Journal of Chromatography, 464 (1989) 365–373
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 123

HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY OF HOMOGENEOUS D-GLUCO-OLIGOSACCHARIDES AND -POLYSACCHARIDES (POLYMERIZATION DEGREE $\geqslant$ 50) WITH PULSED AMPEROMETRIC DETECTION

KYOKO KOIZUMI*, YOKO KUBOTA, TOSHIKO TANIMOTO and YASUYO OKADA
Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 11–68 Koshien Kyuban-cho, Nishinomiya 663 (Japan)
(Received October 21st, 1988)

SUMMARY

High-performance anion-exchange chromatography under alkaline conditions with pulsed amperometric detection was applied to the analyses of (1→2)-, (1→3)-, (1→4)- and (1→6)-linked homogeneous α- or β-D-gluco-oligosaccharides and -polysaccharides up to a degree of polymerization (DP) of $\geqslant$ 50. Each series of homogeneous D-gluco-oligomers and -polymers showed a linear relationship between log k' and DP in isocratic elution using 150 m*M* sodium hydroxide solution containing 100 m*M* sodium acetate as the eluent. An effective separation of individual members of an homologous series of linear glucans was achieved using gradient elution, accomplished by maintaining the sodium hydroxide concentration at 150 m*M* and increasing the sodium acetate concentration during the analysis. The detector response per HCOH group in D-gluco-oligomers (DP 2–7) was almost the same.

INTRODUCTION

In polysaccharide structural studies, partial hydrolysis of polysaccharides followed by identification of the resulting oligosaccharides is a powerful aid. We found previously that high-performance liquid chromatography (HPLC) on a 3-μm NH_2-bonded silica column by using a pump minimized pulsating flow, and a refractive index (RI) detector with high sensitivity is effective for analyses of homogeneous D-gluco-oligosaccharide and -polysaccharide mixtures with a degree of polymerization (DP) up to about 30[1]. However, this method suffers serious disadvantages. One of these is that the RI detector does not easily provide a stable baseline at high sensitivity and, moreover, cannot be used with a gradient. In addition, as the silica-based column must be used at pH < 7.5 and therefore only neutral solutions of samples can be applied, saccharides with higher DPs which are soluble only in alkaline solutions cannot be analyzed on the NH_2-bonded silica column.

Recently, we applied high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection, introduced by Rocklin and Pohl[2], to

0021-9673/89/$03.50

the determination of cyclic glucans (DP 6–25) and obtained excellent resolution and high sensitivity[3]. As this new method is expected to overcome the aforesaid limitations of HPLC of oligo- and polysaccharides on NH_2-bonded silica with RI detection, we studied HPAEC of (1→2)-, (1→3)-, (1→4)- and (1→6)-linked homogeneous D-gluco-oligo- and -polysaccharides using pulsed amperometric detection (PAD).

EXPERIMENTAL

Apparatus and column

HPAEC was conducted with a Dionex BioLC Model 4000i system and a Model PAD II pulsed amperometric detector consisting of an amperometric flow-through cell with a gold working electrode, a silver–silver chloride reference electrode and a potentiostat. The sample loop size was 50 μl. The column used was a Dionex HPIC-AS6 (250 mm × 4 mm I.D.) equipped with an AG6 guard column (50 mm × 4 mm I.D.) (all from Dionex, Sunnyvale, CA, U.S.A.). A Chromatopac C-R3A digital integrator (Shimazu Kyoto, Japan) was used to calculate peak areas.

Chromatographic conditions and measurements

The following pulse potentials and durations were used for analysis of homogeneous linear D-gluco-oligo- and -polysaccharides at range 2 (sampling period, 200 ms): $E_1 = 0.10$ ($t_1 = 300$); $E_2 = 0.60$ ($t_2 = 120$); $E_3 = -0.80$ V ($t_3 = 300$ ms). The response time of the PAD II detector was set to 1.0 s. Eluents prepared daily were degassed by sonication under bubbling of helium gas and kept under a stream of nitrogen. The sample solutions were prepared using 18 MΩ cm deionized water and filtered through a 0.2-μm membrane filter. All separations were carried out at ambient temperature with a flow-rate of 1 ml/min.

