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The Structure of a Galactomannan from *Medicago lupulina* L.

Identification of Oligosaccharides from a Partial Acid Hydrolysate*

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The seeds of *Medicago lupulina* L. (Leguminosae) contain a galactomannan which forms a highly viscous solution in water. The structural features observed, did not deviate from the commonly accepted galactomannan structure (Fig. 1). The galactose content was high, 47.5 %. A partial acid hydrolysate contained the following oligosaccharides; disaccharides, 4-*O*-β-D-mannopyranosyl-D-mannose and 6-*O*-α-D-galactopyranosyl-D-mannose; trisaccharides, *O*-α-D-galactopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-D-mannose and *O*-β-D-mannopyranosyl-(1→4)-*O*-[α-D-galactopyranosyl-(1→6)]-D-mannose (ABC and CDE, Fig. 1), and tetrasaccharides, ABCE and probably CDEG and CEFG (Fig. 1).

This work on the structural determination of a galactomannan from *Medicago lupulina* L. is the third in a serial examination of seeds from different leguminous species of agricultural interest. The exmination was carried out to investigate the possibility of any structural deviation from the commonly accepted structure (Fig. 1), as described in the former publications.[1,2] This particular species, *Medicago lupulina* L. was chosen as its galactomannan gave a much more viscous solution in water than the other two galactomannans examined by us. It is also of interest as it is closely related botanically to the plant producing the most exhaustively examined galactomannan, *Medicago sativa* L. or lucerne. From the analytical data obtained, it may be inferred, however, that its high viscosity is not connected with any exceptional structural features. The only differences from the two species previously examined[1,2] were the high yield of galactomannan from the seeds, 4.2 %, and the high content, 47.5 %, of galactose in the molecule.

* This paper is dedicated to Dr. Berwind P. Kaufmann on the occasion of his seventieth birthday.

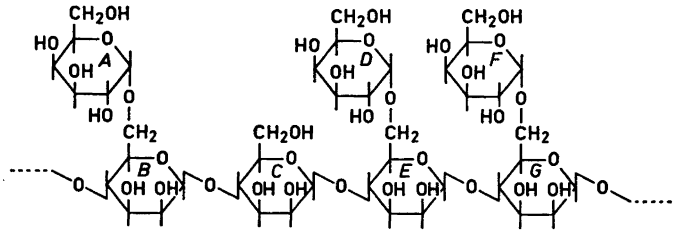

Fig. 1. Section of a galactomannan molecule.

The present examination was carried out with emphasis on the structural determination of the oligosaccharide fractions obtained by partial acid hydrolysis of the galactomannan. The main purpose of the investigation has been the identification of the components in the tri- and tetrasaccharide fractions obtained by the chromatographic separation of the hydrolysate on a carboncelite column. These two fractions, called III and IV, were not completely identified in the former publication.[2]

The galactomannan from *Medicago lupulina* L. was obtained as a white powder, yield 4.2 %, which was highly viscous in water. A hydrolysate of the homogeneous polysaccharide contained only galactose and mannose in the molecular proportion 1:1.10, which corresponds to a content of 47.5 % of galactose in the galactomannan molecule. This is in agreement with the amount of formic acid produced by the periodate oxidation. The liberated formic acid corresponds to 45 % of anhydro hexose as terminal groups, which are probably represented by the galactose units. The amount of formic acid produced would account for the consumption of 0.90 mole periodate per anhydro hexose unit. The uptake of periodate was found to be 1.25 mole which is taken as a proof that vicinal hydroxyl groups other than those of the terminal hexopyranosyl units are present in the polysaccharide molecule.

Fractions I and II were identified as the following compounds: 4-*O*-β-D-mannopyranosyl-D-mannose and 6-*O*-α-D-galactopyranosyl-D-mannose, respectively.

