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Studies on Arabogalactans

III.* Degradation Products of Arabogalactan A from *Larix occidentalis* Nutt. and an Electrophoretic Examination of Arabogalactans from other *Larix* Species

HANS O. BOUVENG

Institutionen för Träkemi, Kungl. Tekniska Högskolan, Stockholm, Sweden

The arabogalactan A obtained from *Larix occidentalis*, on mild hydrolysis to remove the arabinose residues, gave two different galactan fractions which were easily separated by fractional precipitation of their cetyl-trimethylammonium borate complexes. On methylation and hydrolysis of the methylated derivatives, both galactans afforded 2-*O*-methyl-D-galactose, 4-*O*-methyl-D-galactose, 2,4-di-*O*-methyl-D-galactose, 2,6-di-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose and 2,3,4,6-tetra-*O*-methyl-D-galactose though in significantly different proportions. Some arabogalactans from other *Larix* species were examined by electrophoresis and by fractionation and were found in all cases to be of the same complex nature as the *L. occidentalis* polysaccharide.

INVESTIGATION OF DEGRADED ARABOGALACTAN A

In part II of this series [1] an account was given of the fractionation of the arabogalactan fraction from Western larch (*Larix occidentalis* Nutt.) into two fractions (A and B) and of a methylation study of the component A with the higher degree of polymerisation and the higher electrophoretic mobility. Neither sedimentation analysis nor electrophoresis in borate buffer gave any indication of heterogeneity in the two polysaccharides. However, it was later observed that polysaccharide A after brief, mild hydrolysis gave two distinct components on electrophoresis, one with slightly higher electrophoretic mobility than arabogalactan A and one with about the same mobility as arabogalactan B.

In contrast to the behaviour of the material studied by Aspinall *et al.*[2], the arabinose could be removed by prolonged mild hydrolysis without unduly

* Part III and IV of this series were presented at the 136th Meeting of the American Chemical Society in Atlantic City, N. J., September 1959 (abstract 14E).

extensive break-down of the remaining polysaccharide. After removal of the low molecular weight oligosaccharides, a fractional precipitation with cetyltrimethylammonium hydroxide (CTA-OH) — boric acid gave two electrophoretically pure galactans, A'I, $[\alpha]_D^{20}$ +13°, M_G 0.84 and A'II, $[\alpha]_D^{20}$ +19°, M_G 0.66. The arabogalactans A and B had M_G values of 0.76 and 0.63 and rotations of +7° and +10°, respectively. An osmometric determination gave a $\overline{DP}_n$ value of 48 for A'II. An approximate calculation of the degree of polymerisation of A'I from the proportion of terminal groups appearing as tetramethylgalactose on hydrolysis of the methylated polysaccharide gave a value of 20. Arabogalactan B was degraded in the same manner to yield a single component (B') with the same electrophoretic mobility as the original polysaccharide and with a rotation of +21°.

The two galactans were methylated following the procedure described in part II[1] with the exception that A'I after the preliminary methylation with methyl sulphate and sodium hydroxide was methylated once with methyl iodide and barium oxide in dimethyl formamide solution as proposed by Kuhn *et al.*[3] for the methylation of low molecular weight carbohydrates. The barium oxide seemed to give slightly lower methoxyl content than silver oxide but may be useful since the oxidising silver oxide depolymerises more incompletely methylated polysaccharides [4].

The methylated polysaccharides were hydrolysed in a mixture of 30 % glacial acetic acid and 70 % 0.5 N hydrochloric acid and each mixture of methyl ethers obtained was resolved on a carbon-Celite column. Further separation, if required, was done on thick filter paper. The amount of each ether was estimated by hypoiodite oxidation [5]. The results are summarised in Table 1.

Table 1. Methyl ethers (in mole %) obtained from the methylated hydrolysed galactans A'I and A'II from degraded larch arabogalactan A.

