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The Alkali-Soluble Galactomannan-Like Oligosaccharides from the Seed of *Gleditsia triacanthos*

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SUMMARY

Extraction with alkali of the seeds of Gleditsia triacanthos, *after exhaustive extraction with water and neutralization of the extracts by dialysis, permits isolation of the 75% ethanol-soluble products. Gel permeation chromatography of these products allows isolation of an homogeneous galactomannan–protein complex which was further purified by anion-exchange chromatography. The galactomannan has a medium degree of polymerization and a backbone similar to that of the classical galactomannans. The main difference with these polysaccharides lies in the side-chains which are attached to the backbone not only by (1→6)-linkages but also by (1→3)-linkages and which contain, on average, two galactose units, either (1→3), (1→6)-linked or (1→3), (1→3)-linked with α-D and β-D configurations, respectively.*

INTRODUCTION

Galactomannans from the seed of Leguminosae are usually extracted with water but in some cases alkali was necessary to obtain all the galactomannans (McCleary *et al.*, 1976). These alkali-soluble galactomannans were isolated or purified by ethanol precipitation and the

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supernatants were not examined (McCleary *et al.*, 1976). Recent studies showed the presence of galactomannan-like oligosaccharides in the mother liquors of the ethanol precipitation of the water-soluble galactomannans (Manzi & Cerezo, 1984). We now report the isolation of further oligosaccharides, with gross structures related to those of the galactomannans, from the 75% ethanol-soluble products extracted with alkali from the seeds of *Gleditsia triacanthos*, after exhaustive extraction of the water-soluble polysaccharides.

EXPERIMENTAL

General methods

The seeds of *Gleditsia triacanthos* were obtained from ripe pods collected at the Ciudad Universitaria, Buenos Aires. For general procedures and the extraction of the seeds exhaustively with water, 7M urea, 1% ammonium oxalate and 10% potassium hydroxide, see Part II (Mazzini & Cerezo, 1979*a*) and Part III of a previous study (Mazzini & Cerezo, 1982) (where Part I is Mazzini & Cerezo (1979*b*)). The fractionation of the alkaline extracts and the preparation of the fraction containing the low molecular weight compounds (HC) are also described in Part III (Mazzini & Cerezo, 1982).

Monosaccharide analysis was performed according to the method of Reinhold (1972), using a Hewlett-Packard 5830A gas chromatograph as described by Mazzini & Cerezo (1979*b*).

Cellulose acetate electrophoresis was carried out on 8·5 × 2·5 cm strips previously equilibrated with the running buffer. Samples of 4 μl were applied and intensities of 1·5, 2·0 and 3·0 mA per strip for 15–60 min at different pH buffers (7·0 and 8·5) were used. The strips were developed for proteins with 0·5% Amido Black 10B.

Polyacrylamide gel electrophoreses were carried out according to the method of Davis (1964) using 7% polyacrylamide running gels in 0·05M Tris, 0·38M glycine buffer, pH 8·3 (125 × 10 mm). After electrophoresis, proteins were developed with 1% Amido Black 10B and carbohydrate with the periodic acid-Schiff reagent.

Column chromatography

All the chemicals used were from Pharmacia Ltd. The Sephadex G-200 and G-100 analytical columns (30 × 1·8 cm) were run at

7 ml h^{-1}, and 2·7 ml fractions were collected. Water and a solution of 0·05 M NaCl were used as eluants. The Sephadex G-100 preparative columns (75 × 4·5 cm) were run in 0·05M NaCl at 10 ml h^{-1}; solutions (100 mg ml^{-1}, 10 ml) of the samples were applied, and 14 ml fractions were collected. The eluates were monitored for carbohydrate by the phenol–sulphuric acid method (Dubois *et al.*, 1956) and for protein by UV spectrophotometry at 280 nm (Warburg & Christian, 1957); the void volume was controlled with Blue Dextran. Fractions corresponding to the peak were pooled, concentrated and freeze-dried. Two consecutive Sephadex G-15 columns (50 × 1·9 cm and 45 × 3·2 cm), run in water at 15 ml h^{-1}, were used for desalting fraction HC_3; 6 ml fractions were collected and carbohydrate and protein were monitored as above (HC_3, final yield 0·11 g (1·3% of the total soluble galactomannan)).