Fraction III contained trisaccharides and fraction IV tetrasaccharides, all representing one galactose residue (from the side chain) combined with two or three mannose residues (from the main chain); general formula: galactose-(mannose)$_n$. Although it seemed, from chromatographic and electrophoretic data, as if these fractions were homogeneous, it emerged undoubtedly from further experiments that they were mixtures of isomers. The presence of isomers has partly been verified by complete methylation followed by hydrolysis and identification of the methylated hexoses by thin layer chromatography. Supporting evidence has been obtained by reducing the oligosaccharide mixtures by sodium borohydride and examining the partial acid hydrolysates of the reduced forms of the oligosaccharides (Table 3).

By means of the methods mentioned it has been found that fraction III consists of the two possible isomers ABC and CDE (Fig. 1). Fraction IV may consist of three isomers represented by the molecular combinations: ABCE, CDEG and CEFG. In this work it has been proved that ABCE is present.

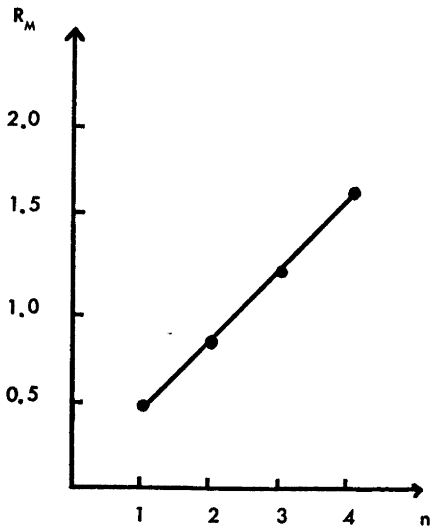

Fig. 2. Relation between R_M-values ($R_M = \log [(1/R_F)-1)]$ and chain length. General formula: galactose-(mannose)$_n$.

None of the analytical data obtained exclude, however, the presence of the two isomers CDEG and CEFG.

It has also been shown, by means of studying the relationship between $R_M\left[R_M = \log\left(\frac{1}{R_F}-1\right)\right]$ and the chain length,[3] which turned out to be strictly linear (Fig. 2), that fraction III and IV together with fraction II apparently belong to a homologous series with galactose at the non-reducing end of the molecule. The series is represented by the general formula: galactose-(mannose)$_n$, where galactose is connected with an α-1,6-binding to the β-1,4-bound mannose chain. The values of n are: 0, 1, 2, and 3. It should be noted that a relationship such as that of Fig. 2 may be found despite the participation of several isomers in one or more of the links forming the homologous series.

Furthermore one would expect to find, in addition to the oligosaccharides described above, two more theoretically obtainable members of the tri- and tetrasaccharide groups. They are; 1,4-β-bound mannotriose and 1,4-β-bound mannotetraose. It seems, however, from the data obtained, especially the values of the galactose:mannose proportions, which were found to be 1:1.90 and 1:2.85, that the oligomannosides, if present, must be in small amounts.

After the identification of the three oligosaccharide fractions and partial identification of the fourth fraction, it can be settled that the galactomannan from *Medicago lupulina* L. also possesses a structure in good agreement with the commonly accepted one for leguminous galactomannans shown in Fig. 1. The results of the periodate oxidation are also in agreement with this conclusion. Galactose molecules are connected to 91 % of the mannose residues of the backbone chain. The fact that all or nearly all oligosaccharides liberated by the partial acid hydrolysis do contain one galactose residue, indicates that the galactosyl groups are evenly distributed along the mannan backbone.

EXPERIMENTAL

Paper chromatograms were, as a routine procedure, run by the descending method on Whatman No. 1 papers in butanol:pyridine:water, 5:3:2, v/v.

