Journal of Chromatography, 321 (1985) 145–157
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,359

ANALYSES OF HOMOGENEOUS D-GLUCO-OLIGOSACCHARIDES AND -POLYSACCHARIDES (DEGREE OF POLYMERIZATION UP TO ABOUT 35) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY

KYOKO KOIZUMI*, TOSHIKO UTAMURA and YASUYO OKADA
Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 4-16 Edagawa, Nishinomiya 663 (Japan)
(Received November 2nd, 1984)

SUMMARY

Conditions for the separation of (1→2)-, (1→3)-, (1→4)- and (1→6)-linked homogeneous D-gluco-oligosaccharides and -polysaccharides were investigated by high-performance liquid chromatography on a 3-μm chemically modified amine column (ERC-NH-1171) and by thin-layer chromatography (TLC) on three kinds of silica gel plates. Saccharide samples were prepared by partial hydrolyses or partial acetolyses of cyclosophoraose [cyclic (1→2)-β-D-glucan], curdlan [(1→3)-β-D-glucan], amylose [(1→4)-α-D-glucan], cellulose [(1→4)-β-D-glucan], dextran [(1→6)-α-D-glucan], and luteose [(1→6)-β-D-glucan]. Each series of saccharides, other than β-D-(1→3)- and β-D-(1→4)-linked ones, whose soluble higher oligomers could not be obtained, was well resolved from glucose to the polymer having a degree of polymerization (DP) of about 35 on the amine column by using simple isocratic elution with acetonitrile–water. TLC analyses were performed on silica gel 70 and silica gel 60 TLC plates with a concentration zone using *n*-butanol–ethanol–water as the developing solvent, and on Si 50000 HPTLC plates using *n*-butanol–pyridine–water. These methods made it possible to separate simultaneously each series of homogeneous saccharides of DP up to 20–30.

INTRODUCTION

Many attempts have been made to separate series of oligosaccharides by chromatography. However, the size range of oligosaccharides that could be conveniently resolved by high-performance liquid chromatography (HPLC) was restricted up to a degree of polymerization (DP) of about 15, as the refractive index (RI) monitor usually employed can only be used satisfactorily in conjunction with isocratic elution. The separation of higher oligomers was generally performed by gradient elution with acetonitrile–water. For example, D'Amboise and co-workers separated oligo- and polysaccharides of DP up to 30 on a Chromosorb LC 9 reversed-phase column[1] and those of DP up to 20 on a LiChrosorb-NH_2 amine-bonded silica column[2]. The de-

0021-9673/85/$03.30

tection method was colorimetry of a reduced form of tetrazolium blue and was not suitable for detecting higher oligomers because of the decrease in their reducing power with increasing molecular mass. Wells and Lester[3] carried out separations of peracetylated linear glucose oligomers containing up to 35 sugar residues on C_{18}-bonded silica columns. However, in this method, derivatization is necessary prior to chromatography and, moreover, the moving wire detector used is not in widespread use.

We found recently that a 3-μm ERC-NH-1171 column with acetonitrile–water (58:42) as the eluent provided excellent separations of partial hydrolysates of cyclic (1→2)-β-D-glucans[4]. Effective resolution of sophorosaccharides with DP up to 33 was achieved in less than 35 min by isocratic chromatography using an RI detector.

In this work, we studied the conditions for the separation of (1→3)-, (1→4)- and (1→6)-linked homogeneous D-gluco-oligosaccharides and -polysaccharides with DP up to about 30, and also an extension of the size range of (1→2)-linked D-gluco-saccharides that could be resolved, up to DP more than 33. In addition, the simultaneous separation of each series of homogeneous D-glucosaccharides of DP up to 20–30 by thin-layer chromatography (TLC) was investigated.

EXPERIMENTAL

Apparatus

HPLC was conducted with a Tri Rotar SR-1 pump, a VL-614 variable-loop injector (both from JASCO, Tokyo, Japan) and an SE-31 RI detector (Showa Denko, Tokyo, Japan) at 1×10^{-5} RI units full-scale. The column used was an ERC-NH-1171 (3 μm) (200 × 6 mm I.D.) (Erma Optical Works, Tokyo, Japan).

Materials

Cyclosophoraose [cyclic (1→2)-β-D-glucan]-N (DP 30) and -S (DP 35) were isolated from a culture filtrate of *Rhizobium meliloti* IFO 13336 according to the method described previously[4]. Amylose [(1→4)-α-D-glucan] and short-chain amylose (DP *ca.* 17) were gifts 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] was a gift. Curdlan [(1→3)-β-D-glucan] and dextran [(1→6)-α-D-glucan] were commercially available. For TLC, silica gel 70 plates (20 × 20 cm) (Wako, Osaka, Japan), silica gel 60 TLC plates with a concentration zone (20 × 20 cm) and Si 50000 HPTLC plates (20 × 10 cm) (both from Merck, Darmstadt, F.R.G.) were used after cutting into 20 × 2.5 or 20 × 10 cm sections. All reagents were of analytical-reagent grade. Reagent-grade organic solvents used for chromatography were dried and freshly distilled before use. Water used in solvent preparations was deionized and distilled.

Preparation of saccharide samples

Each series of homogeneous D-gluco-oligosaccharides and -polysaccharides was prepared by partial hydrolysis or partial acetolysis of cyclosophoraose, curdlan, amylose, cellulose, dextran or luteose.