Materials

Cyclosophoraose [Cys, cyclic (1→2)-β-D-glucan]-P (DP 32) was isolated from a culture filtrate of *Rhizobium meliloti* IFO 13336 according to the method described previously[4]. (1→3)-α-D-glucan was extracted from the fruit body of *Laetiporus sulphureus* with 1 *M* sodium hydroxide for 3 h at room temperature[5]. Short-chain amylose EX-1 ($\overline{DP} \approx 17$) [a mixture of (1→4)-α-D-glucans] was a gift from Hayashibara Biochemical Lab. (Okayama, Japan). Absorbent cotton, of Japanese Pharmacopoeial standard, was used as cellulose [(1→4)-β-D-glucan]. Luteose [(1→6)-β-D-glucan] and curdlan [(1→3)-β-D-glucan)] were gifts. Dextran [(1→6)-α-D-glucan], maltose and gentiobiose were commercially available. Malto- (DP 3–7), sophoro- (DP 2–5), laminara- (DP 2–6) and gentio-oligomers (DP 3–6) were all gifts. These oligomers were individually purified by HPLC on a YMC-Pack PA-03 (250 mm × 4.6 mm I.D.) column (Yamamura Chemical, Kyoto, Japan) with acetonitrile–water as the eluent. All reagents were of analytical reagent grade. The eluent A was 150 m*M* sodium hydroxide solution which was prepared by dilution of carbonate-free 50% sodium hydroxide solution in 18 MΩ cm deionized water. The eluent B was 150 m*M* sodium hydroxide solution, containing 500 m*M* sodium acetate; 300 m*M* sodium hydroxide solution, prepared from carbonate-free 50% sodium hydroxide solution, was diluted in the same volume of 1000 m*M* sodium acetate solution, prepared with 18 MΩ cm deionized water and filtered through a 0.2-μm membrane filter.

Preparation of homologous series of linear glucans

(1→2)-β-D-Glucosaccharides. Cyclosophoraose-P (2.5 mg) was hydrolysed in 1 ml of 0.1 *M* trifluoroacetic acid (TFA) at 100°C for 60 min. After removing TFA by evaporation, the hydrolysate was dissolved in 0.5 ml of deionized water.

(1→6)-α- and (1→6)-β-D-glucosaccharides. Dextran (10 mg) and luteose (10 mg) were individually hydrolysed in 5 ml of 0.3 *M* TFA at 100°C for 30 and 40 min, respectively. Their hydrolysates were dissolved in 1 and 2 ml of deionized water, respectively.

(1→3)-α- and (1→3)-β-D-glucosaccharides. (1→3)-α-D-Glucan (10 mg) of *Laetiporus sulphureus* and curdlan (10 mg) were individually formolysed with 90% formic acid (10 ml) at 90°C for 60 min, and after evaporation of the excess of formic acid the formates were hydrolysed at 100°C under the following conditions; 0.05 *M* TFA (10 ml) for 45 min for the former and 0.1 *M* TFA (10 ml) for 60 min for the latter. The hydrolysates were dissolved in 2 ml of deionized water.

(1→4)-β-D-Glucosaccharides. After formolysis with 90% formic acid at 100°C for 30 min, cellulose was subjected to partial acetolysis using acetic acid–acetic anhydride–sulphuric acid (10:10:1) at 40°C for 10 h. A part of the acetolysate obtained was deacetylated in the usual way. A 0.3% deionized water solution of the deacetylation product was used for an HPAEC analysis. The acetolysate was also used after deacetylation by stirring in 150 m*M* sodium hydroxide solution immediately before HPAEC.

(1→4)-α-D-Glucosaccharides. A sample of short-chain amylose EX-1 (3 mg) was dissolved in 1 ml of deionized water or 150 m*M* sodium hydroxide solution.

