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The Chromatographic Behaviour of a Series of Fructo-oligosaccharides Derived from Levan Produced by the Fermentation of Sucrose by *Zymomonas mobilis*

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ABSTRACT

Levan, a (2→6)-β-D-fructofuranosyl linear polysaccharide has been produced by fermentation of sucrose by Zymomonas mobilis. *Complete hydrolysis of levan and HPLC analysis have shown that the molecular weight of the polymer is in the order of 10^6 daltons. Mild acid hydrolysis of levan produced a series of (2→6)-β-D-fructofuranosyl oligosaccharides of up to DP 14, which were resolved by GPC on Bio-Gel P2.*

The elution positions of these oligosaccharides were different from those of (1→4)-α-D- and (1→6)-β-D-glucopyranosyl oligosaccharides but similar to those of (1→4)-α-D-glucopyranosyl oligosaccharides. These differences have been explained in terms of anomeric configuration for each series of oligosaccharides.

INTRODUCTION

Levan is a polysaccharide consisting of (2→6)-β-D-fructofuranosyl residues that can contain a number of (2→1)-β-D-fructofuranosyl branch points. Levan which is found in some grasses is generally a low

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molecular weight material of degree of polymerisation (DP) 20–30 and may contain a single branch point. Levan, which is produced extracellularly by a large number of bacterial strains (*Aerobacter levanicum, Pseudomonas prunicula, Cornyebacterium* species, *Bacillus subtilis* and *Zymomonas mobilis*) by the action of levansucrase (EC 2.4.1.10) on sucrose in contrast has a high molecular weight, of the order of millions, and can contain branched chains of DP 10–12. The molecular weight of such levans was found to be dependent on the ionic strength of the medium, with low ionic strength medium producing polysaccharides of high molecular weight (Tanaka *et al.*, 1979, 1980). The addition of lower molecular weight levan to such cultures stimulates the production of higher molecular weight levan, by the addition of β-D-fructofuranosyl on to the lower molecular weight material and not by addition of lower molecular weight material on to the existing higher molecular weight material (Tanaka *et al.*, 1980). Thus, sucrose acts as a β-D-fructofuranosyl acceptor to produce the growing chain under the action of levansucrase, and the length of the chain determines the susceptibility of itself as a β-D-fructofuranosyl acceptor. In addition to high molecular weight levan, fructo-oligomers (di-, tri- and tetra-mers) consisting of glucose and fructose have also been detected during the fermentation of sucrose by *Zymomonas mobilis* (Viikari & Linko, 1986). The formation of these transfructosylation products has also been observed by cell free extracts of *A. levanicum* (Feingold *et al.*, 1956), but these oligosaccharides have not been characterised by modern techniques.

In a previous communication (White *et al.*, 1985), we reported the chromatographic behaviour on Bio-Gel P2 of a series of non-reducing oligofructosides from the roots of *Arcania montana* L. These oligofructosides were of the inulin type with D-glucose glycosidically bound to the reducing ends with the structure:

$$[\beta\text{-D-Fru}f\text{-}(2\rightarrow 1)]_n\text{-}\beta\text{-D-Fru}f\text{-}(2\leftrightarrow 1)\text{-}\alpha\text{-D-Glc}$$

The chromatographic behaviour was different from that of malto-oligosaccharides in that the oligofructosides eluted comparatively earlier and therefore appeared larger in size. We now report the chromatographic behaviour of a series of reducing fructo-oligosaccharides derived from levan by acid hydrolysis that have the structure:

$$[\beta\text{-D-Fru}f\text{-}(2\rightarrow 6)\text{-}\beta\text{-D-Fru}f]_n$$

The chromatographic behaviour was similar to that of the isomalto-oligosaccharide series.

EXPERIMENTAL

Materials

Amylose A-A from potato, previously characterised and found to be an essentially linear molecule (Kennedy *et al.*, 1986), was supplied by F. Hoffmann La Roche and Co., Basel, Switzerland. Dextran T110 was obtained from Pharmacia Fine Chemicals, Upsala, Sweden; and pustulan from the Birmingham Collection. All chemicals were of analytical grade.