Sugar	% (A'I)	% (A'II)
4-Methylgalactose	0.6	0.3
2-Methylgalactose	2.5	3.4
2,4-Dimethylgalactose	27.9	20.5
2,6-Dimethylgalactose	2.1	2.7
2,3,4-Trimethylgalactose	13.8	21.8
2,4,6-Trimethylgalactose	13.6	19.6
2,3,4,6-Tetramethylgalactose	39.6	31.7

These results show that A'I and A'II contain the same types of linkages but in different proportions. A'II is a more moderately branched polysaccharide, while A'I has the same heavily branched structure as the original polysaccharide. The 2-*O*-methyl-D-galactose and 2,6-di-*O*-methyl-D-galactose must be assigned at least some structural significance which implies the presence of a small fraction of 1,4-linkages partly combined with doubly branched units, but the 4-*O*-methyl-D-galactose is probably a result either of incomplete methylation or of demethylation during hydrolysis. The amount of 2,4,6-tri-

O-methyl-D-galactose is strikingly larger than that obtained from arabogalactans A and B. This indicates that the easily hydrolysed units of the original polysaccharide (L-arabinose, 3-*O*-β-L-arabopyranosyl-L-arabinose, D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose) are attached to the rest of the molecule mainly through 1,6-linkages as has been suggested by White [6] in the case of the arabinose.

These results indicate a possibility that the arabogalactan A could be heterogeneous. A separation of the material into the hypothetical components corresponding to the galactans A'I and A'II * by fractional precipitation with CTA-OH—boric acid did not appear feasible since no separation could be obtained on electrophoresis in borate buffer or by trial precipitations. On hydrolysis of arabogalactan A in 0.01 N acid at 100°C for 2 h only, it was found possible to isolate a small quantity of electrophoretically pure A II from the main fraction of the A I type (M_G *ca.* 0.85) by fractionation in the usual way. This fraction probaly contained still unchanged arabogalactan A beside A I. Chromatograms of hydrolysates indicated that these two fractions contained about the same proportions of arabinose. Electrophoretic examination of the material obtained from A I on hydrolysis for a further 2 h showed that more A II had been formed. The A I fraction after precipitation as the CTA-borate complex and isolation in the usual manner was subjected to hydrolysis under the same conditions for another 4 h. Further amounts of A II were again found.

It is not yet possible to decide whether A I and A II are fragments of a single molecular species or of two. It is however known that part of the galactopyranosidic linkages in the polysaccharide must be of a weaker type, possibly due to non-bonded steric interaction in the heavily branched structure (*cf.* the preceding paper in this series). If arabogalactan A is homogeneous a possible explanation of the appearance of A I and A II is that they correspond to two molecular fragments of different degrees of branching which are joined by linkages of this type. The observation, that the rates of formation of A I and A II and of galactose and 6-β-galactopyranosyl-galactose under these hydrolytic conditions are of the same order of magnitude, is in agreement with this view. A further indication of the homogeneity of arabogalactan A is the fact that on electrophoresis of the hydrolysed polysaccharides there is little trailing between the two spots corresponding to A I and A II. It therefore seems more probable that A I and A II are released as such during the hydrolysis from a homogeneous polysaccharide and not from separate macromolecules; in the latter case the M_G values should change gradually from the original value (0.76) to the final values (0.84 and 0.66 for A'I and A'II, respectively) with consequent trailing. The small difference between the M_G values for B and B' shows that the removal by hydrolysis of the simple oligosaccharide residues

* The designation A I will be used below to refer to any polysaccharide material obtained by mild hydrolysis of arabogalactan A that has an electrophoretic mobility of the same order as the original arabogalactan A (*ca.* 0.80). A II will be used to refer to any material obtained from arabogalactan A in the same way that has the same electrophoretic mobility as arabogalactan B (*ca.* 0.65). A'I and A'II will be used exclusively for the arabinosefree galactans (M_G values 0.84 and 0.66, resp.) obtained by prolonged mild hydrolysis of arabogalactan A.

containing arabinose and galactose [7] appearently has very little effect on the electrophoretic mobility of this type of polysaccharide.

