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THE USE OF ALKALINE DEGRADATION FOR STRUCTURAL CHARACTERIZATION OF BRANCHED-CHAIN POLYSACCHARIDES

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ABSTRACT

Classical methods of polysaccharide characterization are often inadequate for the evaluation of the number and nature of sugar residues associated with side-branches. This investigation demonstrates that controlled, alkaline degradation may be used to eliminate the backbone of the molecule and to convert branches linked through position 6 of the backbone into individual entities containing saccharinic acid end-groups. Application of alkaline degradation to an arabinogalactan revealed that arabinose residues are present not only in the branches but also in the backbone of the molecule. Furthermore it has been shown that, in addition to single-unit and two-unit branches, a few branches contain as many as ten residues.

INTRODUCTION

The characterization of branching in macromolecules in general, and in polysaccharides in particular, is a problem that thus far has evaded satisfactory solution. This problem is especially acute when a limited number of long branches may be present in the polysaccharides. For example, it has not been possible to establish unequivocally whether the glucomannan of gymnosperms is a linear molecule or contains 1–3 branches[1]. Another example is the water-soluble arabinogalactan of *Larix* species, for which Timell[1] has proposed the tentative structure shown in Fig. 1, based on the results of numerous previous studies[2–7]. It should be noted that the structure assigned in Fig. 1 is actually based on several arbitrary assumptions such as: (*a*) no arabinose residues participate in the make-up of the backbone, (*b*) only single-unit and two-unit branches are present, (*c*) branched residues are located exclusively in the backbone. The structure depicted therefore represents only one of several possible structures. The latter include, for example, amylopectin-like structures in which one branch leads to another, resulting in a spherical molecule. As a matter of

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fact, a spherical structure would be in better conformity with the low viscosity of arabinogalactans in aqueous solution[8] than the structure given in Fig. 1.

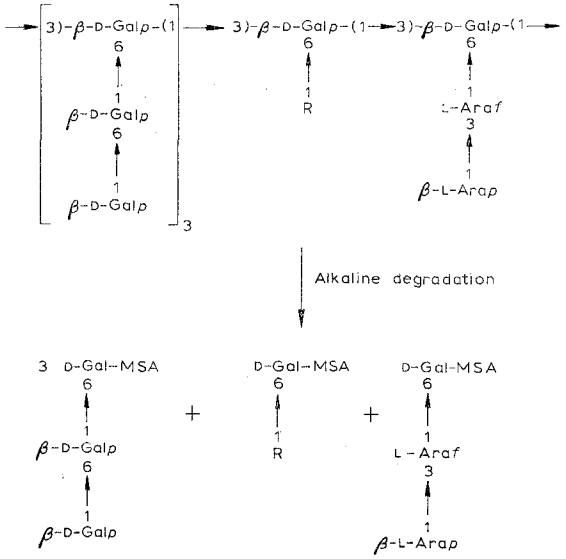

Fig. 1. Structure proposed by previous investigators (compare ref. 1) for the repeating unit in arabinogalactan, and its projected fragmentation on alkaline degradation. MSA = metasaccharinic acid; R = β-D-galactopyranose, or, less frequently, L-arabinofuranose or D-glucopyranosyluronic acid.

Previous work on the alkaline degradation ("peeling") of (1→4)- and (1→3)-linked polysaccharides suggested that this method might be used with advantage for the clarification of the architecture of many branched polysaccharides[9]. This approach has been attempted only once previously, albeit unsuccessfully, for the structural clarification of guaran by Whistler and BeMiller[10]. Arabinogalactan appeared to be a more promising substrate for testing the applicability of this alkaline degradation method, because the (1→3)-linked backbone is anticipated to undergo "peeling" without termination.

Fig. 1 illustrates the products that would be obtained from arabinogalactan by alkaline degradation, were the assigned structure correct. It also illustrates the uniqueness of structural information potentially available from such studies. First of all, the extent of complete degradation may be used to estimate the size of the backbone of the molecule. Secondly, the change in hydrolyzable sugars reveals the nature of monosaccharide residues participating in the make-up of the backbone. Thirdly, the original branches are obtained as oligosaccharides terminated by saccharinic acid groups. Molecular-weight fractionation of these products may reveal reveal the size distribution of the original branches, and the products are also available for further structural elucidation.

DISCUSSION

Arabinogalactan, an extractable polysaccharide abundant in the species *Larix*, has been the subject of numerous structural studies[1–7]. The isolated arabinogalactan is known to contain two fractions, *A* having a molecular weight of 100,000 and *B* having a molecular weight[3–8,11] of 16,000. As the purpose of this investigation was merely to demonstrate the feasibility of alkaline degradation as a structural tool, the entire arabinogalactan as isolated, rather than its individual fractions, was subjected to investigation.