Production of levan

Levan was obtained by fermentation of sucrose by *Z. mobilis* VTT-E-78082 as described elsewhere (Viikari, 1984). The levan produced was precipitated from the fermentation broth by 75% ethanol. The precipitate was centrifuged at 4500 rpm for 10 min, washed with 75% ethanol, ground and dried at 70°C for 1 h.

Hydrolysis of levan for HPLC analysis

A sample of levan (300 mg) was hydrolysed with hydrochloric acid (0·1 M, 10 ml) at 100°C for 1 h and neutralised in Amberlite MB 3 (BDH Chemicals) ion-exchange resin. Sugars were determined by high-performance liquid chromatography (HPLC) (Optilab 931 HSRI) using an Aminex 87-C column (Bio-Rad Laboratories), temperature 85°C, with water as eluent (0·8 ml h^{-1}) and a refractometer (Optilab Multiref 902C) as detector. Only D-fructose and small amounts of ethanol were detected showing that the polysaccharide consisted only of D-fructose (Fig. 1).

Hydrolysis of levan for oligosaccharide component compositional analysis

A weighed amount (20 mg) of levan was partially dissolved in distilled water (0·5 ml) and sulphuric acid (0·05 M, 0·5 ml) added such that the overall acid concentration was 0·025 M. The mixture was heated at 100°C in a boiling water bath for 3 min and immediately neutralised by the addition of barium carbonate. After centrifugation, an aliquot (100 μl) was injected on to a water jacketed column (140 × 2·8 cm i.d.) of Bio-Gel P2, −400 mesh (Bio-Rad Laboratories) maintained at 60°C, to eli-

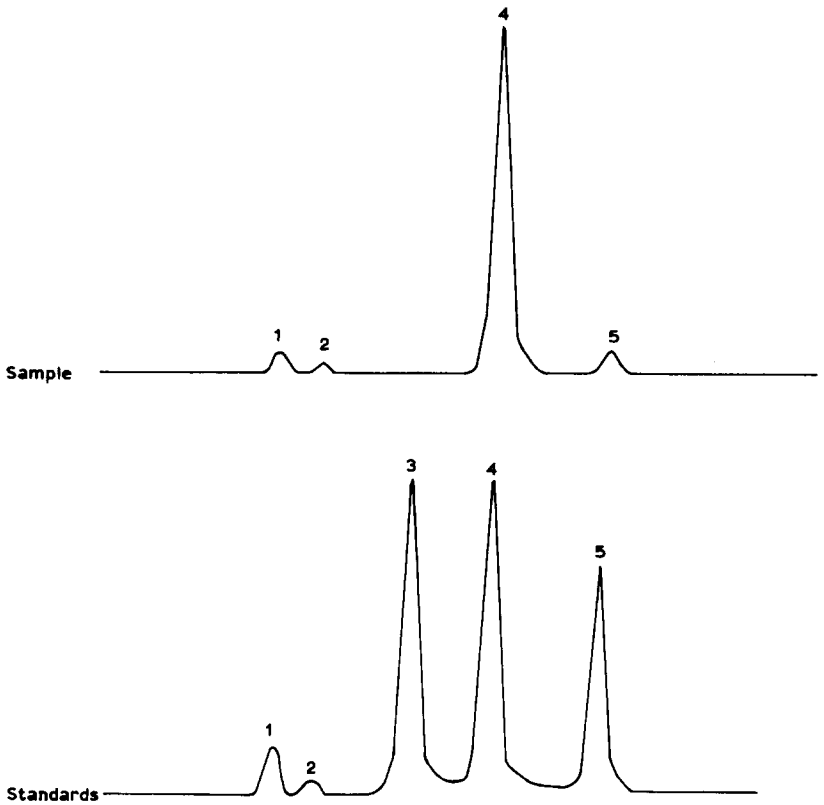

Fig. 1. HPLC analysis of levan hydrolysate peak. 1, system peak. 2, water. 3, D-glucose. 4, D-fructose. 5, ethanol.

