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Note

Isolation of levanoligosaccharides from a partial acid hydrolysate of levan by cellulose column chromatography

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Carbon–Celite column chromatography using aqueous ethanol[1] or 1-butanol[2] for elution has been widely used for the preparative separation of various oligosaccharides. However, for our purpose of preparing a series of levanoligosaccharides from an acid hydrolysate of levan, this type of chromatography appeared not to be sufficiently sensitive to separate a single levanoligosaccharide component from branched oligofructans with the same degree of polymerization (DP) in the hydrolysate, as levan is highly branched. Cellulose powder column chromatography using various 1-butanol–ethanol–water mixtures for elution of homologous glucooligosaccharides has been developed[3], but the method is complicated. Levanbiose and -triose have been isolated by repeated carbon–Celite column chromatography[4], but no other levanoligosaccharide has been isolated.

We report here a simple cellulose column chromatographic method useful for the preparation of a series of levanolilosaccharides (DP = 4–6) from the hydrolysate in combination with carbon–Celite column chromatography.

EXPERIMENTAL

Levan sample and column chromatography

Levan (4 g), which was prepared by cultivating *Bacillus mesentericus* Trevisan (IFO 3034) in a sucrose-containing culture medium as described earlier[5], was hydrolysed in 40 ml of 1 *N* sulphuric acid at 20°C for 2 h under the conditions indicated by Schlubach and Blaschke[4]. The hydrolysate was cooled, neutralized with barium carbonate and filtered.

A 27 × 4.4 cm carbon–Celite column composed of 60 g of Tokusei–Shirasagi carbon (Takeda Pharmaceutical Co., Japan) and 120 g of Celite No. 535 (Johns-Manville, U.S.A.) was prepared as described earlier[6]. After application of the sample of acid hydrolysate and washing with distilled water to remove the monosaccharide fraction, the column was eluted with a water (5 l)–15 % ethanol (5 l) gradient system, and 100-ml fractions were collected at a flow-rate of 100 ml/h. The elution of oligosaccharides was monitored by the resorcinol–hydrochloric acid method[7] and their homogeneity examined by thin-layer chromatography (TLC) on silica gel 60F plates (Merck) by development with 1-butanol–ethanol–water (2:1:1)[8] (solvent I) and spraying with naphthoresorcinol–sulphuric acid reagent[9].

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Whatman CF 11 cellulose powder (40 g) suspended in 400 ml of solvent I was packed to a height of about 25 cm in a 30 × 2.8 cm column, which contained a fritted glass disk in the lower part and was attached to a glass tube with a stopcock. The column was further washed with 400 ml of solvent I. The top surface of the cellulose was covered with a disk of filter-paper. The sample (100–120 mg) was dissolved in 1 ml of distilled water, mixed well with 1 g of silica gel powder (Wakogel C-200; Wako, Japan), which had been washed well with solvent I and dried at 100°C, and dried in a desiccator over silica gel under reduced pressure. This sample was layered evenly on the cellulose column and about 10 ml of solvent I were gently layered on it. After sinking in a disk of filter-paper to cover the surface of the sample layer, Wakogel C-200 soaked in solvent I was layered on it to a height of about 1 cm. The column was eluted at room temperature with solvent I at a flow-rate of 15 ml/h and 10-ml fractions were collected. The elution of oligosaccharides and their homogeneity were examined as described above.

Methylation analysis

The separated oligosaccharide samples (10 mg) were methylated by the method of Hakomori[10] by repeating the procedure two or three times for complete methylation and were hydrolysed as reported earlier[11]. The hydrolysates were analysed quantitatively by the phenol–sulphuric acid method[12] for O-methyl ethers of fructose after separation by TLC on silica gel 60F plates (Merck) by development with a 9:1 mixture of the upper layer of benzene–ethanol–water (200:47:15) and 1-butanol (ascending) and extraction with chloroform–methanol (1:1). 1,3,4,6-Tetra-O-methyl-, 1,3,4-tri-O-methyl- and 3,4-di-O-methylfructoses {$[\alpha]_D^{20}$ in water +29.6° (*c* 0.59), −54.2° (*c* 1.08) and −63.0° (*c* 0.78), respectively}, used as the standards for TLC and quantitative analysis, were prepared by methylation of sucrose and levan by the method of Hakomori[10] and subsequent acid hydrolysis with oxalic acid[13] and separation of the hydrolysates by chromatography on a silica gel column[5].