On complete alkaline degradation of arabinogalactan, all (1→3)-linked backbone residues are converted into metasaccharinic acid derivatives. Thus, the extent of degradation and the average percentage of original backbone residues may be estimated in two independent ways: (*a*) from the decrease in the content of aldose residues (by means of the phenol–sulfuric acid reaction)[12,13], and (*b*) by determining the formation of metasaccharinic acid groups titrimetrically from the decrease in sodium hydroxide concentration during the degradation. As shown in Fig. 2, both methods give concordant results for the proportion of residues degraded to metasaccharinic acids as a function of time in 0.1M sodium hydroxide at 56°. The degradation reaches an end when 38% of the total residues have been converted. Assuming that all original backbone residues have been converted at this point, the percentage of main-chain units in the original arabinogalactan ought to be 38% also. The structure depicted in Fig. 1 has actually 36% of main-chain residues, in excellent accord with the results of the alkaline degradation.

The schematic structure in Fig. 1 proved less successful in predicting the composition and molecular-weight distribution of the oligomeric products obtained

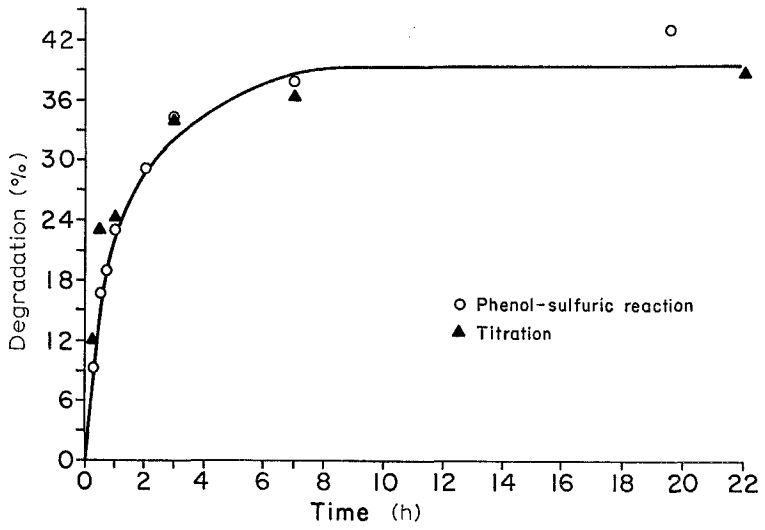

Fig. 2. Degradation of arabinogalactan in 0.1M NaOH at 78°, monitored by two separate methods.

by alkaline degradation. The ratio of galactose to arabinose in the preparation used in this study was 6.5:1, whereas the same ratio for the schematic structure is approximately 6:1. However, if the main chain of the molecule were composed of only galactose residues, as indicated by the structural scheme in Fig. 1, complete alkaline degradation should decrease this ratio to approximately 3.5:1. Instead, the ratio remained almost unchanged (6.4:1) for the oligomeric products obtained after degradation, as shown in Table I. This finding suggests that arabinose residues, instead of being absent from the main chain, are actually distributed almost evenly between the branch- and main-chain residues.

TABLE I

CARBOHYDRATE COMPOSITION OF ARABINOGALACTAN BEFORE AND AFTER ALKALINE DEGRADATION

Sample	*Relative molar amounts of*			*Molar ratios*	
	Arabinose	*Galactose*	*"Galactometasaccharinic" acid*	*Galactose/ Arabinose*	*Sugars/ Saccharinic acids*
Arabinogalactan	0.243	1.580	0.000	6.5/1	—
Degraded arabinogalactan	0.110	0.767	0.337	6.4/1	2.6/1
High mol.wt. fraction (H), 6%	0.010	0.521	0.055	5.2/1	9.7/1
Low mol.wt. fraction (L), 94%	0.204	1.334	0.685	6.5/1	2.2/1

TABLE II

ANALYSIS OF ACID LACTONES AS *O*-TRIMETHYLSILYL DERIVATIVES

Acid	*Retention time (min)*	*Estimated percentages of acids in fraction*	
		Low mol.wt. (L-)	*High mol.wt. (H-)*
Lactic	5.1	0	7
Unknown[a]	14.4	0	5
3-Deoxypentonic (C_5-metasaccharinic) acids			
erythro-[b]	24.9	1	0
threo-[b]	26.0	5	0
Unknown[a]	32.7	4	5
Unknown[a]	33.5	0	2
3-Deoxy-D-*xylo*-hexonic ("α-galactometasaccharinic") acid[b]	34.5	90	81
	Total:	100	100

[a]The absence of the following lactones amongst unknown components was established: glycolic, arabinonic, 3-deoxy-2-*C*-(hydroxymethyl)pentonic (= isosaccharinic) and D-glucuronic acid lactones.
[b]Lactone structures verified by mass spectra.

