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LINKAGE SEQUENCING OF OLIGOSACCHARIDES BY THEIR RATES OF ALKALINE DEGRADATION

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ABSTRACT

D-Glucose oligosaccharides were subjected to alkaline degradation in saturated calcium hydroxide at room temperature. After appropriate time-intervals the calcium hydroxide was neutralized and the oligosaccharides remaining after alkaline degradation were separated on Bio-Gel P-2 with water or boric acid as the solvent. The rates of alkaline degradation and hence the relative amounts of the various oligosaccharides present after alkaline degradation vary, and are dependent on the linkages in the original oligosaccharide.

INTRODUCTION

Learning the structures of the products formed during enzymic hydrolysis of polysaccharides gives valuable information concerning the action patterns of the depolymerases involved. Oligosaccharides having more than one linkage type, such as may be obtained during enzymic hydrolysis of the (1→3), (1→4)-β-D-glucan from barley, are of considerable interest in such studies[1]. However, the unequivocal structural identification of oligosaccharides larger than trisaccharides is tedious.

Advantage has been taken of alkaline degradation reactions to devise a relatively simple procedure for determining linkage sequences in oligosaccharides containing (1→3) and (1→4) linkages. Under alkaline conditions, oligosaccharides are degraded by a β-alkoxylcarbonyl elimination-reaction involving an enolate ion intermediate[2–5], which results in the elimination of successive residues from the reducing end of the oligosaccharide. The rate of the elimination reaction and the structures of the elimination products depend upon the position of the glycosidic linkage. Seventy percent of reducing-terminal, (1→3)-linked residues are removed from D-glucose oligosaccharides after 4 h in saturated calcium hydroxide at 25°, D-glucometasaccharinic acid being the main product[3,5]. In contrast, (1→4)-linked reducing residues are removed much more slowly, and the main product is D-glucoisosaccharinic acid[5,6].

Barker *et al.*[7] have proposed a method of linkage analysis of oligosaccharides based on the t.l.c. identification of the saccharinic acids released and the order of their

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appearance. In the method described in this paper, the order of the (1→3) and (1→4) linkages in oligosaccharides is indicated by the rates of appearance and relative amounts of oligosaccharide degradation-products of successively lower degrees of polymerization. The gel-filtration method of Dellweg *et al.*[8] is used to separate and quantitate the oligosaccharide degradation-products.

MATERIALS AND METHODS

Oligosaccharides. — Laminaratetraose and cellotetraose were prepared by partial acid hydrolysis of laminaran and cellulose, respectively, and were separated from their cogeners by preparative chromatography on charcoal–Celite–stearic acid columns[10]. Each tetraose gave a single peak on Bio-Gel P-2 chromatography, and partial acid hydrolysis yielded only the disaccharides laminarabiose and cellobiose, respectively. 3-*O*-β-cellotriosyl-D-glucose[11], 3-*O*-β-cellotetraosyl-D-glucose[11], and 4-*O*-β-(lamanaribiosyl)cellobiose[12] were isolated from enzyme hydrolyzates of barley β-D-glucan.

Gel filtration. — A column of Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, California, U.S.A., Batch. No. 11712) was prepared essentially as described by John *et al.*[9]. The column (170 cm × 0.9 cm diameter) was maintained at an elevated temperature by circulating water at 60° through the jacket.

The column effluent was monitored by a differential refractometer (Packard Instruments Company Inc., 220 Warrenville Road, Downers Grove, Ill. 60515, Model 7102) coupled to a recorder (Tohshin Electron Co. Ltd., Japan, Model TO2N1) The flow rate was maintained at 0.465 ±0.01 ml/min. Weights of materials in the effluent are reflected as peaks on the recorder chart. Peak heights are given in arbitrary units in the Figures presented in this paper. A peak 10 units high corresponds to approximately 38 μg of carbohydrate, with a precision of ±3 μg. The proportionality applies quite well for peaks higher than 5 units. The responses of carbohydrates present in amounts less than 10 μg often could not be distinguished from short-term baseline drift.

