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FURTHER STUDIES ON THE SEPARATION OF CYCLIC (1→2)-β-D-GLUCANS (CYCLOSOPHORAOSES) PRODUCED BY *RHIZOBIUM MELILOTI* IFO 13336, AND DETERMINATION OF THEIR DEGREES OF POLYMERIZATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Eight pure cyclic (1→2)-β-D-glucans (cyclosophoraoses) varying in size from 17 to 24 residues were previously isolated from culture filtrates of *Agrobacterium* and *Rhizobium*. Thereafter further studies on the separation of cyclosophoraoses by high-performance liquid chromatography (HPLC) with small-particle columns showed an occurrence of many cyclosophoraoses having degrees of polymerization (DPs) of more than 24. One sample prepared from *Rhizobium meliloti* IFO 13336 contained large amounts of higher cyclosophoraoses of up to at least DP 40, which were separated clearly on a 3-μm chemically modified amine column (ERC-NH-1171). Some pure cyclosophoraoses with higher DPs were isolated by liquid chromatography using reversed-phase columns, and their DPs were determined by HPLC of their partial hydrolysates.

INTRODUCTION

Many strains of *Agrobacterium*[1–3] and *Rhizobium*[4–6], when growing in carbohydrate-rich media, produce (1→2)-β-D-glucans of low molecular weight. Previously, the D-glucans were shown to be unbranched, cyclic structures[3,6–8]. Dell *et al.*[8] showed by fast-atom bombardment mass spectrometry that the size of the molecules varies from 17 to at least 24 glucosyl residues. Hisamatsu *et al.*[9] and Koizumi *et al.*[10] isolated eight cyclic (1→2)-β-D-glucans (cyclosophoraoses), and determined the degree of polymerization (DP) of each cyclosophoraoses by high-performance liquid chromatography (HPLC) of its partial hydrolysate. We found recently that cyclosophoraoses with DP > 24 were eluted very slowly or retained in a column under

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the conditions described previously[10]. Moreover, we could obtain a sample containing large amounts of cyclosophoraoses having higher DPs from a culture filtrate of *R. meliloti* IFO 13336, cultivated in a jar fermenter.

In this work, we studied the conditions for the separation of 24 cyclosophoraoses produced by *R. meliloti* IFO 13336 by HPLC on a 3-μm chemically modified amine column, isolated nine pure cyclosophoraoses with DPs > 24 by liquid chromatography (LC) on reversed-phase columns, confirmed their unbranched, cyclic structures by using ^{13}C NMR spectroscopy and determined their DPs by HPLC of their partial hydrolysates.

EXPERIMENTAL

Chromatography

HPLC analyses of cyclosophoraoses and their partial hydrolysates were performed at room temperature with a Tri Rotar SR-1 pump, a VL-614 variable-loop injector (both from JASCO, Tokyo, Japan) and an SE-31 refractive index (RI) monitor (Showa Denko, Tokyo, Japan). Preparative LC was carried out using a Twincle pump and a VL-611 variable-loop injector (JASCO) with an SE-11 RI monitor (Showa Denko) or a KHD-W-104 mini-micro pump (Kyowa Seimitsu, Tokyo, Japan) with a Waters RI R-403 detector (Waters Assoc., Milford, MA, U.S.A.). The columns used for HPLC were an ERC-NH-1171 (3 μm, 200 × 6 mm I.D.), an ERC-ODS-1171 (3 μm, 200 × 6 mm I.D.) (both from Erma Optical Works, Tokyo, Japan), a Hibar LiChrosorb RP-18 (5 μm, 250 × 4 mm I.D.), a Hibar LiChrosorb Si 60 (5 μm, 250 × 4 mm I.D.) (both from Merck, Darmstadt, F.R.G.) and a YMC-Pack AL-312 ODS (5 μm, 150 × 6 mm I.D.) (Yamamura Chemical, Kyoto, Japan). For preparative chromatography an RQ-2 packed column (360 × 24 mm I.D.) (Fujigel Hanbai, Tokyo, Japan) and a column packed with LiChroprep RP-18 (25–40 μm, 300 × 8 mm I.D.) (Merck) were also used. A Chromatopac C-RIA digital integrator (Shimadzu, Kyoto, Japan) was used for quantitative analyses. Acetonitrile and methanol used as the eluent were of analytical-reagent and were dried, then freshly distilled before use. Water used in eluent preparations was deionized and distilled. Eluents were filtered through a 0.45-μm membrane filter and degassed. HPLC using a silica column dynamically modified with 1,4-diaminobutane was conducted according to the method of White *et al.*[11].

