	Control	230	
Fraction B	0.25	65	Floc size ca. 1 mm
	0.50	35	diameter, faster growing
	1.00	35	than sample A. Final floc,
	2.00	29	fast settling.
Strychnos potatorum	0.50	95	Rapid floc formation, floc
polysaccharide fraction	1.00	54	size ca. 1–1.5 mm.
	2.00	58	
	3.00	67	
Strychnos nux-vomica	0.50	67	Rapid floc formation, floc
polysaccharide fraction	1.00	35	size ca. 1.5–2 mm.
	2.00	32	
	3.00	35	
	5.00	48	
Strychnos innocua.	1.00	166	Rapid floc formation, floc
polysaccharide fraction	2.00	42	size 1–1.5 mm.
	3.00	35	
	4.00	34	
	5.00	34	

[a] Initial turbidity, 300 NTU. Samples prepared as 1% solutions.

Extraction of the polysaccharide fraction from Strychnos nux-vomica *and* innocua.—In both cases, seed power (1 g) was treated as above, yielding 100 mg of a polysaccharide fraction. The activity tests were performed on this material without any chromatographic separation.

General.—The ^{1}H and ^{13}C NMR spectra were obtained at 400 and 100 MHz, respectively, with a Bruker AM 400 spectrometer, equipped with a dual probe at 70°C, in the FT mode. The DEPT experiment was performed using a polarisation transfer pulse of 135° and a delay adjusted to an average C,H coupling of 160 Hz. The ^{13}C and ^{1}H chemical shifts were measured using 1,4-dioxane (δ 67.4 from Me_4Si) and sodium 3-(trimethylsilyl)propionate-d_4 as internal standard, respectively. Optical rotations were determined on a Perkin–Elmer 141 polarimeter. HPLC was performed with a Varian 5060 instrument equipped with a UV 100 spectrophotometer as detector. TLC was carried out on Silica Gel F_{254} (Merck). GLC was performed with a Carlo Erba 4160 instrument equipped with a flame-ionisation detector, and GLC–MS with a Hewlett–Packard 5890 instrument.

Samples of the polysaccharide were hydrolysed [3] with 2 M trifluoroacetic acid at 120°C for different times (1, 2, and 4 h). Neutral sugars in the hydrolysates were

analysed as the derived alditol acetates by GLC on an SP 2330 capillary column (Supelco, 30 m × 0.25 mm i.d.) at 235°C, using N_2 as the carrier gas. The molar ratios of the sugars were evaluated by using *myo*-inositol as internal standard and the appropriate response factors.

Samples of the polysaccharide were methylated by a modified Hakomori procedure [4,5], and the methylation products were dialysed, freeze-dried, and hydrolysed with acid. The methylated products in the hydrolysates were reduced with $NaBD_4$, acetylated, and analysed by GLC–MS on an SP-2330 capillary column (Supelco, 30 m × 0.25 mm i.d.; flow rate 0.8 mL/min; He as the carrier gas), with the temperature programme: 80°C for 2 min, 80 → 170°C at 30°C/min, 170 → 240°C at 4°C/min, 240°C for 15 min [6]. GLC of the methylated alditol acetates was carried out on a column identical with that used for GLC–MS, with a flow rate of 1 mL/min, using effective carbon response factors [7], and normalising the peak areas with respect to that of *myo*-inositol hexaacetate used as the internal standard.

Separation of galactomannan from galactan with Fehling's solution.—The PS fraction (100 mg) was dissolved in 8 mL of water and Fehling's solution [8] was added until precipitation of the "copper complex" was just complete. The precipitate was collected by centrifugation, washed with H_2O, and decomposed by maceration for 1 min at 0°C with EtOH containing 5% (v/v) of concd HCl. The residue was washed with EtOH until the washings gave a negative test for chloride. A final washing with acetone, that appeared colourless, showed the absence of Cu(II) chloride in the residue, which, by ^1H NMR spectroscopy, appeared to be still a mixture of galactan and galactomannan. The above procedure was repeated eleven times and the increase of the galactomannan–galactan ratio of the residue was checked each time.