Alkaline degradation. — Oligosaccharides were treated with saturated calcium hydroxide at room temperature. At suitable times, the reaction was stopped by adjusting the pH of the mixture to between 5 and 7 with 0.05M sulphuric acid or by treatment with the acid form of Amberlite IR-120 (Rohm & Haas Ltd., Philadelphia, Pa., U.S.A.). Solutions usually contained ~1 mg/ml of carbohydrate. The amount of solution that needed to be applied to the Bio-Gel P-2 column depended on the sensitivity of the detector and the time of reaction. Replicate samples degraded in vessels flushed with nitrogen or simply degraded in closed vessels gave the same results.

RESULTS AND DISCUSSION

Laminaratetraose is rapidly degraded (peeled) under the alkaline conditions. After 4 h (Fig. 1b), 70% of the initial material had been degraded, whereas after 8 h essentially all of the tetrasaccharide had disappeared. The products at 4 h were

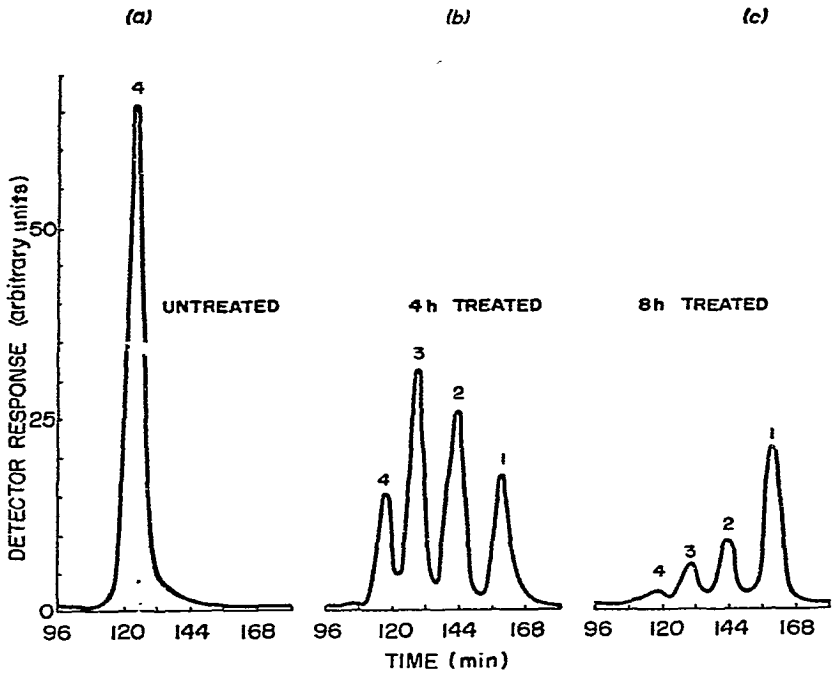

Fig. 1. Elution profiles for laminaratetraose and its alkaline-degradation products: (*a*) untreated starting material, (*b*) products after 4 h, and (*c*) 8 h. The numbers 1, 2, 3, and 4 refer to the degrees of polymerization of the products.