Nuclear magnetic resonance spectroscopy

^{13}C NMR spectra were recorded with a JNM-FX 200 Fourier-transform NMR spectrometer (JEOL, Tokyo, Japan). The samples were dissolved in ^2H_2O at a concentration of 2–3% in a micro cell. Chemical shifts are expressed in ppm downfield from that of tetramethylsilane, using 1,4-dioxane (67.40 ppm) as the internal standard. The machine parameters included a spectrum frequency of 50.10 MHz, broad-band decoupling, 16K memory, 45° pulse, 0.682 sec between pulses, a sweep width of 12,004 Hz, 16,384 data points and a recycle of 2000–25,000. The spectra were recorded at ambient temperature.

Preparation of cyclosophoraoses

The organisms used were *A. radiobacter* IFO 12607, 13127, 13256, *A. rhizo-*

genes IFO 13259, *A. tumefaciens* IFO 3058, *R. phaseoli* AHU 1133, *R. trifolii* AHU 1134, *R. lupini* KLU, *R. japonicum* IFO 13338, *R. trifolii* 4S, *R. trifolii* IFO 13337, *R. meliloti* J7017 and *R. meliloti* IFO 13336. Cells of *Agrobacterium* and *Rhizobium* were cultivated in a synthetic medium described previously[3,6]. Ordinary cultivation took place in 100 ml of the medium in 500-ml erlenmeyer flasks on a rotary shaker at 30°C for 6 days; larger amounts of cultures were obtained with 5 l of the medium in a 10-l jar fermentor at 30°C, which was inoculated with 300 ml of preculture and cultivated for 4 days at an agitation speed of 200 rpm and an aeration rate of 1 volume per volume per min (vvm). Cyclosophoraoses were prepared from the culture filtrates according to the method described previously[3,6].

Partial hydrolysis of cyclosophoraose

A sample (2.5 mg) of cyclosophoraose was hydrolysed in 1 ml of 0.1 *M* trifluoroacetic acid at 100°C for 60–90 min. The solution containing the hydrolysate was neutralized with Dowex WGR (OH^-), filtered and evaporated to dryness under reduced pressure.

Partial acetolysis of cyclosophoraose

A sample (5 mg) of cyclosophoraose was acetylated with 1 ml of acetic anhydride in pyridine (1 ml) at 90°C for 1 h. Excess of reagents was evaporated under reduced pressure. To the residue was added 0.3 ml of acetic acid–acetic anhydride–sulphuric acid (10:10:1) and the mixture was stirred at 45°C for 2 h, then at 20°C for 10 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.

RESULTS AND DISCUSSION

Reinvestigation of cyclosophoraoses produced by Agrobacterium and Rhizobium by HPLC

In recent years, the performance of columns for HPLC has been rapidly improved. A reduced separation time or an increased plate number *N* is desirable and the most effective approach for this purpose is generally to reduce the particle diameter[12,13]. Theory predicts[14] that a particle diameter of about 2 μm will be optimal for many separations and columns with 3-μm particles are now commercially available. This is why we decided to reinvestigate cyclosophoraoses produced by *Agrobacterium* and *Rhizobium* by using a 3-μm particle column.

The earlier separation[10] that had been achieved on a 10-μm NH_2-bonded silica column (μBondapak CH, 300 × 3.9 mm I.D.) was repeated under similar conditions with the new 3-μm NH_2-bonded silica column (ERC-NH-1171, 200 × 6 mm I.D.). Small adjustments in mobile phase composition gave a better resolution than that of the original separation in a separation time of less than half of that originally required with the 10-μm column.