Partial hydrolysis. A glucan sample (10 mg) was hydrolysed in 4–5 ml of trifluoroacetic acid (TFA) at 100°C. The concentration of TFA and the reaction time

for each glucan are summarized in Table I. The solution containing the hydrolysate was neutralized with Dowex WGR (OH^-), filtered and evaporated to dryness under reduced pressure. Curdlan and cellulose hardly dissolved in water and were therefore subjected to formolysis with 90% formic acid at 100°C for 30 min prior to hydrolysis.

TABLE I

CONCENTRATION OF TRIFLUOROACETIC ACID (TFA) AND REACTION TIME FOR PARTIAL HYDROLYSIS OF D-GLUCAN

D-*Glucan*	*Concentration of TFA (M)*	*Reaction time (min)*
(1→2)-β-, (Cyclosophoraose)	0.1	60
(1→3)-β-*, (Curdlan)	0.1	30
(1→4)-α-, (Amylose)	0.1	15–30
(1→4)-β-*, (Cellulose)	0.1	90
(1→6)-α-, (Dextran)	0.3	30
(1→6)-β-, (Luteose)	0.3	60

* After formolysis with 90% formic acid at 100°C for 30 min.

Partial acetolysis. A sample (20 mg) of curdlan was subjected to formolysis using 5 ml of 90% formic acid. The reagent was evaporated under reduced pressure and the residue was dispersed in formamide[5] (3 ml) by shaking at 45°C for 1 day and then acetylated with dry pyridine (2 ml) and acetic anhydride (1 ml) overnight at room temperature. Excess of reagents was evaporated under reduced pressure. To the residue was added 1 ml of acetic acid–acetic anhydride–sulphuric acid (10:10:1) and the mixture was stirred at 40°C for 5 h. The reaction products, after neutralization with sodium hydrogen carbonate, were extracted with chloroform. The chloroform layer was evaporated to dryness under reduced pressure and the acetolysate obtained was deacetylated in the usual way. Partial acetolysis of cellulose was carried out in a similar manner, except the treatment with formamide was omitted. With the other glucans, the formolysis and the treatment with formamide prior to acetylation were not required.

Procedure

HPLC. A 1% aqueous solution of the partial hydrolysate or acetolysate was filtered through a 0.45-μm membrane filter and aliquots of 10–15 μl were analysed by HPLC. Acetonitrile–water with proportions varying from 60:40 to 55:45 was used as the eluent. The eluents were filtered through a 0.45-μm membrane filter and degassed. HPLC analyses were performed at room temperature and the flow-rate was always 1 ml/min.

TLC. The plates were conditioned in an oven at 110°C for 30–60 min and allowed to cool in a desiccator before use. A *ca.* 3% aqueous solution of the partial hydrolysate or acetolysate was applied to the plate with a 2-μl microcapillary attached to the applicator (Merck). Appropriate compositions of the solvent system were used, depending on the separtion desired (Table II). Solvents should be prepared the previous night. The plate was developed at room temperature three to five times in a

TABLE II

OPTIMAL COMPOSITION OF THE SOLVENT SYSTEM

*Saccharide**	*Silica gel 70 plate: n-butanol–ethanol–water*	*Silica gel 60 TLC plate with concentration zone: n-butanol–ethanol–water*	*Si 50000 HPTLC plate: n-butanol–pyridine–water*
1→2	5:5:4	5:5:3	6:5:4
1→3	5:5:3	5:5:2	6:2:2
1→4	5:5:5	5:5:4	6:5:4
1→6	5:5:7	5:5:6	6:8:10

* 1→2, 1→3, 1→4, and 1→6 as in Fig. 8.

closed glass tank thoroughly saturated with the solvent vapour. After each development the plate was dried with air followed by drying *in vacuo*. The spots were revealed by spraying with a reagent consisting of aniline (4 ml), diphenylamine (4 g), acetone (200 ml) and 85% H_3PO_4 (30 ml)[6], then heated in an oven at 80°C for 30 min. Anthrone–sulphuric acid reagent[7] can be also used a colour reagent.

RESULTS AND DISCUSSION

Preparation of saccharide samples for chromatography

Partial hydrolyses of glucans were performed using TFA at 100°C under the conditions shown in Table I. The appropriate choice of TFA concentration and reaction time made it possible to employ partial hydrolysis for the preparation of samples containing a series of oligo- and polysaccharides from higher polysaccharides.

At first it was thought that partial acetolysis, a relatively mild method for the degradation of the glycosidic linkage, would be more suitable than partial hydrolysis for the preparation of a series of saccharide sample, but the disadvantages of acetolysis, namely the tedious and time-consuming procedure, exceed the advantages. As the direct acetolysis of the glucan with acetic acid–acetic anhydride–sulphuric acid led to the preferential production of short-chain oligomers, the glucan was acetylated prior to acetolysis until it became soluble in acetic acid. Further, the product of acetolysis must be deacetylated for this HPLC procedure.

High-performance liquid chromatography

Fig. 1 shows the HPLC elution profile of a partial hydrolysate of cyclosophoraose-S (DP 35), which was recently isolated from a culture filtrate of *R. meliloti* IFO 13336, on an ERC-NH-1171 column using acetonitrile–water (58:42). In less than 45 min a complete separation could be obtained from glucose to linear (1→2)-β-D-glucan of DP 35. The native cyclosophoraose, cyclic (1→2)-β-D-glucan, is usually eluted slightly before the corresponding linear glucan[8].