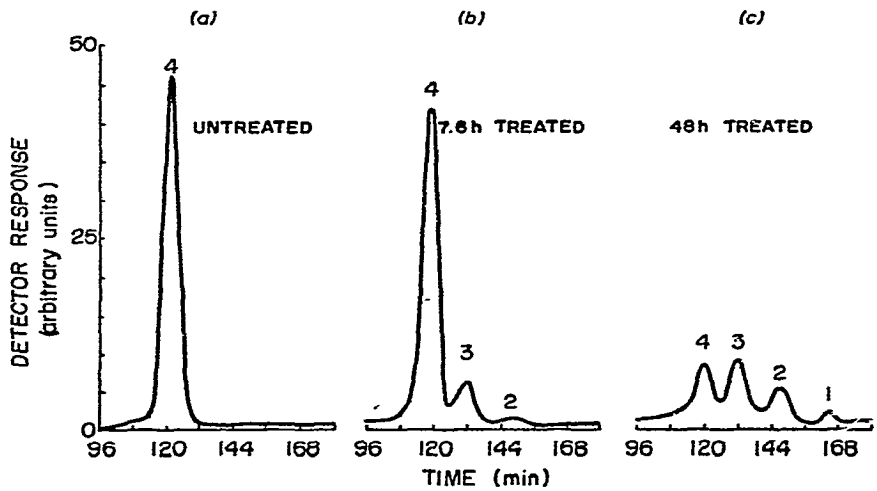

Fig. 2. Elution profiles for cellotetraose and its alkaline-degradation products: (*a*) untreated starting material, (*b*) products after 7.6 h, and (*c*) 48 h. The numbers 1, 2, 3, and 4 refer to the degrees of polymerization of the products.

compounds having elution volumes characteristic of the three lowest members of the D-glucose oligosaccharide series and quantitatively $G_3 > G_2 > G_1$ (Fig. 1b). After 8 h, these three products were still present, but quantitatively $G_1 > G_2 > G_3$ (Fig. 1c). The subscripts 1, 2, and 3 indicate the degree of polymerization of the saccharides.

When interpreting the results, it should be realized that peak heights represent weights of material present and that factors need to be applied to the heights of the peaks to recognize the molar proportion of each compound present. Thus 100 μg of a D-glucose tetrasaccharide corresponds to 0.150 μmoles, 100 μg of a trisaccharide to 0.198 μmoles, 100 μg of a disaccharide to 0.320 μmoles, and 100 μg of glucose to 0.555 μmoles.

Cellotetraose exhibited quite a different pattern of peeling, and, in contrast to laminaratetraose, only about 10% had been degraded after 7.6 h (Fig. 2b). Even after 48 h, approximately 30% of the tetrasaccharide remained (Fig. 2c) and the trisaccharide and disaccharide also persisted. Only a trace of a compound having the elution volume of D-glucose was present at that time (Fig. 2c).

Treatment of the tetrasaccharide 3-*O*-β-(cellotriosyl)-D-glucose with calcium hydroxide for 22.5 h completely removed the (1→3)-linked glucose residue at the reducing terminus and the preponderant product was a trisaccharide, together with some disaccharide (Fig. 3c). The products were themselves degraded only slowly during further alkaline treatment (Fig. 3d), so that at 64 h trisaccharide, disaccharide, and monosaccharide were still present, as would be expected for (1→4)-linked oligosaccharides (Figs. 2b and 2c).