RESULTS AND DISCUSSION

Relationship between log k′ and DP in isocratic elution

HPAEC of each homologous series of D-gluco-oligo- and -polysaccharides on an HPIC-AS6 column was investigated with an isocratic elution using 150 m*M* sodium hydroxide solution containing 100 m*M* sodium acetate as the eluent. Of all D-glucans examined, (1→6)-α-D-glucans were most rapidly eluted and (1→3)-β-D-glucans were retained longest on the column. In each series, a plot of log k' against DP gave a straight line (Fig. 1A). Although these homologous series of D-glucans had an individual linear relationship between log t_R and DP on an amino column (ERC-NH-1171) with acetonitrile–water (Fig. 1B), and between log k' and DP on a reversed-phase column (Asahipak ODP-50) with sodium hydroxide solution (pH 11)[6], too, the elution order of glucans, having different types of linkages and the same DP, on an HPIC-AS6 column was different relative to that not only on an amino column but also on a reversed-phase column. According to Rendleman's review[7], carbohydrates are weak acids having ionization constants in the range of 10^{-12}–10^{-14}; in aqueous media the acidity of the hydroxyl groups in methyl D-glucopyranoside decreases in the order 2-OH ≫ 6-OH > 3-OH > 4-OH, and substitution for an hydroxyl group at C-2 should increase the acidities of the 3- or 4-hydroxyl groups. As it is thought that substitution of the hydroxyl group having stronger acidity in the molecule results in less retention, some of the retention behaviour of D-glucans can be explained on the basis of the acidity of the hydroxyl groups in methyl D-glucopyranoside mentioned above. However, it was not explicable why (1→6)-α-D-glucans are eluted first and much more

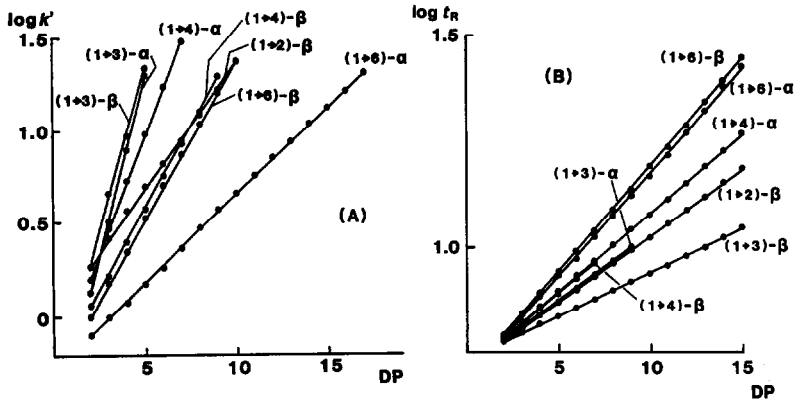

Fig. 1. Relationship between log k' and DP on an HPIC-AS6 column with 150 m*M* sodium hydroxide solution containing 100 m*M* sodium acetate (A), and as a reference relationship between log t_R and DP on an ERC-NH-1171 column with acetonitrile–water (57:43) (B). (1→2)-β, Sophoro-saccharides; (1→3)-α, nigero-saccharides; (1→3)-β, laminara-saccharides; (1→4)-α, malto-saccharides; (1→4)-β, cello-saccharides; (1→6)-α, isomalto-saccharides; (1→6)-β, gentio-saccharides.

rapidly than corresponding (1→6)-β-D-glucans and why (1→4)-β-D-glucans move faster than corresponding (1→4)-α-D-glucans on the column. Further, the acidity of 4-OH seems to be stronger than that of 3-OH.

Separation of each series of homogeneous D-*glucans*

An effective separation of individual members of an homologous series of D-glucans was achieved using gradient elution, which was accomplished by maintaining the sodium hydroxide concentration at 150 m*M* and increasing the sodium acetate concentration during the analysis. In Table I, gradient programmes used for the separation of (1→2)-, (1→3)-, (1→4)- and (1→6)-linked homologous D-gluco-oligosaccharides and -polysaccharides are summarized.