Supporting evidence for the presence of arabinose in the main chain was obtained by the identification of five-carbon metasaccharinic acids (3-deoxypentonic acids) among the products of acid hydrolysis of alkali-degraded arabinogalactan; the C_5 acids were identified by mass-spectral fragmentation patterns (Table II)[14,15]. Some earlier observations by Bouveng[3] also support this idea. He found that mild, acidic hydrolysis of arabinogalactan A (d.p. 600), under conditions such that only furanosidic bonds would be cleaved, decreased the molecular weight substantially, as exemplified by the isolation of fractions in the d.p. range 20 to 48. This extent of degradation would scarcely be possible without the presence of furanosidic sugar residues along the main chain.

Acidic products from the alkaline degradation of the structure in Fig. 1 would be expected to be preponderantly trimeric, with minor proportions of dimeric products. The degradation products obtained in the present study were fractionated by gel (Sephadex G-25) chromatography in order to obtain molecular-weight distribution-patterns. The results are shown in Fig. 3, which also illustrates the curves obtained for the undegraded arabinogalactan and for D-glucose.

The curve obtained shows a principal maximum in the trimeric region, concordant with the proposed structure of the arabinogalactan. In addition, however, a high molecular-weight maximum is present constituting ~6% by weight of the material. For more thorough characterization, the two fractions were isolated in larger quantities by using dialysis through a membrane having 24 Å average pore-size, and subjected to quantitative analysis as outlined in Fig. 4.

The low-molecular weight fraction (L) contained galactose and arabinose in the same ratio (6.5:1) as the original polymer. Ninety percent of the acids obtained by hydrolysis consisted of 3-deoxy-D-*xylo*-hexonic ("galactometasaccharinic") acid, identified as its α-lactone by means of mass spectrometry. In addition, 3-deoxy-*threo*- and *erythro*-pentonic acids were identified as probable products from main-chain, arabinose residues. The ratio of sugars to galactometasaccharinic acid was 2.2:1, supporting the approximate, average molecular-size of a trimer (Table I). Electrophoresis of the fraction displayed, in addition to a principal spot, six additional, distinguishable spots that were not investigated further. Thus, the presence of some dimeric and tetrameric species in this fraction is likely.

The high-molecular weight fraction (H) had a slightly lower galactose-to-arabinose ratio (5:1). 3-Deoxy-D-*xylo*-hexonic acid was again rigorously identified as the main component of acids obtained in hydrolysis. Surprisingly, C_5 metasaccharinic acids were absent, but instead minor proportions of lactic and three unknown acids were found to be present. The origin of the latter acids is not clear.

Fraction H contained sugars and galactometasaccharinic acid in the approximate ratio 10:1, suggesting an average d.p. of 11. The electrophoretic mobility of this fraction is a great deal lower than that of fraction L. It appears clear that this fraction also constitutes an authentic degradation product of an original side branch. Thus, the presence of side-branches, approximately ten residues long, must be postulated for the arabinogalactan.

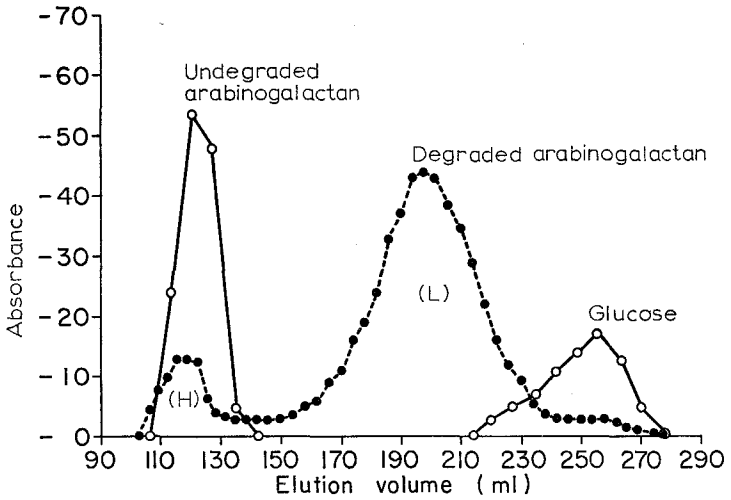

Fig. 3. Sephadex-gel separation of alkaline-degradation products from arabinogalactan.