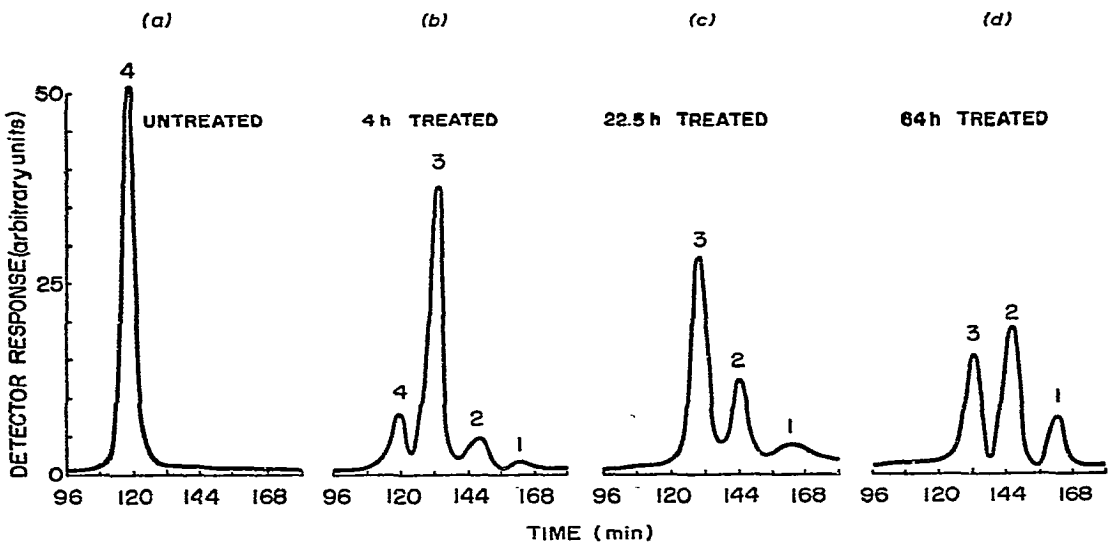

Fig. 3. Elution profiles for 3-*O*-β-cellotriosyl-D-glucose and its alkaline-degradation products: (*a*) untreated starting material, (*b*) products after 4 h, (*c*) 22.5 h, and (*d*) 64 h. The numbers 1, 2, 3, and 4 refer to the degrees of polymerization of the products.

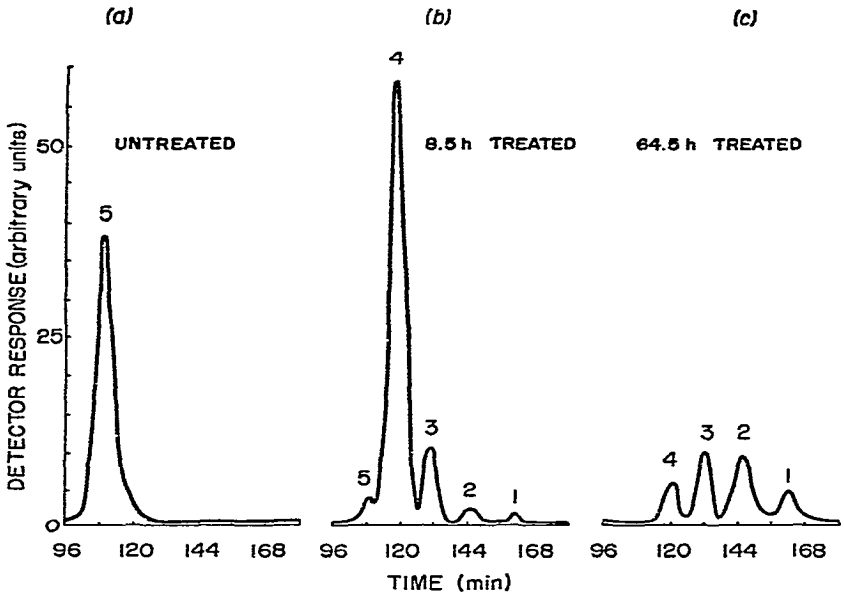

Fig. 4. Elution profiles for 3-*O*-*β*-cellotetraosyl-D-glucose and its alkaline-degradation products: (*a*) untreated starting material, (*b*) products at 8.4 h, and (*c*) 64.5 h. The numbers 1, 2, 3, 4, and 5 refer to the degrees of polymerization of the products.