The anion-exchange column (21 × 0·7 cm) contained DEAE-Sephadex A-25 equilibrated with 2 mM pyridine acetate buffer, pH 5·0. A solution (40 mg ml^{-1}, 0·5 ml) of the sample was applied at the top of the column, and eluted with the same buffer at 17 ml h^{-1}. Fractions of 2 ml were collected, and carbohydrate and protein were monitored as above. The peak fractions were pooled, concentrated and the buffer removed by freeze-drying (HC_{3-1}, yield 10 mg (0·5% of the total soluble galactomannan)). Further elution with increasing concentration (up to 250 mM) pyridine acetate buffer, pH 5·0, did not show any carbohydrate- and/or protein-containing material.

Determination of anomeric configuration

Acetylation of galactomannan HC_{3-1} (1 mg) was performed in dimethyl formamide (200 μl). Pyridine (200 μl) was added, with constant stirring, and later acetic anhydride (200 μl). After 72 h treatment, water was added and the product was extracted with chloroform. The peracetylated derivative (HC_{3-2}) was dissolved in anhydrous acetic acid (300 μl) and submitted to oxidation by chromium trioxide as described by Dmitriev *et al.* (1975). The oxidized product (HC_{3-3}) was extracted with chloroform, washed with water, dried and the chloroform was distilled off.

Methylation analysis

Galactomannan HC_{3-1} (0.8 mg) was dissolved in dimethyl sulphoxide (200 μl) and methylated by the method of Hakomori (Hakomori,

1964; Sandford & Conrad, 1966). The permethylated product was extracted with chloroform and dried. Acetolysis, hydrolysis, reduction and acetylation were carried out as described by Stellner *et al.* (1973).

Analysis of the methylated alditol acetates by combined GLC–MS was performed on a glass column (0·3 × 120 cm) of 3% ECNSS-M on Gas Chrom Q (100–200 mesh), programming from 130°C to 200°C at a rate of 4°C min^{-1} with helium as a carrier gas (12 ml min^{-1}), and a Varian Series 1400 gas chromatograph connected to a Varian MAT CH7A mass spectrometer. Mass spectra were recorded over a mass range of 40–600 atomic mass units, using an ionizing potential of 70 eV. Scans were taken every 4 s. The methylated alditol acetates were identified by a combination of GLC retention times (known standards) and mass spectra.

RESULTS

The 75% ethanol-soluble products (HC, 2·9% of the whole seed) contained 46·1–50·8% carbohydrate, 48·1–53·1% protein and 2·7% ash. The sugar composition of HC is given in Table 1. The major sugars were arabinose (28·3%), xylose (15·3%), mannose (19·0%), galactose (10·0%) and *N*-acetylglucosamine (13·0%). Gel chromato-

TABLE 1
Sugar Composition (Mole %) of the Galactomannans Derived from HC_3 [a]

Sugar	*HC* [b]	HC_3	HC_{3-1}	HC_{3-2}	HC_{3-3}
Arabinose	28·3	4·3	1·0	0·9 (2·1)	—
Xylose	15·3	2·9	0·5	0·4 (1·0)	2·0 (1·2)
Mannose	19·0	62·8	76·0	73·0 (172·3)	48·9 (29·5)
Galactose	10·0	23·5	17·6	18·7 (44·2)	42·2 (25·5)
Glucose	1·3	—	4·1	5·7 (13·5)	4·6 (2·8)
Galacturonic acid	4·5	2·8	—	—	—
N-acetylglucosamine	13·0	4·3	0·6	1·1 (2·6)	2·3 (1·4)

[a] Figures in parentheses indicate μmol (100 mg)$^{-1}$: HC_3, galactomannan isolated from HC; HC_{3-1}, galactomannan obtained after anion-exchange chromatography of HC_{3-1}; HC_{3-2}, peracetylated galactomannan; HC_{3-3}, oxidized acetylated galactomannan.