Under the new conditions the percentage compositions of cyclosophoraoses produced by five strains of *Agrobacterium* and eight strains of *Rhizobium* were determined (Table I). It was found that type III and IV[9] involved cyclosophoraoses

TABLE I

PERCENTAGE COMPOSITION OF CYCLOSOPHORAOSES

Calculations were performed on a digital electronic data processor. Chromatographic conditions: column, ERC-NH-1171 (200 × 6 mm I.D.); eluent, acetonitrile–water (60:40); flow-rate, 1 ml/min; detector, Shodex RI SE-31 at $1 \cdot 10^{-5}$ RI units full-scale; temperature, ambient.

Cyclosophoraoses		*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*	*N*
Type	*Produced by*	*(DP 17)*	*(DP 18)*	*(DP 19)*	*(DP 20)*	*(DP 21)*	*(DP 22)*	*(DP 23)*	*(DP 24)*	*(DP 25)*	*(DP 26)*	*(DP 27)*	*(DP 28)*	*(DP 29)*	*(DP 30)*
I	*R. phaseoli* AHU 1133	82.5	7.6	7.7	2.3										
	R. trifolii AHU 1134	78.2	12.1	8.3	1.4										
	R. lupini KLU	81.7	7.7	7.4	1.6	1.6									
II	*R. japonicum* IFO 13338	6.2	11.6	39.2	22.3	11.8	8.8								
	R. trifolii 4S	12.9	13.3	39.6	20.0	8.9	5.3								
	R. trifolii IFO 13337	6.7	12.5	41.4	22.9	12.2	4.3								
III	*R. meliloti* J 7017	1.6	4.0	5.4	14.4	21.6	28.0	12.8	6.2	2.8	0.8	1.4	0.5	0.3	0.2
	R. meliloti IFO 13336	1.2	3.0	2.1	9.3	16.9	25.0	13.1	17.3	5.8	1.3	3.8	1.0	0.1	0.1
IV	*A. radiobacter* IFO 12607	3.5	6.4	14.7	18.8	22.8	21.6	6.2	4.5	0.7	0.4	0.3			
	A. radiobacter IFO 13127	2.2	7.0	14.3	21.4	25.0	19.4	6.3	3.0	0.7	0.5	0.2			
	A. radiobacter IFO 13256	3.6	6.9	15.2	19.2	22.8	19.7	6.2	4.6	1.0	0.7	0.3			
	A. rhizogenes IFO 13259	3.9	8.5	17.1	18.4	17.9	18.3	6.3	5.8	1.7	1.2	0.8			
	A. tumefaciens IFO 3058	5.8	9.5	16.6	20.4	21.1	16.2	5.3	3.5	0.8	0.5	0.3			

with DP > 24 and especially *R. meliloti* IFO 13336 produced large amounts of higher cyclosophoraoses.

In order to study the higher cyclosophoraoses, a cultivation of *R. meliloti* IFO 13336 in a 10-l jar fermenter was attempted. Fig. 1 shows separation of cyclosophoraoses obtained from the culture. Ten components eluted before A were not cyclosophoraoses; they may be straight-chain glucooligosaccharides containing a (1→2)-α- linkage[15].

Isolation of the higher cyclosophoraoses (DP > 24)

For preparative chromatography the behaviour of cyclosophoraoses on some ODS columns was investigated. Fig. 2 shows the separation of cyclosophoraoses on [1] Hibar LiChrosorb RP-18 (5 μm), [2] ERC-ODS-1171 (3 μm) and [3] YMC-Pack AL-312 ODS (5 μm) columns. In all instances the eluent was 5–6% methanol. Compared with the previous separation on a Dextro-Pak cartridge (10 μm)[10] the three new columns provide faster separations and better resolution.

The elution pattern was different from that obtained on NH_2-bonded silica with acetonitrile–water. That is, there is no definite relationship between DP and retention time (t_R). Moreover, the elution order of C and D, E and F, J and K, and O and P reverse. It is thought that differences in the elution mechanisms[10] result in the differences in elution patterns.