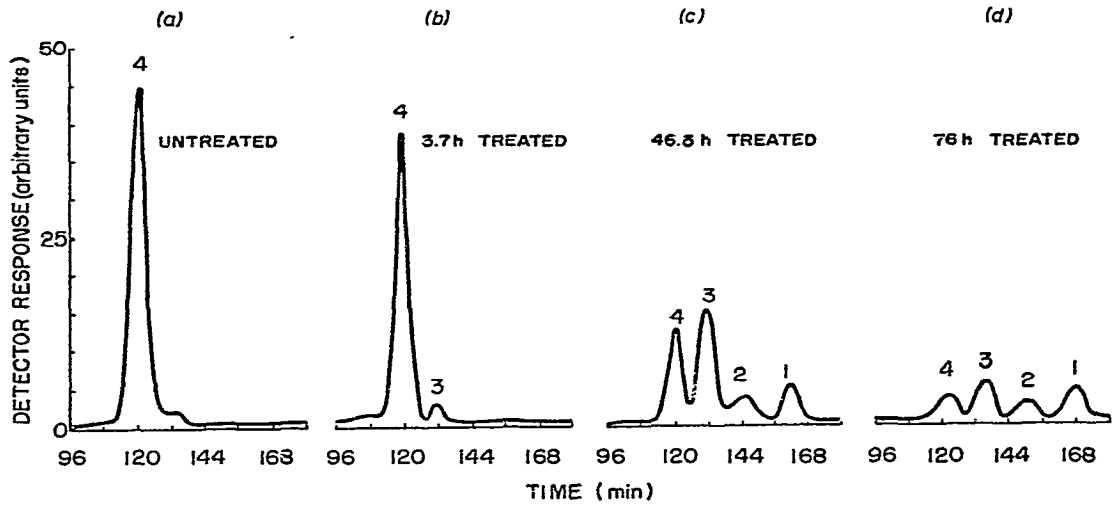

Fig. 5. Elution profiles for 4-*O*-*β*-(laminarabiosyl)cellobiose and its alkaline-degradation products: (*a*) untreated starting material, (*b*) products at 3.7 h, (*c*) products at 46.5 h, and (*d*) 76 h. The numbers 1, 2, 4, and 4 refer to the degrees of polymerization of the products.

The patterns obtained following alkaline treatment of the tetrasaccharide 4-*O*-*β*-(laminarabiosyl)cellobiose for 3.7, 46.5, and 76 h (Figs. 5b, 5c and 5d) demonstrate the slow peeling of the (1→4)-linked glucose residues, followed by rapid peeling of the (1→3)-linked residue, as indicated by the presence of only very low concentrations of disaccharide at all stages of the reaction.

When dealing with linear D-glucose oligosaccharides that contain only (1→3) and (1→4) linkages, the linkage sequence can be deduced directly from the kinetic pattern. Although some formation of isomers resulting from the Lobry de Bruyn and Alberda van Eckenstein reaction would be expected, the compounds give the anticipated pattern on alkaline peeling because the elution volumes of the oligosaccharide products depend mainly on their degree of polymerization.

Application of this technique to unknown oligosaccharides requires that the sample be relatively pure. It is also important to know if the compound is branched or unbranched, as branches may lead to the formation of compounds stable to alkali. Thus, during alkaline peeling of laminaran, those D-glucose residues linked (1→6) may become converted into D-glucometasaccharinic acid, forming a structure that is stable to alkali[6]. Although unbranched polysaccharides of the (1→4)-D-glucan type have been shown to yield stable intermediates during the course of the peeling reaction[13], no evidence was obtained that peeling of the (1→4)-oligo-D-glucosides was being terminated in this way.

In certain circumstances, the method may be used as a means of purification. For example, if an oligosaccharide having (1→4)-linked D-glucose at the reducing end is contaminated with an oligosaccharide of the same degree of polymerization having a (1→3)-linked D-glucose at the reducing end, then a short treatment with alkali will decrease the chain length of the contaminant by one D-glucose residue with only a small loss of the main component. After separation, the oligosaccharide having the terminal, (1→4)-linked D-glucose would be reanalyzed by alkaline degradation.

In addition to information derived from the rate of peeling, identification of oligosaccharides may often be aided by using different solvent systems for the column separations. As shown in Table I, the elution volumes (relative to D-glucose) of the (1→4)-linked oligo-D-glucosides increase when 0.1M boric acid instead of water is used as the solvent, whereas those of the (1→3)-linked oligo-D-glucosides decrease. The difference in elution volumes is sufficient to allow ready resolution of a mixture containing cellobiose and laminarabiose, the respective trisaccharides, or the respective tetrasaccharides.

Salts behaved as if they were macromolecules on the Bio-Gel P-2 column. As shown in Table I, calcium sulphate, sodium chloride, and sodium phosphate (pH 7) appear to be essentially excluded from the column. Consequently, when alkali-treated carbohydrates are neutralized with sulphuric acid, the resulting calcium sulphate appears as a large front that masks products having low elution-volumes. This behavior may be avoided by neutralizing the calcium hydroxide with the acid form of Amberlite IR-120.