Examination of the arabogalactans from a number of larches (*cf.* the preceding section of this paper) showed that the proportions of A I and A II as estimated from electrophoretograms were fairly constant but that the proportion of A to B varied considerably.

INVESTIGATION OF THE ARABOGALACTANS FROM OTHER *LARIX* SPECIES

In view of the complex nature of the arabogalactans from *Larix occidentalis* it seemed desirable to make a brief examination of the arabogalactans of other *Larix* species to see if they were of the same type. The following species were investigated (the yields of arabogalactan calculated on acetone-extracted, airdried heart wood are given in brackets; the results obtained from *L. occidentalis* are included for comparison):

Order *Multiseriales* Patschke [8]:
L. Potaninii Batal. (7.2 %)
L. occidentalis Nutt. (9.3 %)
L. Lyallii Parl. (7.7 %).
Order *Pauciseriales* Patschke:
L. leptolepis (Sieb. & Zucc.) Gord. (8.0 %)
L. laricina (Du Roi) K. Koch (3.9 %)
L. Sukaczevii Djil. (5.3 %)
L. decidua Mill. (3.4 %)
L. Gmelinii (Rupr.) Kuzeneva var. *japonica* (Reg.) Pilger (15.7 %).

EXPERIMENTAL

All melting points are corrected. Evaporations were done under reduced pressure.

Chromatography: Papers: Whatman 1 and Schleicher and Schüll 602 hP. Solvent: Butanol, ethanol, water, 10:3:5. Spray reagent: anisidine hydrochloride.

Electrophoresis. Papers: Whatman 1 and Schleicher and Schüll glass fibre paper. Buffer: 0.1 M borate buffer of pH 10. Spray reagents: anisidine hydrochloride and α-naphtol-sulphuric acid in butanol. In the electrophoresis it was not possible to determine the temperature of the papers accurately and the M_G values are therefore only relative. The variation in different measurements was about ± 0.03 units.

Hydrolysis of the arabogalactan. Arabogalactan A was hydrolysed in 0.01 N hydrochloric acid at 100°C for 18 h. After cooling the solution was neutralised with silver carbonate, filtered, concentrated to a small volume and poured into ethanol. The product was filtered off giving a white powder. This was freed from low molecular weight oligosaccharides by reprecipitation twice with ethanol. A chromatogram of a hydrolysate showed only faint traces of arabinose. On electrophoresis the hydrolysate gave two distinct spots, the faster of which was designated as A′I and the slower as A′II. Hydrolysis of arabogalactan B and isolation of the product in the same way gave the degraded polysaccharide (B′). On electrophoresis this gave only a single spot. The characteristics of these polysaccharides are shown in Table 2.

Table 2. Arabogalactans and corresponding galactans from *Larix occidentalis.*

Polysaccharide	$[\alpha]_D^{20}$	% arabinose (molar)	% galactose (molar)	M_G
A	+ 7°	19	81	0.76
B	+10°	21	79	0.63
A'I	+13°	trace	100	0.84
A'II	+19°	—	100	0.66
B'	+21°	trace	100	0.63

Separation of A'I and A'II. The results of a typical experiment are summarised in Table 3 which shows the fractionation of the galactan mixture (7.51 g = 46 mmole anhydrogalactose) dissolved in water (300 ml) and 0.6 M boric acid solution (50 ml).

Table 3. Fractionation of hydrolysed arabogalactan A (7.51 g).

CTH-OH added (mmoles)	NaOH added (mmoles)	Weight of fraction (g)	Components
2.0	—	2.50	A'I
2.0	1.5	2.08	A'I
4.0	3.0	1.83	A'II
Residue		0.42	A'II

A'I and A'II were obtained in the approximate ratio 2:1. A'I contained traces of arabinose.