The resolutions of three ODS columns are different from each other. This may

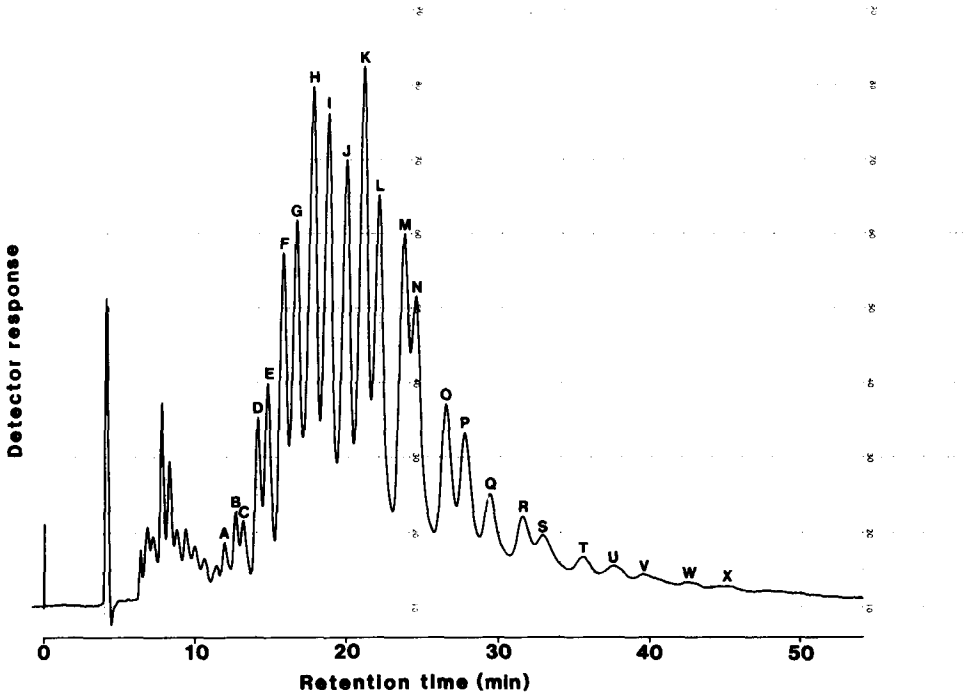

Fig. 1. Separation of cyclosophoraoses obtained from a culture of *R. meliloti* IFO 13336, cultivated in a jar fermenter, on 3-μm NH_2-bonded silica. Chromatographic conditions: eluent, acetonitrile–water (57:43); other conditions as in Table I.

arise from differences in their base materials and chemical modification methods. Overall the resolution is best on a Hibar LiChrosorb RP-18 column, but this column cannot be used for semi-preparative chromatography because of its low column loadability and very limited flow-rate. An ERC-ODS-1171 column for the isolation of I and a YMC-Pack AL-312 ODS column for the separation of J and K and of O and P were useful. M and N, which could be resolved only on a Hibar LiChrosorb RP-18 column, were separated by using a semi-preparative column packed with the larger