minate hydration effects relating to the gel (John *et al.*, 1969; Heyraud & Rinando, 1978). The column was continuously pumped at a constant flow rate (0·86 ml min^{-1}) with sodium chloride solution (0·1 M) to prevent non-specific adsorption phenomena (Kennedy, 1972; White & Kennedy, 1979) using a piston pump (Metering Pumps Ltd, Series II). The column eluent was continuously monitored using an automated L-cysteine-sulphuric acid assay (Barker *et al.*, 1968; White & Kennedy, 1981) incorporating Technicon AA1 modules. The chromatograms were displayed on a chart recorder that ran at a consant speed of 2 in h^{-1} (5·1 cm h^{-1}).

Hydrolysis of other polysaccharides

A weighed amount (20 mg) of amylose A-A was suspended in distilled water (1·0 ml) and sulphuric acid (0·2 M, 1·0 ml) added such that the

overall acid concentration was 0·1 M. The mixture was heated at 100°C on a boiling water bath for 30 min, neutralised with barium carbonate and centrifuged. Similarly weighed amounts (20 mg) of dextran T110 and pustulan were dissolved in distilled water (1·0 ml) and sulphuric acid (18 M, 50 μl) added such that the overall acid concentration was 0·9 M. The solutions were heated at 100°C on a boiling water bath for 15 min, neutralised with barium carbonate and centrifuged. Aliquots (100 μl) of all hydrolysates were analysed for oligosaccharide component composition on a column of Bio-Gel P2, −400 mesh as previously described.

RESULTS AND DISCUSSION

Analysis of levan by HPLC showed D-fructose as the only sugar detected. Since the biosynthesis of levan takes place using sucrose as a template, a proportion of D-glucose would be expected to be present. In order to account for this the levan produced must be of extremely high molecular weight, so that the amount of D-glucose present must have been so low as to escape detection limits on the HPLC system. An estimated molecular weight of 1 million would correspond to a fructose:glucose ratio of about 6000:1. This is also borne out by the low water solubility of the levan, since 20 mg was barely soluble in 0·5 ml of water.

Hydrolysis of levan by sulphuric acid to produce a spectrum of oligosaccharides proved to be very difficult due to high susceptibility of the (2→6)-β-D-fructofuranosyl glycosidic linkage to acid hydrolysis. For instance, 0·1 M acid for 15 min 100°C produced more than 99% fructose. The chromatogram shown in Fig. 2 for levan hydrolysate in which the hydrolysis conditions were extremely mild was the best chromatogram obtained under varying hydrolysis conditions since it contained resolvable component up to the degree of polymerisation (DP) 14 with intermediate and high molecular weight. Within 30 s of the hydrolysis the levan was totally solubilised demonstrating that the original polysaccharide has a very high molecular weight and is rapidly depolymerised by sulphuric acid.

A series of chromatographic peaks were obtained for all polysaccharide hydrolysates which correspond to individual oligomers from DP 1–20, plus unresolved areas of the chromatograms corresponding to intermediate molecular weight and high molecular weight materials as shown in Fig. 2. The elution position of the chromatographic peaks corresponding to a particular DP was found to be different for all oligosaccharide series with the exception of DP 1 (D-glucose and D-fructose