As an application of this HPLC procedure, we analysed a sample of extracellular (1→2)-β-D-glucans from seven strains of *Acetobacter*[9]. By using acetonitrile–water (55:45) as the eluent, the occurrence of a series of linear (1→2)-β-D-glucans,

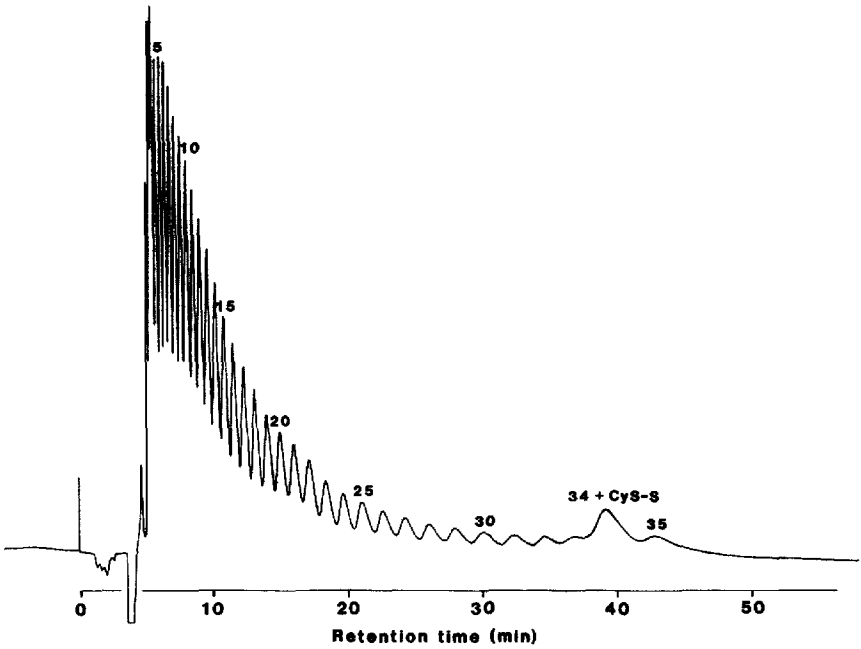

Fig. 1. Chromatogram of partial hydrolysate of cyclic (1→2)-β-D-glucan (DP 35) [cyclosophoraose (CyS)-S]. The number on each peak indicates its DP. Chromatographic conditions: column, ERC-NH-1171 (200 × 6 mm I.D.); eluent, acetonitrile–water (58:42); flow-rate, 1 ml/min; detector, Shodex RI SE-31 at $1 \cdot 10^{-5}$ RI units full-scale; temperature, ambient.

whose molecular size varied from 6 to at least 42 glucosyl residues, in the culture fluid of *Acetobacter* was confirmed.

As can be seen from Fig. 1, there is a relationship between the DPs of a series of sophoro-saccharides [β-(1→2)] and their retention times (t_R). A plot of log t_R against DP gave a straight line. The other series of homogeneous D-gluco-oligomers and -polymers also had an individual linear relationship between log t_R and DP (Fig. 2). Plots of log t_R against DP for each series of cello-saccharides [β-(1→4)] and isomalto-saccharides [α-(1→6)] (not shown in Fig. 2) gave straight lines having almost the same slopes as those for the counterparts, malto-saccharides [α-(1→4)] and gentio-saccharides [β-(1→6)], respectively. Saccharides with 1→4 linkages were retained slightly longer than the corresponding (1→2)-linked saccharides. The retention time of the (1→6)-linked saccharide was the longest and that of the (1→3)-linked saccharide was the shortest.

An effective separation of each series of homogeneous saccharides was achieved using different proportions of acetonitrile and water in the eluent. As a rule, an increasing ratio of water to acetonitrile for more retentive series was used. Figs. 3A, 4, 5 and 6 illustrate separations of the partial hydrolysates of curdlan, amylose, dextran and luteose. About 30 distinct peaks in the Figs. 4, 5 and 6 can be seen. Fig. 3B shows a chromatogram of the partial acetolysate of curdlan. The proportions of higher oligomers in the acetolysate are higher than those in the hydrolysate. However, no distinct peaks of higher polymers with DP > 19 are observed in the chromato-

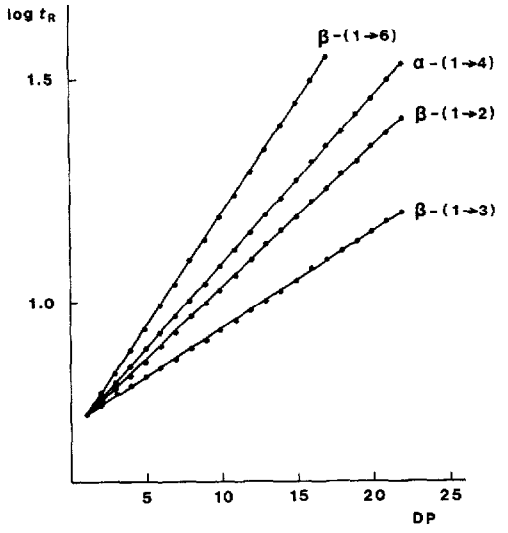

Fig. 2. Relationship between log t_R and DP. β-(1→2), sophoro-saccharides; β-(1→3), laminari-saccharides; α-(1→4), malto-saccharides; β-(1→6), gentio-saccharides. Eluent, acetonitrile–water (57:43); other conditions as in Fig. 1.