Fig. 2 shows the HPAEC elution profile of short-chain amylose EX-1 ($\overline{DP} \approx 17$). The number on each peak, indicating its DP, was confirmed by adding malto-oligosaccharides of known DPs. Amylose tends to precipitate from the solution and to retrograde on the column during liquid chromatography, but these phenomena are avoidable in high pH eluents such as 150 m*M* sodium hydroxide solution. Although amylose EX-1 dissolved more easily in 150 m*M* sodium hydroxide solution and the alkaline sample solution gave essentially the same chromatogram as that of the aqueous solution, after storage for 3 days in a refrigerator small peaks of epimerization products appeared in the chromatogram of the alkaline sample solution.

In the chromatogram of partial hydrolysates of cyclic (1→2)-β-D-glucan (Cys-P, DP 32), the residual cyclic glucan appears just behind the linear glucan of DP 21 (Fig. 3). This phenomenon suggested that the separation mode on an HPIC-AS6 column was not only simple anion exchange, but also involved some hydrophobic interactions. In the case of cyclic (1→4)-α-D-glucan (cyclodextrin, CD), α-CD (DP 6) and γ-CD (DP 8) were eluted together with maltotetraose (DP 4) and maltooctaose (DP 8), respectively, and β-CD (DP 7) appeared just prior to maltodecaose (DP 10). On an amino column, cyclic glucans moved together with the linear glucans having one or

TABLE I

GRADIENT PROGRAMMES USED FOR THE SEPARATION OF (1→2)-, (1→3)-, (1→4)- AND (1→6)-LINKED HOMOGENEOUS D-GLUCO-OLIGOSACCHARIDES AND -POLYSACCHARIDES

Eluents: A, 150 m*M* sodium hydroxide solution; B, 150 m*M* NaOH solution containing 500 m*M* sodium acetate. Eluent composition is changed linearly over a specified time.

Programme for	*Time (min)*	*Eluent A (%)*	*Eluent B (%)*	*Concn. of sodium acetate in eluent (mM)*
(1→2)-β-D-Glucans	0	75	25	125
	15	60	40	200
	40	50	50	250
(1→3)-α-D-Glucans	0	80	20	100
	1	40	60	300
	15	0	100	500
(1→3)-β-D-Glucans	0	70	30	150
	2	40	60	300
	20	0	100	500
(1→4)-α-D-Glucans	0	60	40	200
	10	40	60	300
	30	20	80	400
(1→4)-β-D-Glucans	0	80	20	100
	10	50	50	250
	30	40	60	300
(1→6)-α-D-Glucans	0	90	10	50
	1	80	20	100
	10	70	30	150
	30	60	40	200
(1→6)-β-D-Glucans	0	80	20	100
	3	70	30	150
	30	50	50	250

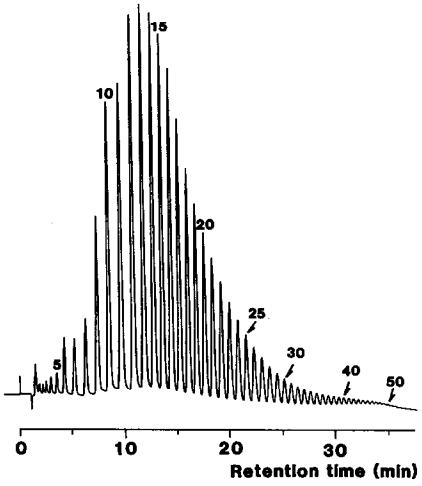

Fig. 2. Chromatogram of (1→4)-α-D-glucans [short-chain amylose EX-1 ($\overline{DP} \approx 17$)]. The number on each peak indicates its DP. Chromatographic conditions: column, HPIC-AS6 (250 mm × 4 mm I.D.); eluent and gradient programme as shown in Table I; flow-rate, 1 ml/min; detector, PAD II; meter scale, 10 K nA; temperature, ambient.