RESULTS AND DISCUSSION

Fig. 1 shows the elution profiles of oligosaccharides contained in the acid hydrolysate of *B. mesentericus* levan obtained by carbon–Celite column chromatography with a water–15% ethanol gradient elution system after washing out the monosaccharide fraction with distilled water. The oligosaccharides were eluted with six peaks, designated A–F in order of elution, were pooled each within the range indicated by the bars, concentrated at 40°C, lyophilized (A) or precipitated with ethanol and washed with diethyl ether and dried in a desiccator over silica gel under reduced pressure (B–F). Fraction F was excluded from the study as it was present in only a small amount. Fractions C, D and E were purfied by fractionation on a cellulose column. The elution profiles of the oligosaccharides are presented in Fig. 2. The fractions indicated by the bars were pooled, treated as described above and obtained as a white powder. Each sample thus obtained gave a single spot on TLC.

Table I shows the results of the characterization of the purified samples examined for the ratio of reducing sugar to total ketohexose (DP) and specific rotation, and those of methylation analysis, showing that the samples of C, D and E represented levantetraose, -pentaose and -hexaose, respectively.

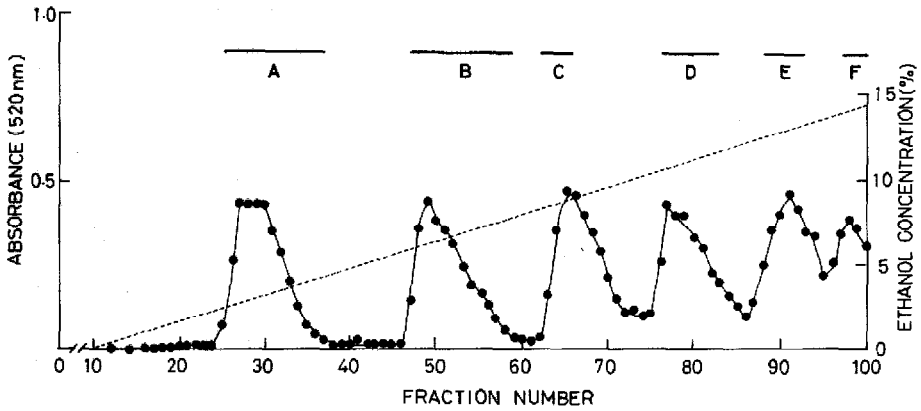

Fig. 1. Fractionation of a levan acid hydrolysate on a carbon–Celite column using a water–15% ethanol gradient elution system. The fractions were monitored by the resorcinol–hydrochloric acid method and the fractions indicated by the bars were collected.

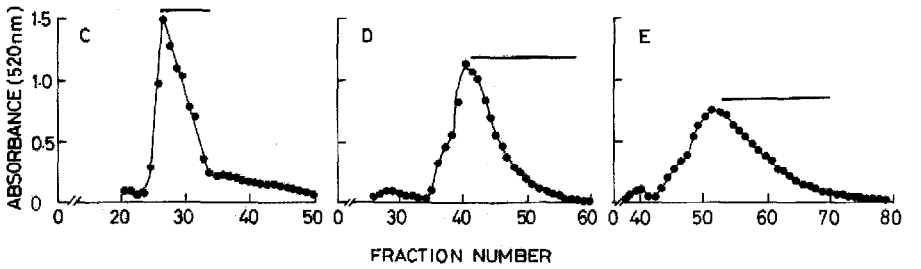

Fig. 2. Fractionation of the fractions C, D and E on a cellulose powder column by development with 1-butanol–ethanol–water. The fractions were monitored as in Fig. 1, and those showing a single sugar component on TLC (indicated by the bars) were collected.

TABLE I

IDENTIFICATION OF SEPARATED OLIGOSACCHARIDES

Fraction (yield from 4 g of levan, mg)	*Total ketohexose/ reducing sugar**	$[\alpha]_D^{20}$ *in* H_2O *(c)*	*Lit.* $[\alpha]_D^{20}$**	*Composition of O-methyl-fructoses*** (theoretical)*
C (110)	4.01	−31.0° (0.77)	−31.8°	1:2.73:trace (1:3:0)
D (60)	4.75	−32.8° (0.60)	−34.0°	1:3.64:trace (1:4:0)
E (90)	5.70	−34.8° (1.04)	−35.5°	1:4.70:trace (1:5:0)

* Determined by the resorcinol–hydrochloric acid method[7] and the method of Nelson[14]–Somogyi[15] using fructose as the standard.

** Calculated value by Schlubach and Blaschke[4] according to a relationship between molecular rotation and DP.

*** Molar ratio of 1,3,4,6-tetra-O-methylfructose, 1,3,4-tri-O-methylfructose and 3,4-di-O-methylfructose.

Fractions A and B obtained by carbon–Celite column chromatography were determined to be a crude levanbiose and -triose preparation, respectively, by TLC and by determinations of DP and optical rotation. Schlubach and Blaschke[4] isolated the two oligosaccharides from an acid hydrolysate of leven by repeated carbon–Celite column chromatography. The present cellulose column chromatography was also effective for the purification of these levanbiose and -triose preparations. The preliminary treatment on a carbon–Celite column was useful for concentration of oligosaccharide components and also for repeated use of the same cellulose column.

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