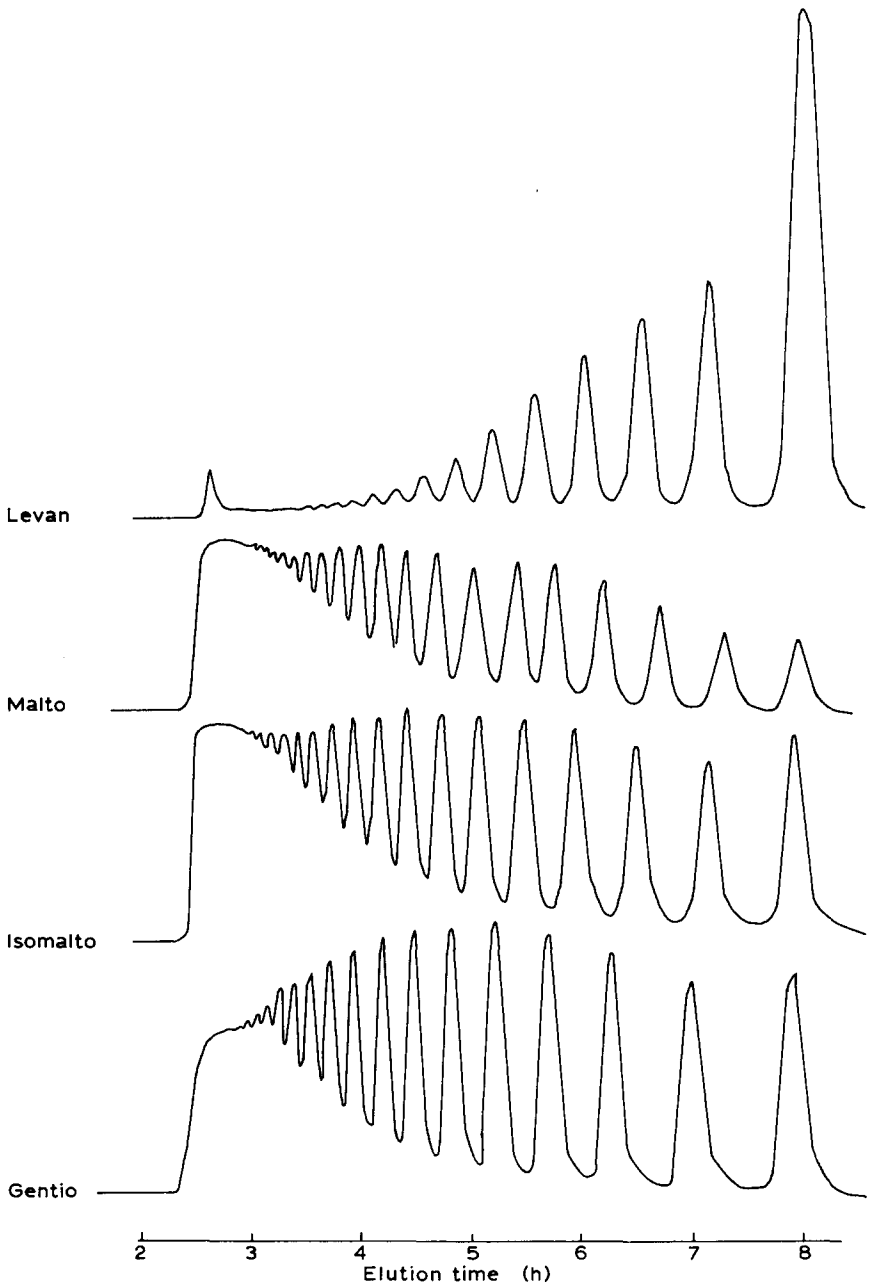

Fig. 2. Gel permeation chromatograms for the hydrolysates of levan, amylose, dextran, and pustulan.

are not separated on Bio-Gel P2). When the elution volume (V_e) of a particular component is expressed in terms of the total column volume (V_t), which in this case is equivalent to the elution position of either D-glucose or D-fructose, then the relative elution volume (V_e/V_t) is independent of the parameters of a particular column. The relative elution volumes for all oligosaccharide series are shown in Table 1. Taking the malto-oligosaccharide series as a standard and plotting relative elution volumes against $\log_{10}$ (mol wt) gives a curve that is linear in the middle of the fractionation range (Fig. 3). However, when the relative elution volumes are plotted against DP, a non-linear relationship is found (Fig. 3). Since gel permeation chromatography separates molecular mixture according to molecular size, the relative size of all other oligosaccharide series can be computed using the relative elution volume for each oligosaccharide (Table 1) and the non-linear relationship between the relative elution positions and the DP for the malto-oligosaccharide