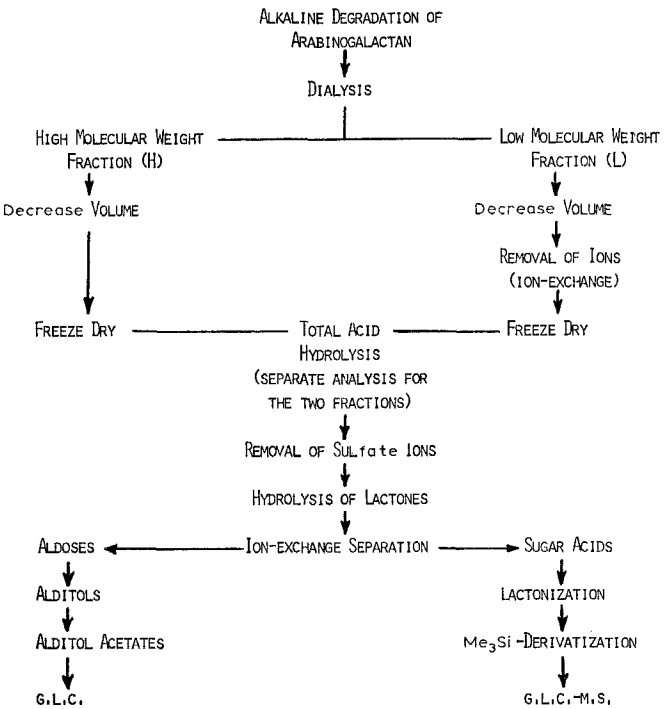

Fig. 4. Generalized analytical procedure adopted for analysis of arabinogalactan.

EXPERIMENTAL

Apparatus. — The gas chromatograph used was a Perkin–Elmer Model F-30 (flame-ionization detector) connected to an Infotronics Model CRS-104, electronic digital integrator for determination of retention times and integrated peak-areas. The following conditions were used for g.l.c. analysis: (*1*) alditol acetates[16,17], a column of 3% ECNSS-M on Gas Chrom Q, 100–120 mesh, operated isothermally at 190°, injector and detector temperatures at 250°; and (*2*) Me_3Si derivatives of sugar acids[14,15], column of 3% OV-17 on Gas Chrom G, 100–120 mesh, programmed from 70–170° at 3°.min^{-1}, injector and detector temperatures at 200°. The mass spectrometer was a PE-270 instrument operated at 20 eV and was used in combination with a gas chromatograph as just described.

Materials. — Reagent-grade ion-exchange resins Dowex-50 (H^+), 50–100 mesh and Dowex-1 X4, 50–100 mesh were utilized for separations. The Dowex-1 X4 was converted into the acetate form by eluting with a 2M solution of sodium acetate, with subsequent elution with distilled water. Analytical-grade acetic acid adjusted to 10% was used for elution of the retained lactones. The pyridine used for acetylation and trimethylsilylation was distilled, and dried over potassium hydroxide pellets.

Commercial monosaccharides of pure grade were used. A sample of purified arabinogalactan was kindly supplied by Professor T. E. Timell, State University of New York, College of Environmental Science and Forestry, Syracuse. Samples of pure 3-deoxy-D-*xylo*-hexonic ("galactometasaccharinic") acid, 3-deoxy-D-*ribo*-hexonic ("α-glucometasaccharinic") acid and 3-deoxy-D-*arabino*-hexonic ("β-glucometasaccharinic") acid were similarly supplied by Dr. O. Theander, Department of Chemistry, Agricultural College of Sweden, Uppsala, and Dr. R. Rowell, U.S. Department of Agriculture, Forest Products Laboratory, Madison. The 3-deoxy-L-*threo* (and L-*erythro*)-pentonic acids were prepared by treatment of L-arabinose with sodium hydroxide (0.1M) for 15 min at 95°. Their retention times were recorded and their mass spectra were used for identification[14,15]. The 3-deoxy-2-*C*-(hydroxymethyl)pentonic acid was similarly prepared, by alkaline treatment of cellobiose (0.1M sodium hydroxide, 2 h, 100°, nitrogen atmosphere), and identified likewise.

Procedure. — A generalized analytical procedure is shown in Fig. 4. This denotes the preferred total procedures, based on the detailed, total analyses described here. The major part of this procedure was developed from methods described in the literature[16–19].