Thin layer chromatography was carried out on silica gel in cyclohexanol:benzene: ethanol, 10:10:3, v/v. Aniline oxalate was used as a spray to detect the sugar components on the chromatograms. Periodate and starch were used to detect the alcohols on paper chromatograms.[6]

The polysaccharide

Extraction, purification and characterisation of the galactomannan. The milled seeds (100 g) were extracted with cold water (3000 ml) under continuous stirring for 2 h. After centrifugation the copper complex of the galactomannan was precipitated by adding Fehling's solution (100 ml) and purified further by ethanol precipitations.[4] The polysaccharide preparation was a white powder, yield 4.2 %; N, nil; sulfated ash, 0.3 %; the highly viscous aqueous solution was neutral and gave no coloration with iodine, showing that no starch was present. The product was shown to be homogeneous as it moved as a single spot examined by electrophoresis in borate buffer and 2 N NaOH (120 mA, 18 h).

The further characterisation of the galactomannan was performed as explained in the previous publications.[1,2] The completely hydrolyzed product consisted of D-galactose and D-mannose in the proportion galactose:mannose, 1:1.10, which corresponds to 47.5 % of anhydro-galactose in the polysaccharide.

The results from a typical series of periodate oxidation experiments of the galactomannan are given in Table 1.

The anomeric configuration of the galactose was confirmed to be the α-form as galactose was liberated by α-galactosidase only.

The oligosaccharides

Acid hydrolysis and fractionation. The partial acid hydrolysis of the galactomannan and the separation of the hydrolyzed product was carried out as explained previously.[2] In addition, the fractions were purified by thick filter paper chromatography (Whatman No. 3). The oligosaccharide fractions obtained (fractions I, II, III, IV) were examined by paper chromatography, thin layer chromatography, and electrophoresis. They were found to behave as homogeneous fractions with the same mobilities as the corresponding fractions obtained from *Anthyllis vulneraria* L.[2]

Methylation analysis.[5] The oligosaccharide (5—40 mg) was dissolved in DMF (dimethylformamide) (1.25 ml) in a ground glass stoppered test tube. A mixture of BaO (25

Table 1. Oxidation by periodate.

Time h	Moles of periodate consumed per $C_6H_{10}O_5$	Acid, equivalents per $C_6H_{10}O_5$
1	0.73	—
4	0.90	—
24	1.10	0.311
48	1.17	0.368
72	1.23	0.403
96	1.22	0.425
120	1.25	0.450

parts) and $Ba(OH)_2 \cdot 8\ H_2O$ (1 part) was added. Methyl iodide (0.25 ml) was added and the test tube shaken at room temperature for approximately 20 h. The clarified solution was diluted with chloroform (5 ml), washed with 1 ml sodium thiosulfate solution (1 N) followed by water (8 × 2 ml). The chloroform solution was dried with Na_2SO_4 and evaporated to dryness under reduced pressure (40° in the water bath). Complete methylation was verified by means of the IR spectrum of the product.

The fully methylated product was hydrolyzed by the addition of 1 N H_2SO_4 and kept at 100° overnight. The neutralized product ($BaCO_3$) was examined by thin layer chromatography.

Reduction by sodium borohydride. The oligosaccharide (5 mg) and sodium borohydride ($NaBH_4$) (10 mg) were dissolved in water (10 ml). This mixture was left at room temperature for 3 h. The reduction was complete after this treatment as no aniline positive spots could be detected on the chromatograms of the reaction mixture. The solution was neutralized by the addition of Amberlite IR 120 (H^+). After evaporation to dryness, the product was dissolved in methanol which was removed again by evaporation under reduced pressure. This was repeated three times to remove the borate. The final product was dissolved in water (0.4 ml) and used for further examinations, that is, partial acid hydrolysis in a sealed test tube at 100°, followed by paper chromatographic examinations. Two identical chromatograms were irrigated simultaneously, one was sprayed with aniline oxalate to detect the sugar components and the other by periodate and starch which also detected the alcohol components.