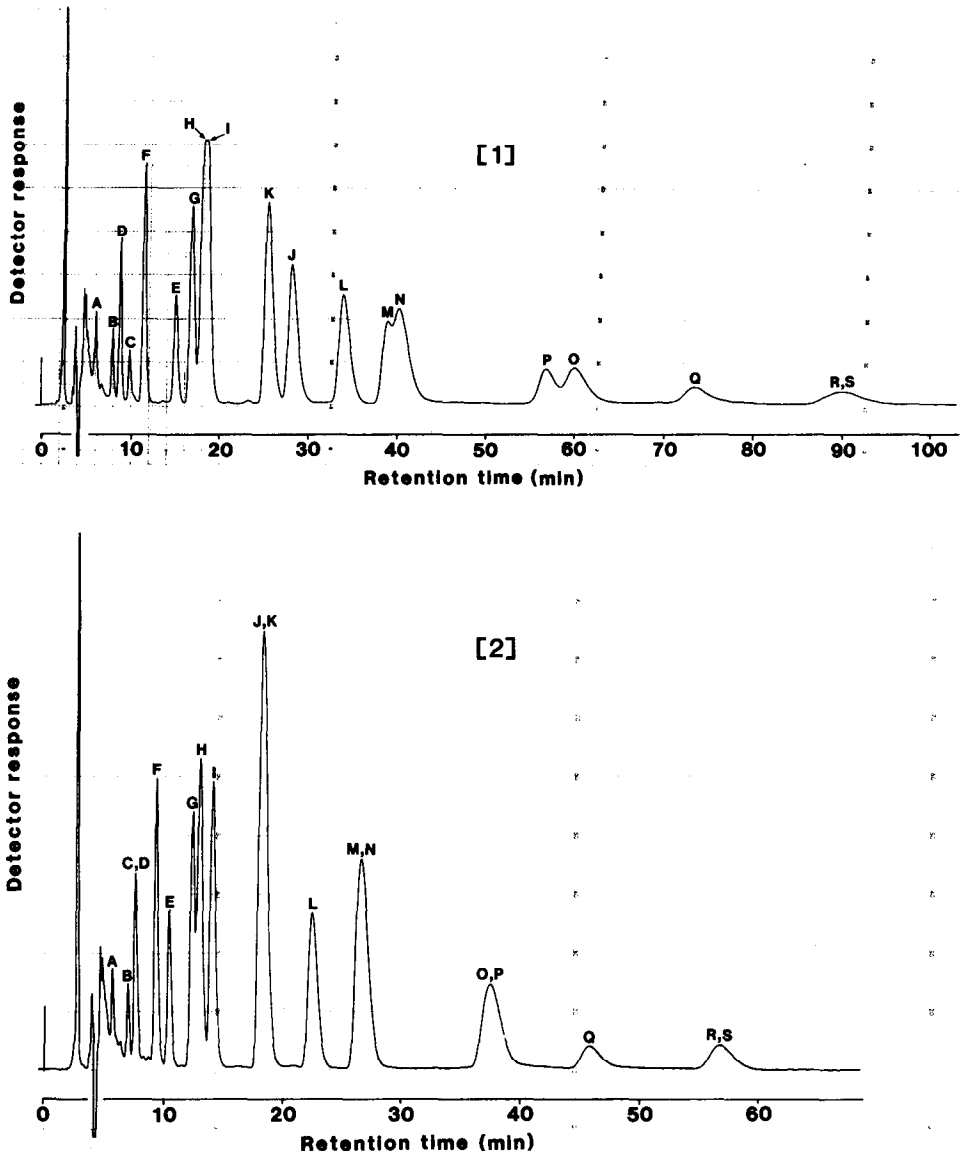

Fig. 2.