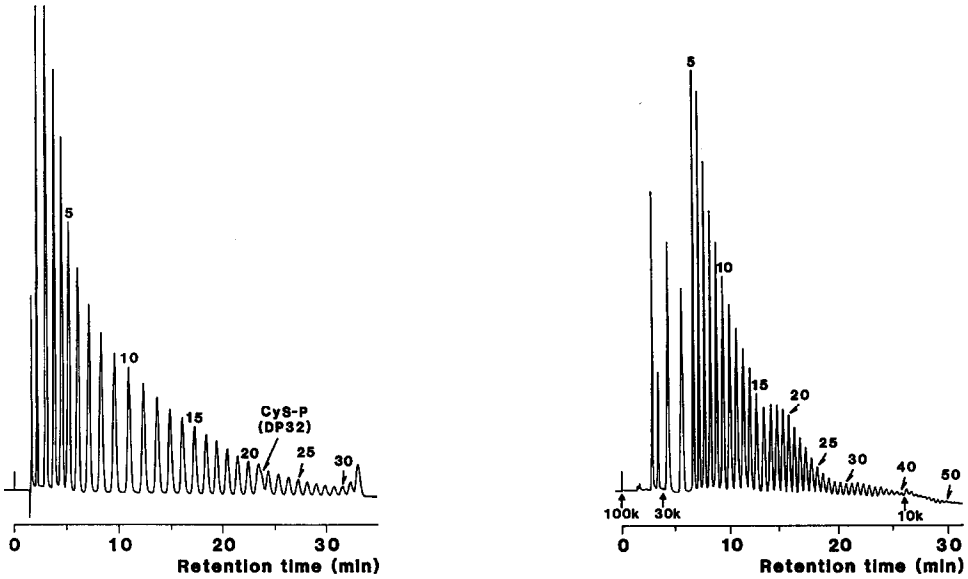

Fig. 3. Chromatogram of (1→2)-β-D-glucans [partial hydrolysates of CyS-P (DP 32)]. Chromatographic conditions: meter scale, 30 K nA; other conditions as in Fig. 2.

Fig. 4. Chromatogram of (1→6)-α-D-glucans (partial hydrolysates of dextran). Chromatographic conditions: meter scale, 100, 30 and 10 K nA; other conditions as in Fig. 2.

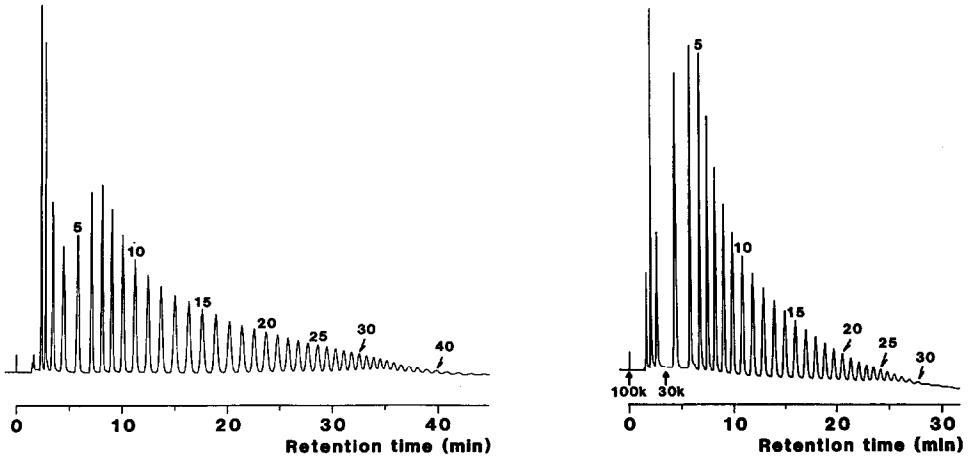

Fig. 5. Chromatogram of (1→6)-β-D-glucans (partial hydrolysates of luteose). Chromatographic conditions as in Fig. 3.