Methylation of the galactans A'I and A'II. The galactans were methylated first with methyl sulphate and sodium hydroxide. The product from methylation of A'I was then subjected to a methylation with methyl iodide and barium oxide in dimethyl formamide as described by Kuhn *et al.*[3]. After a reaction time of 20 h the unreacted barium oxide was removed by filtration and the dimethyl formamide was evaporated. The product was freed from barium iodide by treatment with freshly precipitated silver acetate in alcoholic solution. This treatment raised the methoxyl content from 25.3 to 38.7 %. The partially methylated galactans were exhaustively methylated with methyl iodide and silver oxide in dimethyl formamide following the procedure described in part II [1]. No trace of unmethylated monomer was found on chromatograms of hydrolysates of the methylated derivatives.

Hydrolysis of the methylated galactans and fractionation of the hydrolysates. The two galactans were each hydrolysed at a concentration of about 1.5 % in a mixture of glacial acetic acid and 0.5 N hydrochloric acid (1:2) at 100°C for 18 h. The reaction mixture was treated with excess silver acetate, concentrated to a small volume, taken up in ethanol and filtered. The mixture of ethers obtained on evaporation of the ethanol was dissolved in water and adsorbed on a carbon-Celite column 3.5 × 46 cm.

A'I (0.47 g) was eluted with 4 l 6 → 25 % ethanol + 2 l 25 → 40 % ethanol + 1 l 50 % acetone, and A'II (0.65 g) with 5 l 6 → 35 % ethanol + 1 l 50 % acetone. Both were collected in 25 ml fractions. The amount of reducing sugar in each fraction was determined by hypoiodite oxidation [5] and the mixed fractions were further resolved on Schleicher and Schüll 602 hP filter papers for quantitative estimation as described in part II [1]. The results of the fractionations are summarised in Table 4.

*Characterisation of the methyl ethers. 2-O-Methyl-*D*-galactose, 4-O-methyl-*D*-galactose* and *2,6-di-O-methyl-*D*-galactose* were indistinguishable from authentic material by chromatography or electrophoresis.

Table 4. Fractionation of the methylated hydrolysed galactans A'I and A'II.

Fract. No.	mmoles(total)	Sugars	% of fraction	mmoles
Galactan A'I				
24—38	0.065	4-Methylgalactose	18	0.012
		2-Methylgalactose	82	0.053
52—100	0.558	2,4-Dimethylgalactose		
101—135	0.065	2,4-Dimethylgalactose	34	0.022
		2,6-Dimethylgalactose	66	0.043
136—174 *	0.287	2,3,4-Trimethylgalactose		
175—220	0.192	2,4,6-Trimethylgalactose		
Residue	0.915	2,4,6-Trimethylgalactose	10	0.092
		2,3,4,6-Tetramethylgalactose	90	0.823
Galactan A'II				
24— 30	0.098	4-Methylgalactose	9	0.009
		2-Methylgalactose	91	0.089
48— 76	0.518	2,4-Dimethylgalactose		
77— 84	0.091	2,4-Dimethylgalactose	23	0.021
		2,6-Dimethylgalactose	77	0.070
85—119	0.576	2,3,4-Trimethylgalactose		
120—164	0.391	2,4,6-Trimethylgalactose		
Residue	0.960	2,4,6-Trimethylgalactose	13	0.124
		2,3,4,6-Tetramethylgalactose	87	0.836

* Contained traces of arabinose methyl ethers.

2,4-Di-O-methyl-D-galactose was crystallised from acetone, m. p. 101°, sintering at 85°, aniline derivative m. p. 213—215° and mixed m. p. 212—215°.

2,3,4-Tri-O-methyl-D-galactose. Aniline derivative, m. p. and mixed m. p. 169—170°.

2,4,6-Tri-O-methyl-D-galactose was obtained in a crystalline state from galactan A'II, m. p. 96—98° after recrystallisation from ethanol-ethyl acetate. Aniline derivative, m. p. 176—178°, in both cases undepressed on admixture with authentic samples.