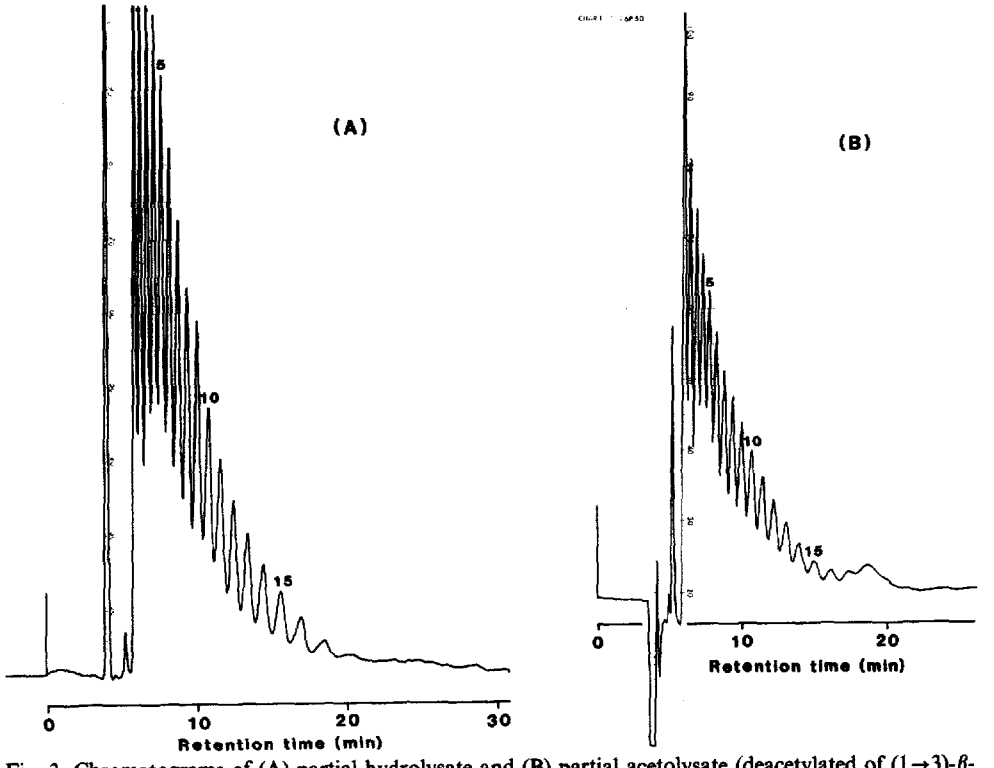

Fig. 3. Chromatograms of (A) partial hydrolysate and (B) partial acetolysate (deacetylated of (1→3)-β-D-glucan (curdlan). Eluent, acetonitrile–water (60:40); other conditions as in Fig. 1.

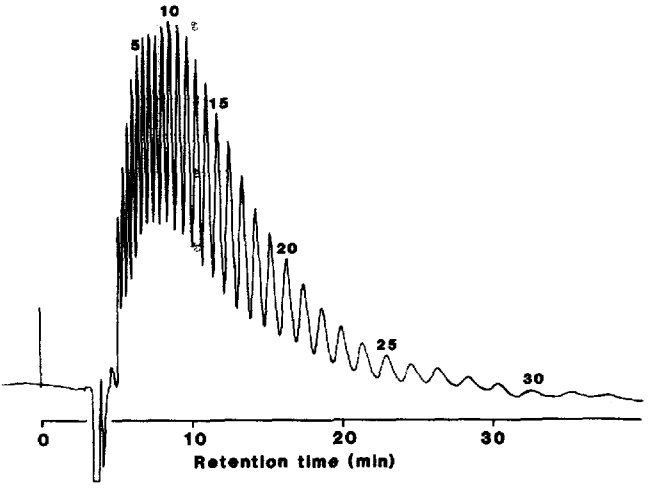

Fig. 4. Chromatogram of partial hydrolysate of (1→4)-α-D-glucan (amylose). Eluent, acetonitrile–water (57:43); other conditions as in Fig. 1.

grams of the acetolysate or the hydrolysate, because the β-(1→3)-linked higher polymers are not sufficiently soluble in water. The β-(1→4)-linked saccharides are more insoluble and hence only ten distinct peaks were observed in the chromatogram of the partial acetolysate of cellulose.

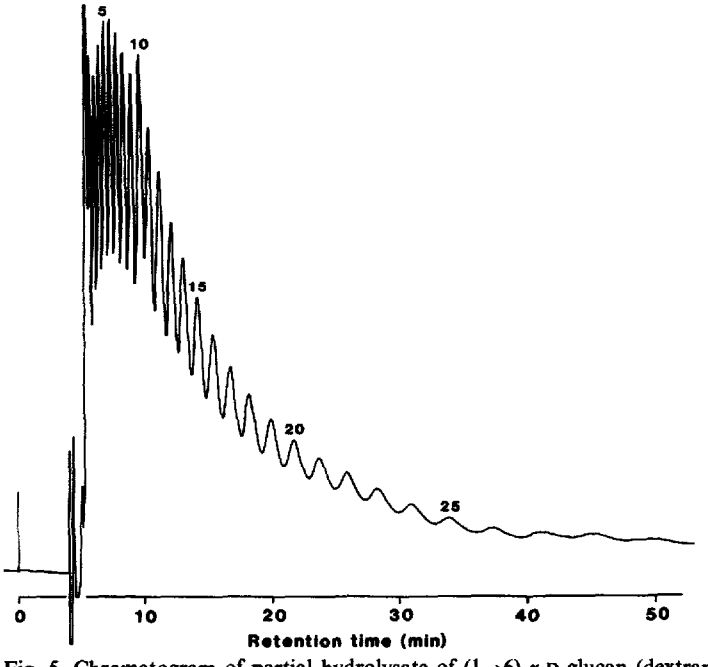

Fig. 5. Chromatogram of partial hydrolysate of (1→6)-α-D-glucan (dextran). Eluent, acetonitrile–water (56:44); other conditions as in Fig. 1.