[b] HC also contains fucose (5·9%) and glucuronic acid (2·7%).

graphy of HC on Sephadex G-200 showed an incipient fractionation but a better fractionation was achieved on Sephadex G-100 using water or 0·05 M NaCl as eluants. Two preparative chromatographies under the latter conditions allowed the separation of four fractions (Fig. 1) (yields respect to HC): HC_1 (8–21%), HC_2 (5–11%), HC_3

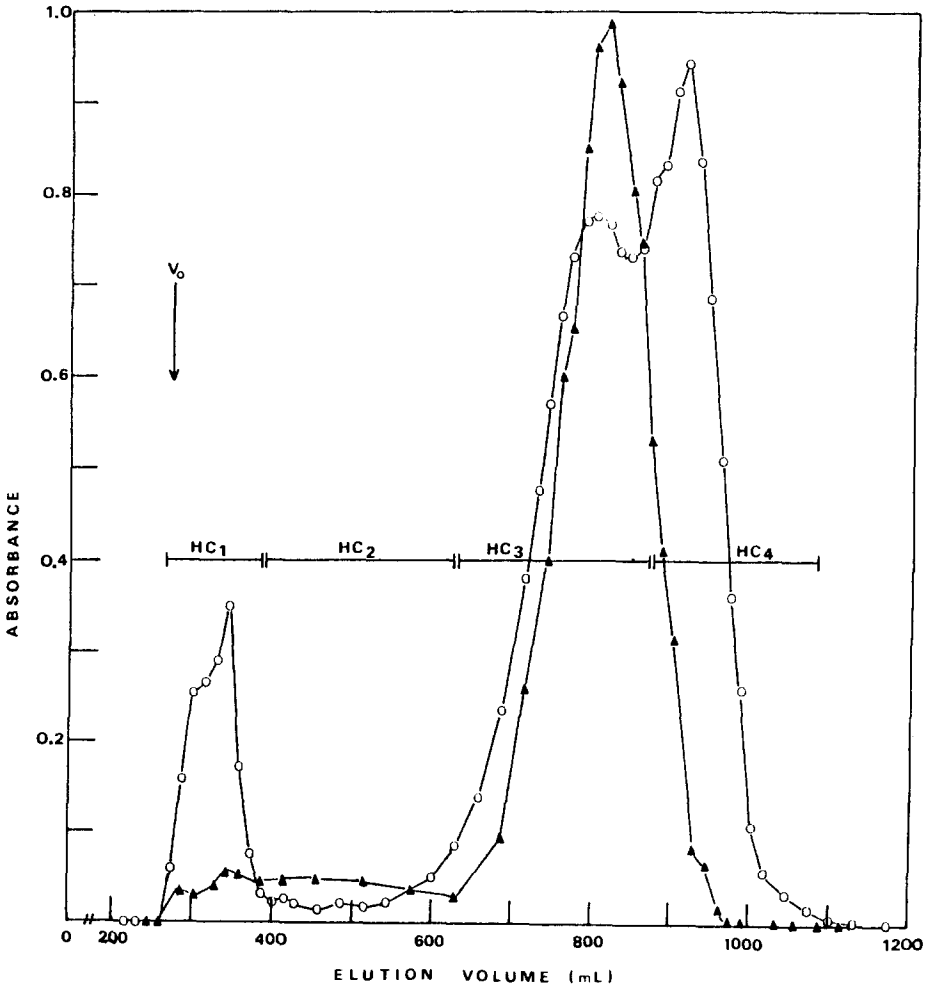

Fig. 1. Preparative column chromatography on Sephadex G-100 of fraction HC: Protein, o——o; carbohydrates, ▲——▲. For yields and composition of the peaks see text.

(53–65%) and HC_4 (6–7%). Fraction HC_3 was controlled for homogeneity on cellulose acetate and polyacrylamide gel electrophoresis and HC_1, HC_2 and HC_4 fractions were reserved for further work.