2,3,4,6-Tetra-O-methyl-D-galactose. Aniline derivative in both cases, m. p. 199—201° and mixed m. p. 198—200°.

Estimation of the degree of polymerisation of A'I and A'II. For *A'II* the estimation was made osmometrically using the same type of osmometer as Lindberg and Meier [9]. The measurements were made in aqueous solution with "Ultrafilter allerfeinst" membranes from Membrangesellschaft Göttingen, Germany. This gave a $\overline{DP_n}$ value of 48 but this is of course subject to inaccuracies due to the use of water as solvent. The methylation data did not permit any reliable estimate of the DP value.

A'I. This galactan leaked too much through the membrane to allow osmometric measurement. Calculation from the excess of endgroups found from the methylation data gave a DP value of 20. Although precautions were taken, small losses due to the volatility [10] of the tetramethylgalactose corresponding to these endgroups were probably unavoidable. Allowing for losses of the order of 5 % (*cf.* Part II of this series) would give a DP value of 17. These DP values of course depend on the hydrolysis time used in the preparation of the galactans. It appears however that the ratio of the DP values for A'I and A'II is about 1:3.

Isolation and electrophoresis of the arabogalactans from other larchwoods. The polysaccharides were isolated mainly as described in Part I [7]. Extraction 3—4 times with water at room temperature afforded about 95 % of the arabogalactan accessible to cold water extraction.

Electrophoresis on glass fibre sheets in borate buffer and fractionation experiments showed that the arabogalactans from the closely related *L. occidentalis* and *L. Lyallii* by containing a larger proportion of the arabogalactan B (25—30 %) differed distinctly from all the other species investigated, which contained only 5—10 % of arabogalactan B. Due either to lack of material or to the small amounts present, arabogalactan B was only isolated in a pure state from *L. Lyallii* and in about 90 % purity from *L. Gmelinii*.

Investigation of the A fractions. The A fractions were isolated as described in Part II [1] of this series. They were analysed for arabinose and galactose according to Saeman *et al.*[11] The results of these analyses and of the analyses of the B fractions isolated are summarised in Table 5.

Table 5. The composition of the arabogalactans from some *Larix* species.

Species	Fraction	Galactose (weight %)	Arabinose (weight %)	Moles galactose per mole arabinose
L Potaninii	A	89.7	10.3	7.3
L occidentalis	A	83.9	16.1	4.3
»	B	81.6	18.4	3.7
L. Lyallii	A	85.5	14.5	4.9
»	B	75.7	24.3	2.6
L. leptolepis	A	90.3	9.7	7.8
L. laricina	A	86.7	13.3	5.4
L. Sukaczevii	A	86.7	13.3	5.4
L. decidua	A	87.2	12.8	5.7
L. Gmelinii var. *japonica*	A	92.2	7.8	9.8
»	B	83.7	16.3	4.3

From these results it is evident that the arabinose content of larch arabogalactans from different sources is subject to large variations. The higher arabinose content of the B fractions may be partly due to a contaminating araban (*cf.* the preceeding paper of this series). The detection of traces of a slow-moving component on an electrophoretogram heavily spotted with the B fraction of *L. Lyallii* is in agreement with this view.

The M_G values of the A fractions lay within the range 0.81—0.86 except for the fraction from *L. occidentalis* which had $M_G = 0.76 \pm 0.03$. The low M_G value of this polysaccharide can be attributed to its comparatively high arabinose content, the main part of which is present as terminal arabofuranosidic residues incapable of forming borate complexes [12]. The B fractions had M_G values of 0.63 ± 0.03.

Hydrolytic degradation of the A fractions. Samples (1.5 g) of the A fractions were subjected to hydrolysis in 0.005 N hydrochloric acid at 100°C for 18 h, and the polysaccharide material in the reaction mixture was precipitated with ethanol. Estimations from electrophoretograms indicated that all samples gave both A I and A II in proportions varying between 2:1 and 3:1. These variations are probably not significant since a slight fractionation of the galactans may occur during isolation.

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