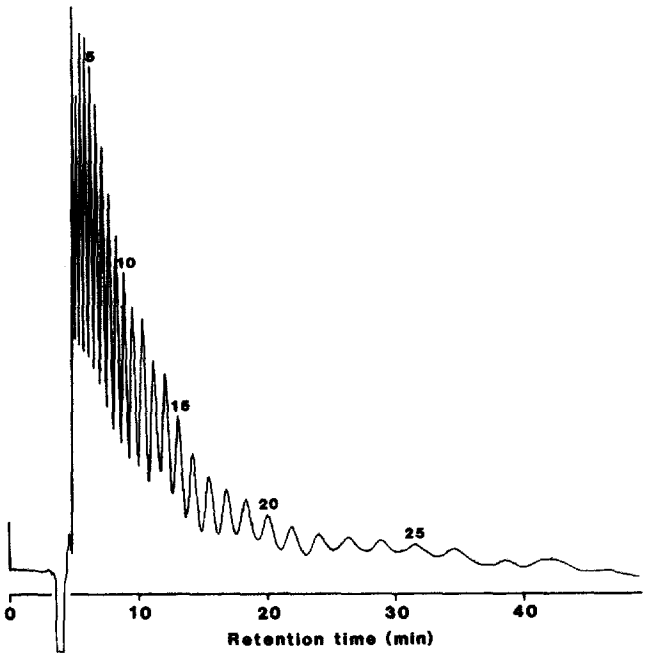

Fig. 6. Chromatogram of partial hydrolysate of (1→6)-β-D-glucan (luteose). Eluent, acetonitrile–water (55:45); other conditions as in Fig. 1.

According to the classification generally accepted, saccharides with DP > 10 are polysaccharides and their separation is usually carried out by gel permeation chromatography. A commercially available short-chain amylose, EX-1 (DP *ca.* 17) showed a single peak in the elution profile on Sephadex G-100 (Fig. 7A). Fig. 7B illustrates the chromatogram of the same sample, amylose EX-1, on an ERC-NH-1171 column. This HPLC analysis demonstrated that the chain length distribution of amylose EX-1 is from 2 up to 30 glucose units.

Thin-layer chromatography

As methods more convenient than HPLC for the separastion of oligosaccharides, paper chromatography (PC) and TLC have frequently been used. Umeki and Kainuma[10] achieved the quantitative fractionation of malto-saccharides with DP up to 25 using multiple descending PC. In general, TLC is superior to PC with regard to resolution, sensitivity and especially the time required for analysis. However, malto-oligosaccharides so far separated by HPTLC using multiple development are of DP up to 10[11] and those determined quantitatively by HPTLC are of DP up to about 12[12]. We therefore attempted to separate higher oligomers and polymers of D-glucosaccharides by TLC.

At first ordinary silica gel 60 (Merck) and silica gel 70 TLC plates (Wako) were used for the separation of partial hydrolysates of D-glucans. According to the manufacturers, the mean grain size and the pore size of silica gel 60 are 15 μm and 60 Å, respectively, and those of silica gel 70 are 10 μm and 70 Å, respectively. Arising from