TABLE 1
Relative Elution Positions (V_e/V_t) for Oligosaccharide Series

DP	*Oligosaccharide series*			
	Malto-	*Isomalto-*	*Gentio-*	*Levan*
1	1·000	1·000	1·000	1·000
2	0·917	0·904	0·885	0·888
3	0·844	0·823	0·794	0·809
4	0·778	0·754	0·723	0·744
5	0·721	0·695	0·662	0·687
6	0·680	0·645	0·613	0·640
7	0·631	0·601	0·571	0·598
8	0·589	0·564	0·534	0·561
9	0·557	0·532	0·505	0·531
10	0·528	0·502	0·478	0·504
11	0·501	0·480	0·456	0·479
12	0·479	0·458	0·436	0·459
13	0·460	0·441	0·419	0·442
14	0·443	0·424	0·404	0·429
15	0·428	0·409	0·392	ur
16	0·416	0·397	0·380	ur
17	0·403	0·387	0·373	ur
18	0·381	0·377	ur	ur
19	0·374	ur	ur	ur
20	ur	ur	ur	ur

ur = unresolved chromatographic area.

series (Fig. 3). Plots of apparent molecular size relative to malto-oligosaccharides against actual DP produce straight lines (Fig. 4) from which the gradients (Table 2) give the relative molecular size to the malto-oligosaccharide series. From Table 2 and Fig. 4, it is apparent that the series of fructo-oligosaccharides derived from levan resemble the isomalto-oligosaccharide series.

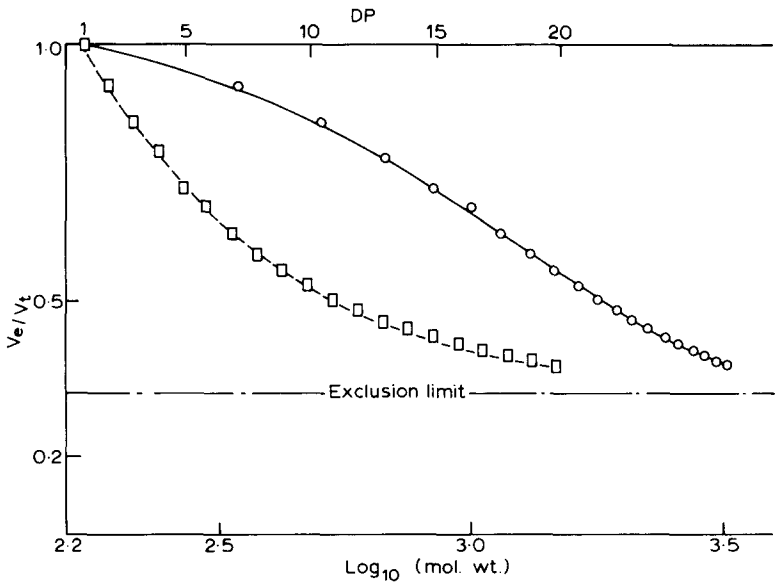

Fig. 3. Relationship between relative elution volume (V_e/V_t) and $\log_{10}$ molecular weight (o——o) and DP (□- - -□) for malto-oligosaccharides on Bio-Gel P2 (−400 mesh).

TABLE 2
Apparent Size of Oligosaccharide Series Relative to Malto-oligosaccharide Series

Oligosaccharide series	*Size relative to malto-oligosaccharide series* (1→4)-α-D-Glc
Levan (2→6)-β-D-Fru	1·09
Isomalto- (1→6)-α-D-Glc	1·11
Gentio- (1→6)-β-D-Glc	1·24

Since all these linear oligosaccharide series contain either D-glucose or D-fructose, which are not separated by gel permeation chromatography, and differ only in the nature of the glycosidic linkage, differences in elution position can only be due to differences in glycosidic linkage. As gel permeation chromatography separates molecular mixtures according to molecular size, differences in elution position can only be due to differences in apparent hydrodynamic volume brought about by differing glycosidic linkages.