At the end, a galactomannan (20 mg) was obtained which, by integration of anomeric signals in the ^1H NMR spectrum, contained ca. 8% of galactan. An apparent molecular weight of 80 000 was estimated by gel-filtration; $[\alpha]_D$ + 23° (*c* 0.1, H_2O).

The supernatant liquors of the eleven treatments with Fehling's solution were collected, neutralised with 2 M HCl, and dialysed against tap water for 24 h. The dialysate was freeze-dried to give a galactan (38 mg) that, by integration of the anomeric signals in the ^1H NMR spectrum, appeared to contain 9% of a galactomannan, as confirmed by GLC analysis of alditol acetates. An apparent molecular weight of 80 000 was estimated by gel-filtration; $[\alpha]_D$ + 45° (*c* 0.1, H_2O).

Determination of the absolute configuration of the monosaccharides.—The mixture of mannose and galactose from the total acid hydrolysis of the galactomannan was chromatographed on an HPLC column (Lichrospher 100 NH_2, 10 μm; 3:1 CH_3CN–H_2O; λ 192 nm). The configuration of each monosaccharide, identified by HPLC, was established by polarimetry. The configuration of the galactose from the galactan was established in the same way.

Partial acid hydrolysis of galactan.—The galactan (25 mg) was treated with 0.1 M CF_3CO_2H (0.2 mL) at 100°C for 1 h. The crude material was dialysed against water, using a cellulose tube with a cut-off of 12 000–14 000 Da (Spectrapor 4).

Freeze-drying of the dialysate yielded a residue (17 mg), which was chromatographed on Bio-Gel P 2 (Bio-Rad), using Millipore water as eluent. The collected fractions were freeze-dried and pooled on the basis of TLC analysis on silica gel (4:1 2-PrOH–H_2O). In addition to a galactose fraction (2 mg), four further fractions were collected: **a** (4 mg), **b** (2 mg), **c** (2 mg), and **d** (4 mg), in increasing order of polarity.

Fraction **a**, on the basis of ^1H and ^{13}C NMR spectra [9], comprised β-D-galactopyranosyl-(1 → 4)-D-galactopyranose. As such, the ^1H NMR spectrum of the alditol obtained by reduction with $NaBH_4$ showed only one anomeric signal at δ 4.52 (d, 7.8 Hz), assignable to the β-galactopyranosyl unit. Moreover, methylation analysis confirmed the presence of a 4-sustituted galactopyranose.

Fractions **b** and **c** showed the ^1H NMR spectra of complex mixtures and they were therefore both reduced with $NaBH_4$. As we were unable to separate the alditol mixtures, they were acetylated with pyridine–Ac_2O at room temperature overnight and purified by TLC. (silica gel; 7:3 EtOAc–$CHCl_3$). Pure fractions were hydrolysed with 0.1 M NaOMe–MeOH at room temperature for 2 h. The ^1H NMR spectrum of the product from fraction **b** showed two doublets (1:1 ratio), both with a coupling constant of 7.8 Hz, at δ 4.53 and δ 4.58, while fraction **c** gave a product whose ^1H NMR spectrum showed three doublets (1:1:1 ratios), each with a coupling constant of 7.8 Hz, at δ 4.54, 4.60, and 4.62. As the methylation analysis of both these products showed the presence of 4-substituted galactopyranose, it could be concluded that fractions **b** and **c** contained the trisaccharide and the tetrasaccharide, respectively, made up of (1 → 4)-linked β-D-galactopyranose residues.