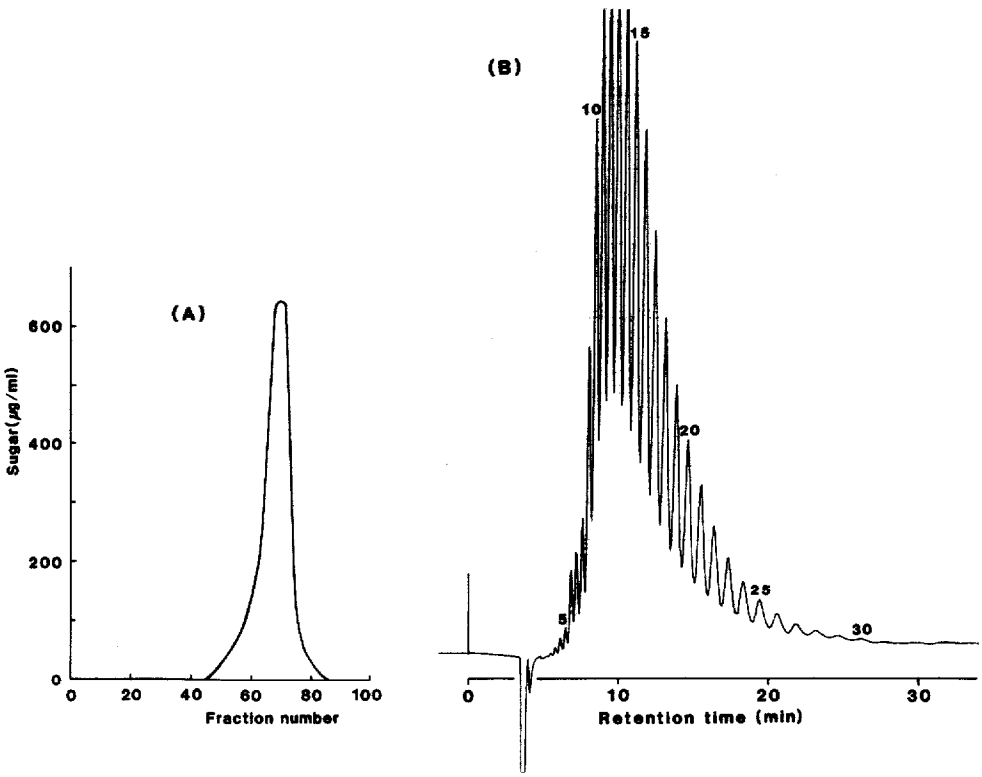

Fig. 7. Elution profiles of short-chain amylose EX-1 (DP *ca.* 17) on (A) Sephadex G-100 and (B) ERC-NH-1171. Chromatographic conditions: (A) sample fractionated on a 90 × 5 cm I.D. column; fraction size 20 ml; (B) eluent, acetonitrile–water (55:45); other conditions as in Fig. 1. * Reproduced from Hayashibara Research Products Catalogue by permission of Hayashibara Biochemical Laboratories.

these natures, although the migration rates of solvents on the latter plate are slower than those on the former, on the latter plate four replicate developments with the solvent system *n*-butanol–ethanol–water (5:5:4) resulted in a sufficient separation of the each series of D-gluco-saccharides with DP up to about 20 (Fig. 8). On the other hand, polymers of DP > 15 could not be separated on the former plate. Several solvent systems, *e.g.*, isobutanol–ethanol–water, *n*-butanol–pyridine–water, *n*-butanol–acetone–water, *n*-propanol–acetone–water and n-butanol–ethanol–acetone–water, were also examined, but the best solvent system was *n*-butanol–ethanol–water. For the analysis of the partial hydrolysate of curdlan, TLC is better than HPLC, as there are only 18 distinct peaks in HPLC (see Fig. 3), whereas in TLC 22 distinguishable spots are seen on a silica gel 70 plate (Fig. 8). This is because the detection limit of higher oligomers in TLC is lower than that in HPLC.

Next, silica gel 60 TLC plates with a concentration zone (Merck) were tested. It is said that when a solvent system containing a large proportion of water is used, the concentration zone of the silica gel plate is not very effective. However, the plate with a concentration zone was satisfactory for the separation of the partial hydro-

lysate of cyclosophoraose-S, *i.e.*, over 30 distinct spots were observed on the plate with four replicate developments with *n*-butanol–ethanol–water (5:5:3) (Fig. 9). The chromatographic behaviour of D-gluco-saccharides on this plate was analogous to that on a silica gel 70 plate. The mobilities of (1→3)-linked saccharides were the highest, followed by (1→2)- and then (1→4)-linked saccharides, and the mobilities of (1→6)-linked saccharides were the smallest. The most satisfactory results were obtained by decreasing the water content in the developing solvent for the separation of (1→3)-linked saccharides and by increasing the water content for that of (1→6)-linked saccharides.