Degradation and fractionation. — Distilled water was boiled to remove dissolved oxygen and carbon dioxide prior to preparation of the alkaline solution. Arabinogalactan (2.25 g) was dissolved in 800 ml of 0.1M sodium hydroxide, in a glass container that was thoroughly deaerated with nitrogen gas, heat sealed, and immersed in a constant-temperature bath for 24 h at 78°. The alkaline solution of polysaccharide was then dialyzed against 20 liters of distilled water. The solution retained in the dialysis sack, and also the dialyzate, were evaporated to low volume and the retained fraction, denoted as (H), was freeze dried; it weighed 130.6 mg. Sodium

ions were removed from the dialyzate (**L**) by Dowex-50 resin (H^+) and this fraction was similarly freeze dried and weighed (2.17 g).

Hydrolysis and separation of neutral sugars and acids. — The procedures of Blake and Richards[16] were used, except that the aldose and acid fractions were separated on a column of Dowex-1 X4 (acetate) resin and xylose was used as an internal standard.

Trimethylsilylation. — The Me_3Si derivatives were prepared from the lactones of the corresponding acids. The lactones were formed by treatment of the acids with 1 ml of 0.1M hydrochloric acid and evaporation to dryness. The lactone (1–10 mg) was suspended in 0.5 ml of anhydrous pyridine and 0.2 ml of *N,O*-bis(trimethylsilyl)-trifluroacetamide and 0.1 ml of chlorotrimethylsilylsilane were added. After a few h at room temperature, the solution was evaporated in a rotary evaporator and the residue dissolved in hexane to give a final concentration of about 1 mg/ml for g.l.c.–m.s. analysis.

The mass spectrum of 3-deoxy-D-*xylo*-hexonic acid lactone ("α-galactometasaccharinic" acid lactone) exhibited distinct peaks for *m/e* 378 (M^+), 363 (0.5), 322 (0.5), 292 (4), 273 (4), 258 (2), 246 (10), 219 (4), 205 (15), 189 (10), 147 (29), 129 (40), 117 (10), 103 (25), and 73 (100).

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REFERENCES

1 T. E. TIMELL, *Adv. Carbohydr. Chem.*, 20 (1965) 409–483.
2 E. V. WHITE, *J. Am. Chem. Soc.*, 63 (1941) 2871–2875; 64 (1942) 302–306, 1507–1511, 2838–2842.
3 H. O. BOUVENG AND B. LINDBERG, *Acta Chem. Scand.*, 12 (1958) 1977–1984.
4 H. O. BOUVENG, *Acta Chem. Scand.*, 13 (1959) 1869–1876.
5 H. O. BOUVENG, *Acta Chem. Scand.*, 13 (1959) 1877–1883.
6 H. O. BOUVENG AND B. LINDBERG, *Acta Chem. Scand.*, 10 (1956) 1515–1519.
7 H. O. BOUVENG, *Acta Chem. Scand.*, 15 (1961) 78–86.
8 H. A. SWENSON, H. M. KAUSTINEN, J. J. BACHHUBER, AND J. A. CARLSON, *Macromolecules*, 2 (1969) 142–145.
9 R. L. WHISTLER AND J. N. BEMILLER, *Adv. Carbohydr. Chem.*, 13 (1960) 289–329.
10 R. L. WHISTLER AND J. N. BEMILLER, *J. Org. Chem.*, 26 (1960) 2886–2892.
11 H. MOSIMANN AND T. SVEDBERG, *Kolloid-Z.*, 100 (1942) 99–105.
12 M. DUBOIS, K. A. BILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
13 R. A. YOUNG, K. V. SARKANEN, P. G. JOHNSON, AND G. G. ALLAN, *Carbohydr. Res.*, 21 (1972) 111–122.
14 G. PETTERSON, O. SAMUELSON, K. ANJOU, AND E. SYDOW, *Acta Chem. Scand.*, 21 (1967) 1251–1256.

15 G. PETTERSON, *Tetrahedron*, 26 (1970) 3413–3428.
16 J. D. BLAKE AND G. N. RICHARDS, *Carbohydr. Res.*, 14 (1970) 375–387.
17 E. P. CROWELL AND B. B. BURNETT, *Anal. Chem.*, 39 (1967) 121–124.
18 R. MALINEN AND E. SJÖSTRÖM, *Paperi Puu*, 54 (1972) 451–468; 55 (1973) 5–7, 10–13.
19 E. SJÖSTRÖM, D. HAGLUND, AND J. JANSSON, *Sv. Papperstidn.*, 69 (1966) 381–385.