Fig. 6. Chromatogram of (1→3)-β-D-glucans (partial hydrolysates of curdlan). Chromatographic conditions: meter scale, 100 and 30 K nA; other conditions as in Fig. 2.

two DP units less[1], because the cyclic glucan has two hydroxyl groups less than the corresponding linear glucan.

Figs. 4–7 illustrate separations of the partial hydrolysates of dextran [α-(1→6)], luteose [β-(1→6)] and curdlan [β-(1→3)], and partial acetolysates of cellulose [β-(1→4)]. Around 45–50 distinct peaks can be seen in Figs. 4 and 5. By previous HPLC on NH_2-bonded silica with acetonitrile–water, using a sensitive RI detector, (1→6)-linked glucans were detected only up to about DP 30. Furthermore, no distinct peaks of higher polymers with DP > 19 were observed in the chromatogram of the hydrolysates of curdlan, because the β-(1→3)-linked higher polymers are not sufficiently soluble in water, and the β-(1→4)-linked D-glucans are more insoluble and hence only ten distinct peaks were observed in the HPLC chromatogram of the partial acetolysates of cellulose[1]. On the other hand, by HPAEC on an HPIC-AS6 column with alkaline eluents, using PAD, β-(1→3)-linked D-glucans of DP up to 30 and β-(1→4)-linked D-glucans of DP up to 22 were observed as distinct peaks in each chromatogram (Figs. 6 and 7). When the hydrolysates of curdlan were dissolved in 150 m*M* sodium hydroxide solution, the number of distinct peaks in the chromatogram increased up to 35. Fig. 7 shows a chromatogram of an aqueous mixture of β-(1→4)-linked D-glucans, prepared by deacetylation of partial acetolysates of cellulose. When the partial acetolysates of cellulose were dissolved in 150 m*M* sodium hydroxide solution by stirring for 30 min, the resulting deacetylates mixture in alkaline

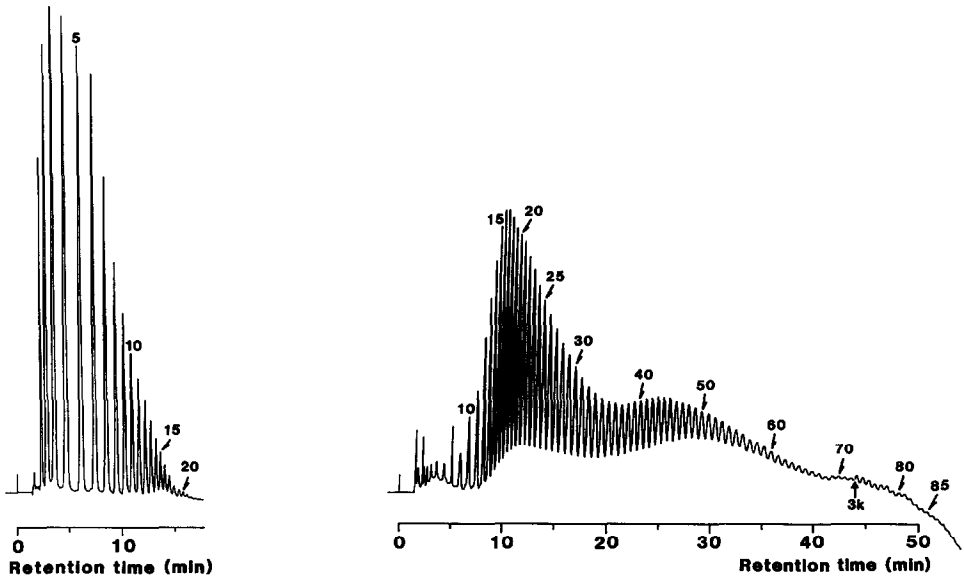

Fig. 7. Chromatogram of (1→4)-β-D-glucans (partial acetolysates of cellulose). Chromatographic conditions as in Fig. 3.