HC_3 was rechromatographed on Sephadex G-100 and further on Sephadex G-15 producing, in both cases, only one symmetrical peak with a maximum coincident for carbohydrate and protein. The final product contained 40·9% carbohydrate and 42·9% protein and showed only one band which developed for carbohydrate and protein when analysed for homogeneity on polyacrylamide gel electrophoresis. The carbohydrate moiety was mainly composed (Table 1) of mannose (62·8%) and galactose (23·5%) in a molar ratio of 2·7. The amino acid composition of HC_3 is given in Table 2.

Anion-exchange chromatography on DEAE-Sephadex A-25 (AcO^-) eluted with 2 mM pyridine acetate buffer, pH 5·0, produced

TABLE 2
Amino Acid Composition (g (16 g N)$^{-1}$) of the Galactomannans[a]

Amino acids	*HC_3*	*HC_{3-1}*
Asp	10·6	11·4
Thr	3·8	4·3
Ser	5·2	8·4
Glu	20·3	19·5
Pro	9·3	4·3
Gly	4·2	4·9
Ala	5·6	7·2
Val	7·0	5·3
Met	—	—
Ile	5·6	4·0
Leu	9·5	6·9
Tyr	3·9	3·0
Phe	4·9	3·7
Lys	4·9	6·5
His	—	1·4
Arg	5·4	9·2

[a] HC_3, galactomannan isolated from HC; HC_{3-1}, galactomannan obtained after anion-exchange chromatography.

only one fraction (HC_{3-1}) which represented 43% of the starting material and contained 58·2% carbohydrate and 40·7% protein; the rest of the sample was retained in the column even when the molarity of the buffer was increased up to 250 mM. Sugar and amino acid compositions are given in Tables 1 and 2, respectively. The major sugars were again mannose and galactose but the molar ratio changed to 4·3.

Galactomannan HC_{3-1} was acetylated and the peracetylated product (HC_{3-2}) extracted with chloroform and analysed for sugar composition (Table 1) giving a Man:Gal ratio of 3·9. Oxidation of the peracetylated derivative with chromium trioxide in acetic acid produced an oxidized acetylated galactomannan (HC_{3-3}) extractable with chloroform, whose sugar composition is given in Table 1. The oxidation destroyed 83% of mannose together with 42% of galactose and 79% of glucose; the Man:Gal ratio in the degraded galactomannan was 1·16.

Galactomannan HC_{3-1} was methylated by the method of Hakomori (1964). Hydrolysis of the permethylated derivative produced the partially methylated sugars shown in Table 3. The degree of polymerization calculated by using the percentage of tetra-*O*-methylmannose was 30.

TABLE 3

Relative Proportions (Mole %) of the Methylated Sugars from the Galactomannan HC_{3-1} [a]

Mannose				*Galactose*	
2,3,4,6-Tetramethyl	*2,3,6-Trimethyl*	*2,6-Dimethyl*	*2,3-Dimethyl*	*2,3,4,6-Tetramethyl*	*2,4,6-Trimethyl*
3·3	49·1	8·4	10·5	14·8	12·9

[a] Small amounts of 2,4-di-*O*-methylgalactose (1·2%) were also detected by GLC and characterized by GLC–MS. Peaks with the retention time corresponding to 3,4,6-tri-*O*-methylmannose, 2,4,6- or 3,4,6-tri-*O*-methylglucose (1·7%), 2-*N*-methyl-3,6-di-*O*-methyl-*N*-acetylglucosamine (0·3%) and 2-*N*-methyl-4,6- or 2-*N*-methyl-3,4-di-*O*-methyl-*N*-acetylgalactosamine (0·7%) were also detected by GLC.

DISCUSSION

The 75% ethanol-soluble products obtained in the alkaline extractions of plant cell walls are usually discarded due to the difficulty of removing large amounts of salts. Neutralization of the alkaline extracts by dialysis before precipitation of the higher molecular-weight polymers permits isolation of the low molecular-weight, 75% ethanol-soluble compounds.