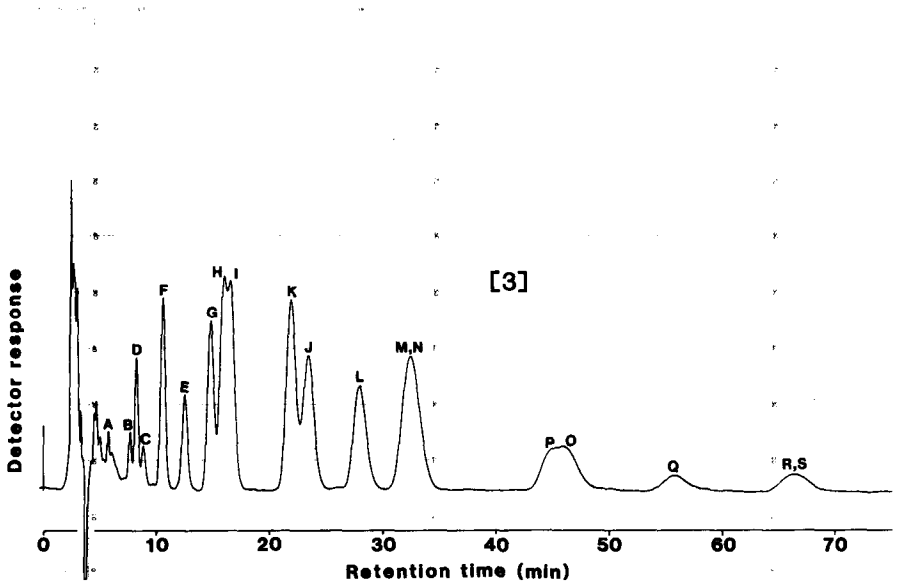

Fig. 2. Separation of cyclosophoraoses obtained from a culture of *R. meliloti* IFO 13336, cultivated in a jar fermenter, on C_{18}-bonded silica. Chromatographic conditions: [1] Hibar LiChrosorb RP-18 column (5 μm, 250 × 4 mm I.D.), eluent methanol–water (5.5:94.5), flow-rate 0.7 ml/min; [2] ERC-ODS-1171 column (3 μm, 200 × 6 mm I.D.), eluent methanol–water (5:95); [3] YMC-Pack AL-312 ODS column (5 μm, 150 × 6 mm I.D.), eluent methanol–water (6:94). Other conditions as in Table I.

particle counterpart (LiChroprep RP-18, 25–40 μm). L and Q were isolated by the first rough separation on an ODS column for preparative chromatography (RQ-2 packed column) with aqueous methanol whose concentration was increased stepwise from 2% to 5%, and were purified by HPLC on a YMC-Pack AL-312 ODS column. R and S were obtained as a mixture in a ratio of *ca.* 4:3. The amounts of cyclosophoraoses eluted later than R and S were too small to use for further investigation.

^{13}C NMR spectroscopy

The ^{13}C NMR spectrum of each component isolated showed only six signals, which indicates that each component is homogeneous in molecular size and is an unbranched, circular molecule. The ^{13}C NMR chemical shifts of cyclosophoraoses I–S are shown in Table II (those of A–H have already been reported[9]). The chemical shifts of R and S were determined in a mixture (R:S≈4:3). The spectrum showed two signals for C-2 and the more intense signal was assigned to C-2 of R.

Determination of DP

The DP of newly isolated each cyclosophoraose was determined by HPLC of its partial hydrolysate. Partial hydrolyses of cyclosophoraoses I–Q were performed as described previously[10]. An ERC-NH-1171 3-μm column with acetonitrile–water (58:42) as the eluent provided excellent separations of sophorosaccharides with DP up to 33. Fig. 3 illustrates the HPLC elution profiles of Q and of a partial hydrolysate of Q. The last distinguishing peak in the chromatogram of the partial hydrolysate of

TABLE II

^{13}C NMR CHEMICAL SHIFTS (ppm) OF CYCLOSOPHORAOSES IN $^{2}H_2O$ SOLUTION

Cyclosophoraose	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
I	102.75	83.02	76.38	69.72	77.17	61.62
J	102.94	83.40	76.31	69.59	77.15	61.59
K	102.88	83.23	76.36	69.65	77.18	61.59
L	102.82	83.23	76.33	69.65	77.15	61.59
M	102.96	83.40	76.31	69.59	77.18	61.59
N	102.94	83.20	76.31	69.59	77.15	61.59
O	102.94	83.40	76.33	69.62	77.18	61.59
P	102.94	83.40	76.33	69.62	77.18	61.59
Q	102.91	83.28	76.31	69.59	77.18	61.56
R	102.96	83.43	76.31	69.56	77.18	61.56
S	102.96	83.31	76.31	69.56	77.18	61.56

Q was the 33rd peak, counting from the glucose peak, and consequently the DP of Q is 33. The circular form was always eluted earlier than the open form, and Q was eluted at almost the same position as the 32nd peak of the hydrolysate. In the same way, the DPs of I, J, K, L, M, N, O and P were unambiguously estimated as 25, 26, 27, 28, 29, 30, 31 and 32, respectively.