Identification of the oligosaccharide fractions

Fraction I, mannobiose, 4-*O*-β-D-mannopyranosyl-D-mannose; degree of polymerisation (DP) 2; $[\alpha]_D^{27}$ −7° (*c* 1.5, water), giving exclusively mannose after hydrolysis. By paper chromatography it was indistinguishable from an authentic sample of mannobiose. It proved to be 1,4-linked by the test of formaldehyde formation after periodate oxidation of the corresponding sugar alcohol.[7] Borohydride reduction followed by acid hydrolysis gave mannose and mannitol. These data confirm the identification of fraction I as mannobiose.

Fraction II, 6-*O*-α-D-galactopyranosyl-D-mannose; DP 2; $[\alpha]_D^{23}$ + 110° (*c* 1.0, water); mannose and galactose were obtained in equimolecular amounts [8] by complete hydrolysis. The disaccharide was attacked by α-galactosidase only. It proved to be 1,6-linked when submitted to the periodate oxidation of the corresponding sugar alcohol.[7] Borohydride reduction followed by hydrolysis gave galactose and mannitol. By paper chromatography it was identical with an authentic sample. These data confirm the identification of fraction II as 6-*O*-α-D-galactopyranosyl-D-mannose.

Fraction III. This fraction is a mixture of two isomeric trisaccharides: *O*-α-D-galactopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-D-mannose and *O*-β-D-mannopyranosyl-(1→4)-*O*-[α-D-galactopyranosyl-(1→6)]-D-mannose (ABC and CDE, Fig. 1). It was

Table 2. Paper chromatography of the hydrolysis products of the fractions I, II, III, and IV.

Reference substances	R_G	Total hydrolysis of fractions No.				Partial hydrolysis of fractions No.			
		I	II	III	IV	I	II	III	IV
Galactose	0.84		+	+	+		+	+	+
Mannose	0.15	+	+	+	+	+	+	+	+
Fraction I	0.60					+		+	+
Fraction II	0.48						+	+	+
Fraction III	0.25							+	+
Fraction IV	0.08								+

Table 3. Hydrolysis of the reduced trisaccharide fraction. Paper chromatographic analysis.

R_G	Total hydrolysis 1 N H_2SO_4 20 h	Partial hydrolysis 0.2 N H_2SO_4 2 h	0.2 N H_2SO_4 1 h	0.1 N H_2SO_4 2 h	0.1 N H_2SO_4 1 h
0.84	galactose	galactose	galactose	galactose	galactose
1.15	mannose	mannose	mannose	mannose	mannose
0.48					fraction II [c]
0.57		reduced fraction I [a]	reduced fraction I	reduced fraction I	reduced fraction I
0.45		reduced fraction II [b]	reduced fraction II	reduced fraction II	reduced fraction II
0.24		reduced fraction III	reduced fraction III	reduced fraction III	reduced fraction III
1.01	mannitol	mannitol	mannitol	mannitol	mannitol

[a] Reduced fraction I = 4-*O*-β-D-mannopyranosyl-D-mannitol.
[b] Reduced fraction II = 6-*O*-α-D-galactopyranosyl-D-mannitol.
[c] Fraction II = 6-*O*-α-D-galactopyranosyl-D-mannose.

obtained as a white powder; DP 3; $[\alpha]_D^{20}$ + 40.5° $\xrightarrow{24\text{ h}}$ + 64.5° $\xrightarrow{72\text{ h}}$ unchanged (*c* 1.5, water). Galactose and mannose were obtained in the proportion 1:1.9 after total hydrolysis. The five expected components were present in a partial hydrolysate (Table 2). The trisaccharides were attacked by α-galactosidase and not by β-galactosidase. After the reduction of the trisaccharides by sodium borohydride only one alcohol spot was detected by chromatographic examinations. Total hydrolysis of the alcohols gave galactose, mannose, and mannitol. A partial hydrolysate contained several components (Table 3). One of these was an alcohol corresponding to reduced fraction II (R_G 0.45), which is only obtainable if the trisaccharide fraction contains a substance with the molecule combination CDE. By examination of another hydrolysate of the same fraction, hydrolyzed to a lesser extent, small amounts of a disaccharide (R_G 0.48), identical with fraction II, were obtained (Table 3). This disaccharide is only obtainable if the molecule combination ABC is present in the fraction. By the paper chromatographic examination of the last mentioned hydrolysate, a very small amount of mannobiose was also observed. This may be due to the presence of a small amount of mannotriose in the trisaccharide fraction. These data were confirmed by the methylation experiments (Table 4) which, besides 2,3,4,6-Me_4Gel, gave 2,3,4-Me_3Man and 2,3,6-Me_3Man characteristic of ABC, further 2,3,4,6-Me_4Man and 2,3-Me_2Man characteristic of CDE.