Recently an unusual stationary phase for TLC has been developed. Si 50000

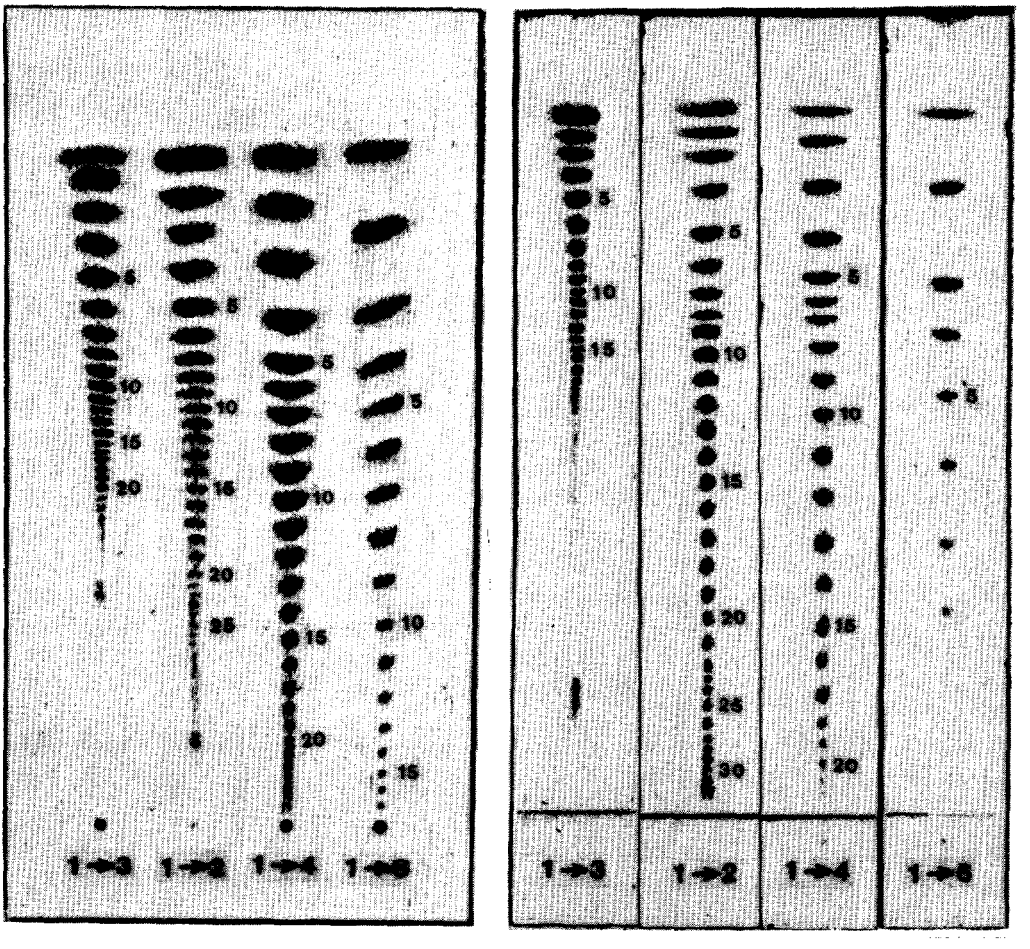

Fig. 8. Chromatograms of partial hydrolysates of D-glucans on a silica gel 70 plate developed with *n*-butanol–ethanol–water (5:5:4). The development was repeated four times. The number on each spot indicates its DP. 1→3, Laminari-saccharides; 1→2, sophoro-saccharides; 1→4, malto-saccharides; 1→6, gentio-saccharides.

Fig. 9. Chromatograms of partial hydrolysates of D-glucans on silica gel 60 TLC plates with a concentration zone developed with *n*-butanol–ethanol–water (5:5:3). Others as in Fig. 8.

HPTLC plates (Merck) are coated with synthetic porous silica originally prepared for the use as the concentration zone of silica gel 60 TLC plates, with a uniform large pore size of 50000 Å. As Si 50000 has a very low surface activity, it can be used as a stationary phase support for partition chromatography. On this plate saccharides show chromatographic behaviour analogous to that on filter-paper, but the time required for analysis is much shorter and the resolution is much better. Moreover, some aggressive reagents, *e.g.*, concentrated H_2SO_4 can be used for the colour development of spots on this plate. Each series of D-gluco-saccharides having β-(1→2)- and α-(1→4)-linkages showed excellent resolution for DP up to 30 and 26, respectively, on this plate using four replicate developments with *n*-butanol–pyridine–water (6:5:4) (Fig. 10). Better resolution of α-(1→4)-linked lower oligomers (DP 5, 6 and 7) is possible by decreasing the number of replicate developments to 2 or 3. The separation of a series of β-(1→6)-linked saccharides with DP up to about 20 could be achieved by four replicate developments with the same solvent system but with higher pyridine and water contents (6:8:10). On this plate β-(1→3)-linked saccharides

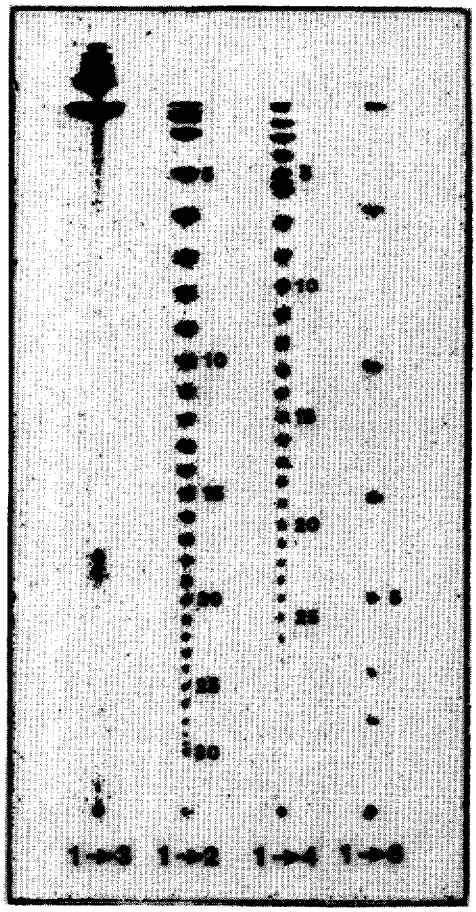

Fig. 10. Chromatograms of partial hydrolysates of D-glucans on Si 50000 HPTLC plates developed with *n*-butanol–pyridine–water (6:5:4). Others as in Fig. 8.

could not be separated even when the contents of pyridine and water were decreased as low as possible. Development with other solvent systems often employed in PC, *e.g.*, *n*-butanol–acetic acid–water and *n*-propanol–ethyl acetate–water, did not give better results.

Although the thin-layer chromatograms of α-(1→6)-linked saccharides are not shown in Figs. 8, 9 and 10, their chromatographic behaviour was almost identical with that of β-(1→6)-linked saccharides on all three kinds of plates.

The best compositions of the solvent systems for the separation of four individual series of D-gluco-saccharides on the three kinds of plates are summarized in Table II.

Fig. 11 illustrates the separation of α-(1→4)-linked saccharides on a silica gel 70 plate by two-dimensional multiple-development chromatography in comparison with the separation of the same sample on the same kind of plate by one-dimensional, four replicate development chromatography. The number of replicate developments in the two-dimensional TLC was five in the first direction and three in the seccond direction. The results demonstrate that two-dimensional multiple-development TLC is very time consuming, but it makes possible a better resolution of higher polymers.