The crude material (fraction HC) that remains after the precipitation of the high-molecular weight compounds contained nine different sugars (Table 1) which suggested it was heterogeneous. In addition it was heavily 'contaminated' with protein. Gel permeation chromatography allows the isolation of an homogeneous galactomannan (HC_3) with a Man:Gal ratio of 2·7, slightly contaminated with a xylan and a peptin (Table 1). This contamination is usual in legume seed galactomannans extracted with alkali and the material can be purified by further anion-exchange chromatography (McCleary *et al.*, 1976).

The galactomannan (HC_{3-1}) was recovered from this last chromatography only in 66% yield and the Man:Gal ratio changed to 4·3, suggesting a Man:Gal ratio dispersion. It still contained as much protein as the starting material; the amino acid composition of the protein (Table 2) was different from that found in the protein which accompanies the seed galactomannans extracted with water (Manzi *et al.*, 1984), but similar to the amino acid composition of the water-insoluble residues of the embryo and the endosperm (Mazzini & Cerezo, 1979*b*). The resistance of the galactomannan–protein complex to fractionation suggests a covalent linkage between both products and this is consistent with the presence of small amounts of *N*-acetylglucosamine which accompanies the galactomannan and the protein through all the processes of fractionation. Carefully purified legume seed galactomannans contain small amounts of protein and it has been suggested that these are glycopeptides (Manzi *et al.*, 1984).

Methylation analysis of the permethylated derivative showed that the galactomannan has a backbone similar to that found in legume seed galactomannans, namely a β-(1 → 4)-linked D-mannose backbone. The percentage of tetra-*O*-methylmannose (Table 3) indicated a low degree of polymerization (≃ 30) which could explain its ethanol–water solubility. Considering that a similar amount of protein is attached to the galactomannan it could also explain why the galactomannan was

not lost during the dialysis. No trimethylated xylose or arabinose residues were found indicating that, in contrast to the galactomannans extracted with water (Manzi *et al.*, 1984), in these molecules there are no lateral chains terminated by these sugars. The detection of traces of 2,5-di-*O*-methylarabinose is consistent with the existence of traces of a contaminating arabino-xylan.

Major deviations from the usual galactomannan structure are indicated by the following. (a) The presence of 3-linked galactose units in amounts similar to that of the terminal galactose — this would suggest that the lateral chains consist, on average, of two galactose units. A less probable hypothesis is that the 3-linked galactose units are inserted in the backbone producing a major change in the conformation of the molecule. (b) The presence of 3,4-linked mannose residues would suggest that about half of the lateral chains could be linked to the backbone through C-3. Some of these 'non-usual' units have been detected previously in legume seed galactomannans extracted with water, but only in minor, or trace, amounts (Manzi *et al.*, 1984).

The acetylated galactomannan has mannose and galactose in a molar ratio of 3·9 in agreement with the previously mentioned Man:Gal ratio dispersion. Chromium trioxide oxidation (Dmitriev *et al.*, 1975) of this derivative showed that most of the mannose units have the β-D anomeric configuration in agreement with the proposed structure. The fact that 42% of the galactose units were oxidized suggested that about half of these units have also the β-D configuration. This is not usual, as in the classical galactomannans the non-reducing, end-chain galactose units have the α-D configuration. Considering this result as well as the fact that the amount of galactose oxidized is similar to the amount of 3-linked galactose, it is probable that these last residues have the β-D configuration.

In summary, the galactomannan isolated from the cell walls of the seed of *Gleditsia triacanthos* is a glycopeptide whose carbohydrate part has a medium degree of polymerization and a backbone similar to that of the classical galactomannans extracted with water from legume seeds. The main difference with these polysaccharides lies in the side-chains which are attached to the backbone not only by (1 → 6)-linkages but also by (1 → 3)-linkages and which contain, on average, two galactose units, either (1 → 3), (1 → 6)- or (1 → 3), (1 → 3-linked with α-D- and β-D-anomeric configurations, respectively.

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