When the products of alkaline degradation of oligo-D-glucosides were tested

TABLE I

Compound	*Relative elution volumes*[a] *Solvent*	
	Water	*0.1M boric acid*
Sucrose	0.91	0.94
Laminarabiose	0.90	0.86
Cellobiose	0.91	0.94
Gentiobiose	0.88	
Raffinose	0.81	0.83
Laminaratriose	0.80	0.77
Cellotriose	0.81	0.84
Laminaratetraose	0.73	0.70
Cellotetraose	0.74	0.78
Ethanol	0.96	
Dextran-40 (Pharmacia)	0.34	0.39
Sodium chloride	0.37	0.41
Sodium phosphate (pH 7)	0.34	
Calcium sulphate	0.34	
Ferrous nitrate	0.35	
Ferric nitrate	0.36	
Sodium dihydrogen arsenate	0.35	
Boric acid	1.14	1.21
D-Glucoisosaccharinic acid	0.35	
	0.41	
	0.51	
D-Glucometasaccharinic acid (lactone)	0.35	
	0.41	
	0.52	

[a]Elution volumes are relative to glucose. The elution volume of D-glucose with water as solvent was 75 ±2 ml, and with a 0.1M boric acid solvent, 73 ±2 ml, from a 170 × 0.9 cm column of Bio-Gel P-2.

after neutralization with cation-exchange resins, peaks having low relative elution-volumes could then be observed on chromatograms. These were presumably due to saccharinic acids, as D-glucoisosaccharinic acid and D-glucometasaccharinic acid (lactone) (Table I) gave multiple peaks having low relative elution-volumes.

In contrast to the salts and the saccharinic acids, boric acid had a very high relative elution-volume, higher in fact than ethanol or D-glucose. This is presumably caused by a reversible interaction with the polyacrylamide matrix because of its weakly basic character. Stronger bases, such as calcium and sodium hydroxides, were observed to react irreversibly with the column matrix.

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REFERENCES

1 D. R. BARRAS, A. E. MOORE AND B. A. STONE, *Enzyme–Substrate Relationships Among β-Glucan Hydrolases*, in C. HJNY (Ed.), *Cellulases and their Applications, Advan. Chem. Ser.*, 95 (1969) 105–138.
2 H. S. ISBELL, *J. Nat. Bur. Stand.*, 32 (1944) 45–59.
3 W. M. CORBETT AND J. KENNER, *J. Chem. Soc.*, (1954) 3274–3277.
4 R. L. WHISTLER AND J. N. BEMILLER, *Advan. Carbohydr. Chem.*, 13 (1958) 289–329.
5 W. B. CORBETT, in J. HONEYMAN (Ed.), *Recent Advances in the Chemistry of Starch and Cellulose*, Heywood, London, 1959, 106–133.
6 W. M. CORBETT AND J. KENNER, *J. Chem. Soc.*, (1955) 1431–1435.
7 S. A. BARKER, J. M. EDWARDS, P. J. SOMERS AND A. REPAS, *Carbohydr. Res.*, 6 (1968) 341–346.
8 H. DELLWEG, M. JOHN AND G. TRENEL, *J. Chromatogr.*, 57 (1971) 89–97.
9 M. JOHN, G. TRENEL AND H. DELLWEG, *J. Chromatogr.*, 42 (1969) 476–484.
10 G. L. MILLER, J. DEAN, AND R. BLUM, *Arch. Biochem. Biophys.*, 91 (1960) 21–26.
11 W. W. LUCHSINGER, S. C. CHEN, AND A. W. RICHARDS, *Arch. Biochem. Biophys.*, 112 (1965) 531–536.
12 D. J. MOFFA AND W. W. LUCHSINGER, *Cereal Chem.*, 47 (1970) 54–63.
13 G. MACHELL AND G. N. RICHARDS, *J. Chem. Soc.*, (1957) 4500–4506.