Fig. 8. Elution profile of a mixture of maltodextrins prepared from starch of Amylo-Waxy maize. Chromatographic conditions: gradient programme (% of eluent B), 40 at 0 min; 60 at 5 min, 70 at 20 min, 80 at 45 min and 90 at 70 min. Attenuation rose from 10 to 3 K nA full scale for the last 10 min; other conditions as in Fig. 2.

TABLE II

RELATIVE DETECTOR RESPONSES OF D-GLUCO-OLIGOMERS

Chromatographic conditions as in Fig. 2. The amounts of D-gluco-oligomers used were 5 nmol each.

DP	*No. of HCOH*	*Relative detector response*[a]			
		(1→4)-α-	*(1→2)-β-*	*(1→3)-β-*	*(1→6)-β-*
2	8	1.00	1.00	1.00	1.00
3	11	1.39	1.36	1.38	1.21
4	14	1.72	1.70	1.63	1.51
5	17	2.06	2.04	2.03	1.78
6	20	2.33		2.28	2.07
7	23	2.59			

[a] (1→4)-α-, Malto-oligomers; (1→2)-β-, sophoro-oligomers; (1→3)-β, laminara-oligomers; (1→6)-β-, gentio-oligomers.

solution was filtered and was directly chromatographed, 28 distinct peaks being detectable.

These results indicate that if polymers having higher DPs and soluble in alkaline solution are available, the numbers of detectable peaks should be increased. Fig. 8 shows an elution profile of a mixture of maltodextrins prepared from starch of Amylo-Waxy maize[8] by debranching with isoamylase and removing most of the water-soluble components by repeated extraction with hot water. The sample was dissolved in a small amount of 1 *M* sodium hydroxide solution and diluted five times in deionized water. The elution programme for the short-chain amylose (Table I) was modified for an effective separation of the maltodextrins (see the legend of Fig. 8).

Relative detector response

From the individual chromatogram of malto- (DP 2–7), sophoro- (DP 2–5), laminara- (DP 2–6) and gentio-oligomers (DP 2–6) (5 nmol each), obtained by the respective elution programme shown in Table I, the relative detector response was evaluated. The results indicated that the sensitivity of detection does not decrease with increasing molecular weight. The detector response per HCOH group in those oligosaccharides was almost the same (Table II). This is favourable for analysis of higher oligomers and polymers, and one of the principal advantages of the new method with PAD.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. A. Amemura (Osaka University) for gifts of luteose, curdlan, sophoro- (DP 2–5), laminara- (DP 2–6) and gentio-oligomers (DP 3–6) and to Professor S. Hizukuri (Kagoshima University) for gifts of malto-oligomers (DP 3–7), and also thank Professors M. Taki and T. Yamada, Dr. M. Hisamatsu and Dr. K. Takeo for gifts of Amylo-Waxy maize starch, cyclosphoraose and the fruit body of *Laetiporus sulphureus*, respectively.

REFERENCES

1 K. Koizumi, T. Utamura and Y. Okada, *J. Chromatogr.*, 321 (1985) 145–157.
2 R. D. Rocklin and C. A. Pohl, *J. Liq. Chromatogr.*, 6 (1983) 1577–1590.
3 K. Koizumi, Y. Kubota, T. Tanimoto and Y. Okada, *J. Chromatogr.*, 454 (1988) 303–310.
4 K. Koizumi, Y. Okada, T. Utamura, M. Hisamatsu and A. Amemura, *J. Chromatogr.*, 299 (1984) 215–224.
5 K. Takeo and S. Matsuzaki, *Carbohydr. Res.*, 113 (1983) 281–289.
6 K. Koizumi and T. Utamura, *J. Chromatogr.*, 436 (1988) 328–333.
7 J. A. Rendleman, Jr., *Adv. Chem. Ser.*, 117 (1973) 51.
8 M. Taki, M. Hisamatsu and T. Yamada, *Die Stärke*, 28 (1976) 153–157.