The geometry of individual sugar rings in an oligosaccharide is essentially rigid, but the relative orientation of each sugar component brought about by rotation around the glycosidic linkages determines the overall conformation of the oligosaccharide. The range of rotational

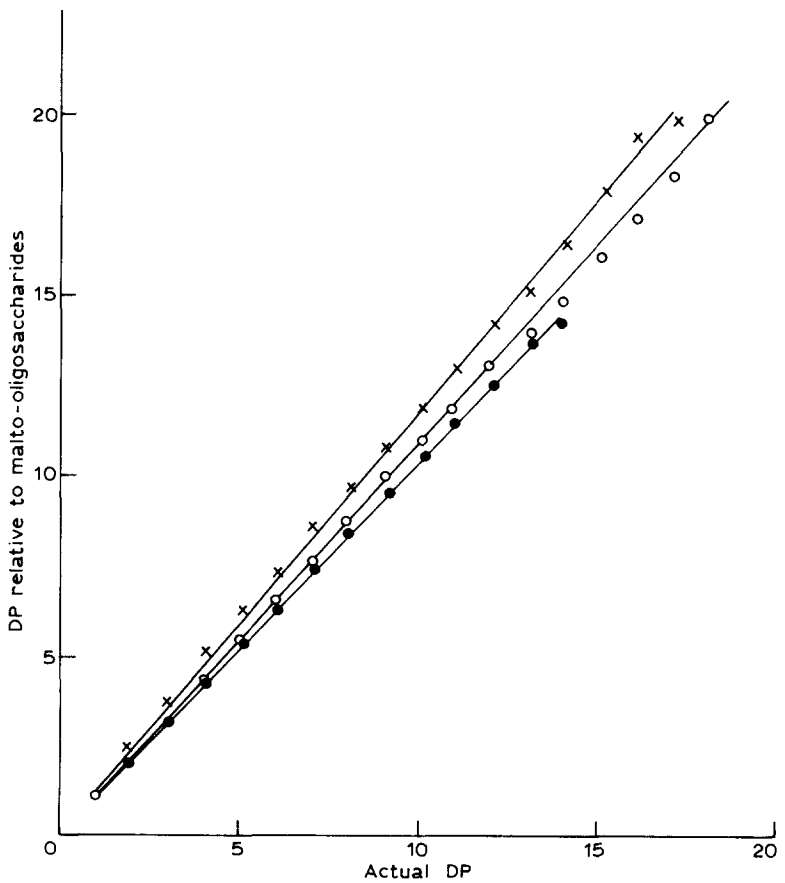

Fig. 4. Apparent DP of oligosaccharides derived from pustulan (×——×), dextran (○——○) and levan (●——●), relative to malto-oligosaccharides versus actual DP.

angles about the glycosidic linkage can be severely restricted by steric hindrance between adjacent sugar rings and by non-bonding interactions between functional groups in adjacent sugar rings. In order for oligosaccharides to have minimum energy, different angles of rotation about the glycosidic linkage are adopted and the overall tertiary structure of the oligosaccharide is formed (Rees, 1977). In the case of the oligosaccharides under study the tertiary structure of malto-oligosaccharides is that of an α-helix (French & Murphy, 1973), whereas that for all other oligosaccharides under study is a random coil with differing degrees of rotational freedom about the glycosidic linkages and methylene bridges.

Thus malto-oligosaccharides elute later than all other oligosaccharides under study and therefore 'appear' smaller since the α-helix is a very compact structure and therefore can enter the pores of the gel more easily than the other oligosaccharides. The oligosaccharides from levan, which have both a glycosidic linkage and a methylene bridge, are structurally similar to those of the isomalto- and gentio-oligosaccharide series and differ only in the nature of the monosaccharide repeating unit. From Table 2 it is clear that fructo-oligosaccharides from levan resemble closely isomalto-oligosaccharides and not gentio-oligosaccharides, so therefore it can be concluded that the tertiary structures are also similar. Thus, a $(1\rightarrow6)$-α-D-glycosidic linkage between D-glucopyranosyl residues brings about the same tertiary structure and observed chromatographic behaviour of a $(2\rightarrow6)$-β-D-glycosidic linkage between D-fructofuranosyl residues. The small difference in elution behaviour is almost certainly due to the differing monosaccharide repeating units.

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