In general, acetolysis is a milder method than hydrolysis for the degradation of glycosidic linkages. Therefore, partial acetolysis of L was also attempted for the determination of DP. As direct acetolysis of L with acetic acid–acetic anhydride–

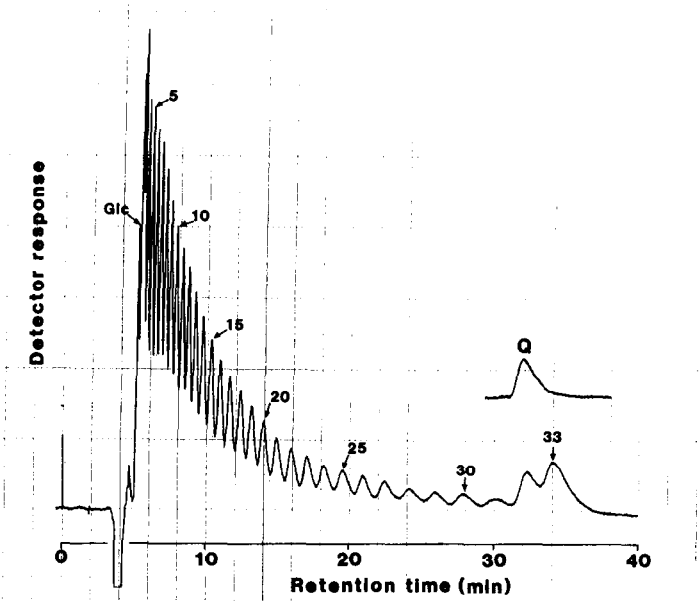

Fig. 3. Separation of D-glucose and sophorosaccharides present in partial hydrolysate of cyclosophoraose Q. The number beside each peak indicates its DP. The upper trace shows the chromatogram of native Q. Chromatographic conditions: eluent, acetonitrile–water (58:42); other conditions as in Table I.

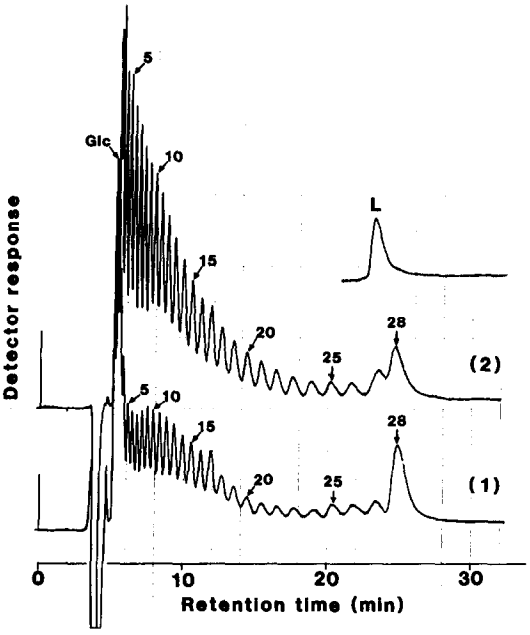

Fig. 4. Chromatograms of (1) partial acetolysate (deacetylated) and (2) partial hydrolysate of cyclosophoraose L and of native L. Chromatographic conditions as in Fig. 3.