Table 4. R_F values of methyl derivatives obtained from the fractions III and IV. Thin layer chromatography.

Methyl derivatives	Fraction III	Fraction IV
2,3-Me_2Man [a]	0.09	0.09
2,3,6-Me_3Man	0.17	0.17
2,3,4-Me_3Man	0.19	0.19
2,3,4,6-Me_4Man	0.31	0.31
2,3,4,6-Me_4Gal	0.25	0.25

[a] 2.3-Dimethyl-D-mannose, etc.

Thin layer chromatography of the methylated trisaccharide fraction before hydrolysis revealed four spots which may indicate the presence of two isomers. The four spots would be the fully methylated α- and β-forms of the methyl glycosides of each of the trisaccharides.

Assembling the analytical data, they all tend to confirm the conclusion that fraction III consists of the two isomeric trisaccharides, ABC (Fig. 1), *O*-α-D-galactopyranosyl-(1→6)-β-D-mannopyranosyl-(1→4)-D-mannose and CDE (Fig. 1), *O*-β-D-mannopyranosyl-(1→4)-*O*-[α-D-galactopyranosyl-(1→6)]-D-mannose.

Fraction IV. This fraction is probably a mixture of the three isomeric tetrasaccharides with the molecule combinations ABCE, CDEG, and CEFG. It was obtained as a white powder: $[\alpha]_D^{22}$ + 38° (*c* 1.0, water). After total hydrolysis it gave galactose and mannose in the proportion 1:2.85. The anomeric galactose configuration was the α-form as judged by the enzymic hydrolysis. Partial hydrolysis revealed the six expected components (Table 2). This layer chromatography of the fully methylated fraction before hydrolysis contained six components, which may indicate the presence of three isomers. The six spots would be the α- and β-forms of the methyl glycosides of each of the three isomers. Hydrolysis of the methylated product (Table 4) revealed five components. The identification of 2,3,4-Me_3Man proves that the molecule combination ABCE must be present. 2,3,4,6-Me_4Man reveals the presence of either CDEG or CEFG or both. After the reduction of the tetrasaccharide fraction by sodium borohydride one alcohol spot only was detected by chromatographic examinations. Total hydrolysis and paper chromatographic examination of the reduced fraction gave galactose, mannose, and mannitol.

REFERENCES

1. Sømme, R. *Acta Chem. Scand.* **20** (1966) 589.
2. Sømme, R. *Acta Chem. Scand.* **21** (1967) 685.
3. French, D. and Wild, G. M. *J. Am. Chem. Soc.* **75** (1953) 2612.
4. Andrews, P., Hough, L. and Jones, J. K. N. *J. Am. Chem. Soc.* **74** (1952) 4029.
5. Kuhn, R., Egge, H., Brossmer, R., Gauhe, A., Klesse, P., Lochinger, W., Röhm, E., Trischmann, H. and Tschampel, D. *Angew. Chem.* **72** (1960) 805.
6. Metzenburg, R. L. and Mitchell, H. K. *J. Am. Chem. Soc.* **76** (1954) 4187.
7. Clancy, M. J. and Whelan, W. J. *Chem. Ind. (London)* **1959** 673.
8. Sunderwirth, S. G., Olson, G. G. and Johnson, G. *J. Chromatog.* **16** (1964) 176.

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