TLC (silica gel; 75:25:10 2-PrOH–H_2O–CH_3OH) of fraction **d** afforded a pure fraction, whose ^1H and ^{13}C NMR spectra displayed signals assignable to a structure containing only (1 → 4)-linked β-D-galactopyranosyl units. Accordingly, the ^1H NMR spectrum of the corresponding alditol showed four doublets, each with a 7.8 Hz coupling constant, at δ 4.54, 4.60, 4.62, and 4.63. The GLC analysis of acid hydrolysates of this fraction confirmed the presence of only galactose, confirming that fraction **d** contained a pentasaccharide built up of (1 → 4)-linked β-D-galactopyranose residues.

Partial acid hydrolysis of galactomannan.—The galactomannan (10 mg) was hydrolysed as described for the hydrolysis of the galactan, and the dialysate was chromatographed on Bio-Gel P 2 (Bio-Rad) using deionised H_2O as the eluent. The collected fractions were freeze-dried and pooled on the basis of TLC analysis (silica gel; 4:1 2-PrOH–H_2O). In addition to a fraction (3 mg) made up of a mixture of galactose and mannose, a disaccharide fraction was isolated, which was confirmed as β-D-mannopyranosyl-(1 → 4)-D-mannopyranose, on the basis of its ^1H and ^{13}C NMR spectra [10] and methylation analysis.

Determination of the coagulant activity.—This was evaluated using an artificial turbid water: Kaolin clay in distilled water. An ionic background was provided by adding $NaHCO_3$ to a final alkalinity of 250 mg/mL $CaCO_3$ as determined by the Palintest total alkalinity method (Wilkinson and Simpson Ltd., Palintest House, Team Valley, Gateshead, Tyne and Wear, England). Initial turbidity of raw water:

300 NTU. Test conditions: rapid mix 300 rpm, 2 min; slow mix 40 rpm, 20 min; settling time, 30 min. The data are reported in Table 1.

3. Results and discussion

The results from the purification of the polysaccharide sample PS (see Experimental) indicated that fractions A and B were likely to be different M_w fractions of the same polysaccharide, made up of galactose and mannose in a 2.6:1 ratio.

The ^{13}C NMR spectrum of PS showed six strong and sharp signals (δ 105.2, 78.5, 75.3, 74.1, 72.6, and 61.6) whose chemical shifts were in agreement with a $\rightarrow$ 4)-β-D-Gal$p$-(1 $\rightarrow$ structure [9]; in addition to weaker and broader signals in the anomeric region, at δ 101.1, 100.9, and 99.8. A DEPT experiment showed a downfield-shifted methylene group at 67.5 ppm, suggesting a glycosidic linkage at a C-6 oxymethylene group.

The ^1H NMR spectrum displayed, in the anomeric region, a doublet at δ 5.02 (3.5 Hz), a broad singlet at δ 4.76, and a very strong doublet (7.8 Hz) at δ 4.62 in 1:2.5:6 ratios. These signals were correlated, by a one-bond 2D H,C NMR heterocorrelated experiment, to ^{13}C signals at 99.8, 101.1–100.9, and 105.2 ppm, respectively. The $^1J_{C,H}$ values of these signals, measured by a DEPT coupled experiment, were 173.6 Hz, for the signal at δ 99.8, and 163.3 Hz, for the signal at δ 105.2, showing, in an unambiguous way, the α configuration for one galactopyranosyl unit and the β configuration for the other galactopyranosyl unit [11], in agreement with the $^3J_{H,H}$ values of the corresponding anomeric protons. For the very broad signals at δ 101.1–100.9, a $^1J_{C,H}$ value of 166–163 Hz could be measured, suggesting the β configuration for mannopyranose [11].

These spectral features appear to be without precedent for any known mannogalactan and suggested, contrary to what was indicated by the purification results, the possibility that the PS was actually made up of a mixture of a galactan and galactomannan. The usual structure of the former consists of a backbone of (1 $\rightarrow$ 4)-linked β-D-galactopyranose [12] and that of the latter of a backbone of (1 $\rightarrow$ 4)-linked β-D-mannopyranose bearing α-D-galactopyranosyl units linked at the C-6 position of some mannose residues [12].