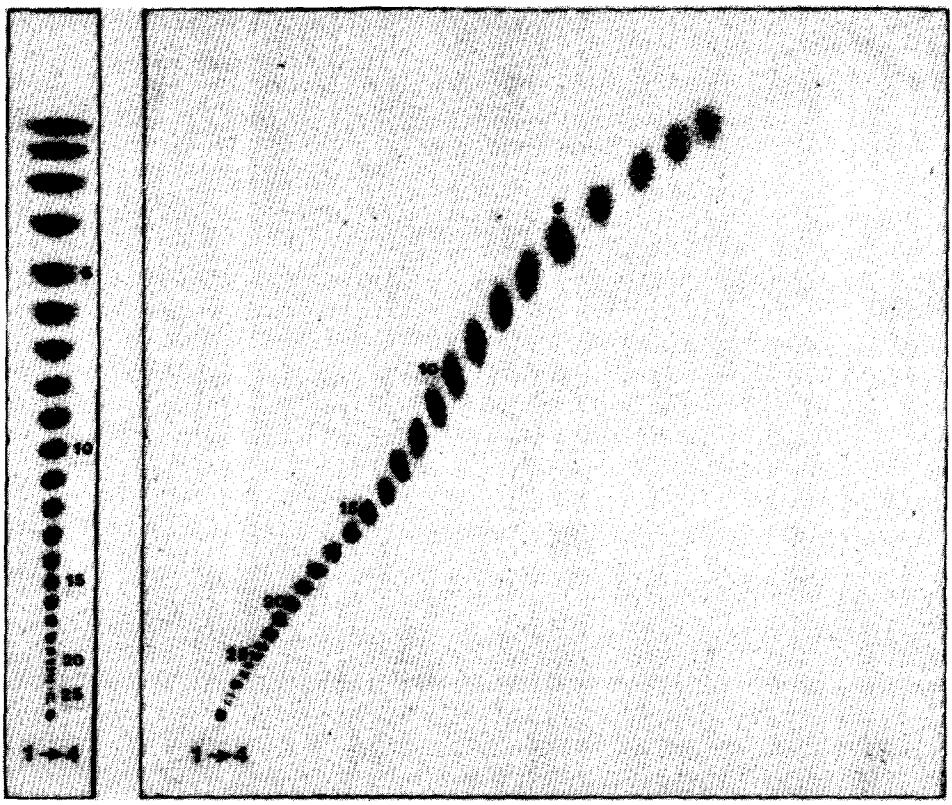

Fig. 11. Separation of α-(1→4)-linked D-gluco-saccharides on a silica gel 70 plate by two-dimensional multiple-development chromatography (right) and by one-dimensional multiple-development chromatography (left). Eluent, *n*-butanol–ethanol–water (5:5:5); number of developments, 5 and 3 (right), 4 (left).

CONCLUSION

Partial hydrolysis of polysaccharides followed by identification of the resulting oligosaccharides is a powerful aid in polysaccharide structural studies. The results presented in this paper demonstrate that HPLC on a 3-μm NH_2-bonded silica column and TLC on a silica gel plate with or without a concentration zone and an Si 50000 plate are effective and rapid procedures for analyses of homogeneous D-gluco-oligosaccharide and -polysaccharide mixtures. If water-soluble polymers, a pump minimized pulsating flow and an RI detector with high sensitivity are available, homogeneous D-gluco-oligomers and -polymers with DP up to 35 and probably more can be readily separated by using simple isocratic elution with acetonitrile–water. TLC with multiple development also can be conveniently used for the simultaneous separation of homogeneous D-gluco-oligomers and -polymers of DP up to about 30. The retention time in HPLC and the R_F value in TLC are useful for establishing the linkage structure of D-gluco-saccharides.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. A. Amemura (Osaka University) for a gift of luteose and also thank Professor A. Misaki (Osaka City University) and Dr. A. Amemura for their helpful advice with respect to the preparation of the saccharide samples.

REFERENCES

1 D. Noël, T. Hanai and M. D'Amboise, *J. Liq. Chromatogr.*, 2 (1979) 1325.
2 M. D'Amboise, D. Noël and T. Hanai, *Carbohydr. Res.*, 79 (1980) 1.
3 G. B. Wells and R. L. Lester, *Anal. Biochem.*, 97 (1979) 184.
4 K. Koizumi, Y. Okada, T. Utamura, M. Hisamatsu and A. Amemura, *J. Chromatogr.*, 299 (1984) 215.
5 E. B. Larson and F. Smith, *J. Amer. Chem. Soc.*, 77 (1955) 429.
6 S. A. Hansen, *J. Chromatogr.*, 105 (1975) 388.
7 R. Dreywood, *Ind. Eng. Chem., Anal. Ed.*, 18 (1946) 499.
8 K. Koizumi, Y. Okada, S. Horiyama, T. Utamura, M. Hisamatsu and A. Amemura, *J. Chromatogr.*, 265 (1983) 89.
9 A. Amemura, T. Hashimoto, K. Koizumi and T. Utamura, *J. Gen. Microbiol.*, 131 (1985) in press.
10 K. Umeki and K. Kainuma, *J. Chromatogr.*, 150 (1978) 242.
11 D. Nurok and A. Zlatkis, *Carbohydr. Res.*, 81 (1980) 167.
12 P. Würsch and Ph. Roulet, *J. Chromatogr.*, 244 (1982) 177.