In this light, the ratio of galactan and galactomannan in the PS appeared to be 1.7:1 (^1H NMR integration). Mixtures of these polysaccharides are not easy to separate and, in some cases, the formation of copper complexes has been exploited [13].

In this case, the use of Fehling's solution allowed us to separate a galactan contaminated by 9% of galactomannan, and a galactomannan contaminated by 8% of galactan. Chemical, spectroscopic, and activity analyses were performed on these samples.

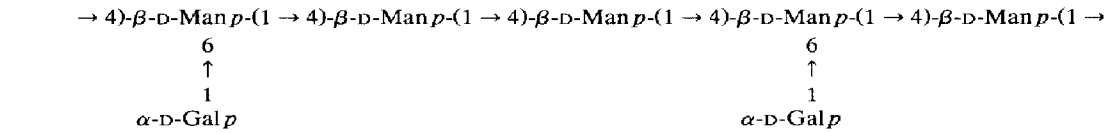
The ^1H NMR spectrum of the galactan mainly showed only one anomeric proton signal (δ 4.62, d, 7.8 Hz). The ^{13}C spectrum displayed an anomeric signal at δ 105.2 and five signals at δ 78.5, 75.4, 74.3, 72.9, and 61.7, suggesting a linear structure built up of only β-galactopyranosyl residues linked through 1 $\rightarrow$ 4 bonds.

Table 2
Molar ratios of methylated monosaccharides from the hydrolysis of methylated galactomannan

Methylated monosaccharide	Molar ratio
2,3,4,6-Tetra-*O*-methylgalactopyranose	1.0
2,3,6-Tri-*O*-methylmannopyranose	1.5
2,3-Di-*O*-methylmannopyranose	1.0

This suggestion was confirmed by the data from the partial acid hydrolysis (see Experimental) and from the methylation analysis of the galactan, that indicated the presence of (1 → 4)-linked β-D-galactopyranosyl oligomers, up to pentasaccharide, and the presence of only 2,3,6-tri-*O*-methylgalactopyranose, respectively.

The ^{1}H NMR spectrum of the galactomannan showed two main anomeric signals at δ 5.02 (d, 3.5 Hz) and 4.76 (bs) for α-galactopyranose and β-mannopyranose, respectively, in addition to a very weak anomeric signal for the galactan at δ 4.62. The integration of the first two signals indicated a mannose–galactose ratio of 2.5 : 1, confirmed by total acid hydrolysis. The ^{13}C NMR spectrum showed three anomeric signals at δ 101.1, 100.9, and 99.8, the first two assignable to β-mannopyranose and the latter to α-galactopyranose, in agreement with the 2D NMR experiment for the whole polysaccharide fraction, discussed above The methylation data (Table 2) indicated a ratio of inner–nodal units of 1.5 : 1, suggesting the following average repetitive unit, irrespective of the distributions of the galactosyl stubs along the chain:



This seems to be the first report on the monosaccharide composition of a galactomannan from any *Strychnos* species, although the finding of a galactomannan in the seeds of *Strychnos nux-vomica* has been quoted [13], without any indication of the ratio between monosaccharides. There have also been reports of a galactan in the seeds of *S. nux-vomica* [14] and a mannogalactan in the seeds of *S. potatorum* [1].

As far as the coagulant activity is concerned, the data of Table 1 indicate that the polysaccharide fractions isolated from *Strychnos nux-vomica* and *S. innocua* show an activity similar to that of *Strychnos potatorum*. Preliminary ^{1}H NMR data also suggest the presence of a mixture of galactan and galactomannan in the seeds of the *S. nux-vomica* and *innocua* species. Work is in progress to define details of the polysaccharide compositions of these species for chemotaxonomic purposes.

To date, coagulant activity tests of the galactan and the galactomannan from *Strychnos potatorum* have not been conclusive in defining whether one polysaccharide, and which, or only the mixture of polysaccharides is active. Owing to the contamination of each polysaccharide with 8–9% of the other, work is continuing to establish unequivocally the basis of the coagulant activity.

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