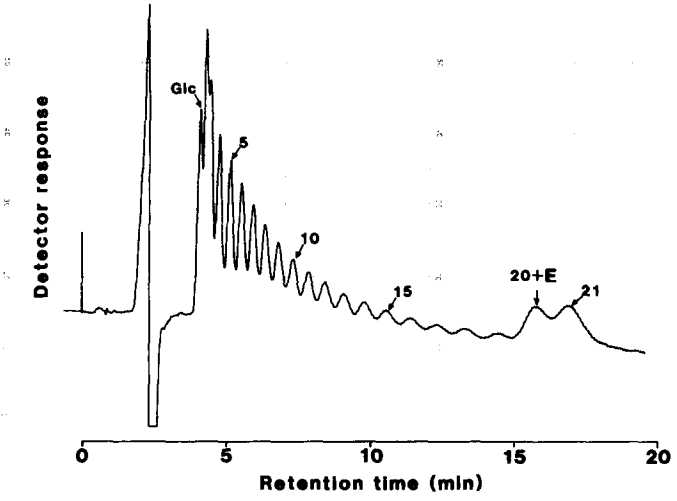

Fig. 5. Separation of D-glucose and sophorosaccharides present in partial hydrolysate of cyclosophoraose E on a silica column, dynamically modified with 1,4-diaminobutane. Chromatographic conditions: column, Hibar LiChrosorb Si 60 (5 μm, 250 × 4 mm I.D.); eluent, 45% aqueous acetonitrile containing 0.01% of modifier; detector sensitivity, $4 \cdot 10^{-5}$ RI units full-scale; other conditions as in Table I.

sulphuric acid led to the preferential production of short-chain sophorosaccharides, L was acetylated prior to acetolysis. After partial acetolysis, the products were deacetylated and chromatographed. In conclusion, acetolysis, which required much more complicated operations, had no merit for the determination of the DP of cyclosophoraose, because on hydrolysis under the appropriate conditions the longest straight-chain sophorosaccharide was present in a sufficient amount for it to be recognized in the partial hydrolysate (Fig. 4).

White *et al.*[11] reported that silica columns, dynamically modified with polyfunctional amines, provide a rapid, inexpensive method of separating oligosaccharides up to DP 20. A hydrolysate of E (DP 21) was analysed according to their procedure (Fig. 5). Its resolution was better than that on a 10-μm chemically modified amine column[10], but was inferior to that on a 3-μm chemically modified amine column. Moreover, it was found that their method required a long time for conditioning of a column prior to analysis and the pH of an eluent containing 1,4-diaminobutane modifier exceeded the limit of the optimal pH for a silica column, and therefore deterioration of column was considerable.

According to the classification generally accepted, cyclosophoraoses are polysaccharides. Most polysaccharides contain 80–100 sugar residues, carbohydrates containing 5–15 sugar residues rarely occur naturally and a few natural polysaccharides contain 25–75 sugar residues[16]. From this point of view, the occurrence of cyclosophoraoses containing 17–40 sugar residues is noteworthy.

REFERENCES

1 E. W. Putman, A. L. Potter, R. Hodgson and W. Z. Hassid, *J. Amer. Chem. Soc.*, 72 (1950) 5024.
2 E. Barreto-Bergter, C. R. Camargo, L. R. Hogge and P. A. J. Gorin, *Carbohydr. Res.*, 82 (1980) 366.
3 M. Hisamatsu, A. Amemura, T. Matsuo, H. Matsuda and T. Harada, *J. Gen. Microbiol.*, 128 (1982) 1873.
4 W. S. York, M. McNeil, A. G. Darvill and P. Albersheim, *J. Bacteriol.*, 142 (1980) 243.
5 L. P. T. M. Zevenhuizen and H. J. Scholten-Koerselman, *Antonie van Leeuwenhoek; J. Microbiol. Serol.*, 45 (1979) 165.
6 A. Amemura, M. Hisamatsu, H. Mitani and T. Harada, *Carbohydr. Res.*, 114 (1983) 277.
7 L. P. T. M. Zevenhuizen and A. R. W. Neerven, *Carbohydr. Res.*, 118 (1983) 127.
8 A. Dell, W. S. York, M. McNeil, A. G. Darvill and P. Albersheim, *Carbohydr. Res.*, 117 (1983) 185.
9 M. Hisamatsu, A. Amemura, K. Koizumi, T. Utamura and Y. Okada, *Carbohydr. Res.*, 121 (1983) 31.
10 K. Koizumi, Y. Okada, S. Horiyama, T. Utamura, M. Hisamatsu and A. Amemura, *J. Chromatogr.*, 265 (1983) 89.
11 C. A. White, P. H. Corran and J. F. Kennedy, *Carbohydr. Res.*, 87 (1980) 165.
12 J. J. Kirkland, *J. Chromatogr. Sci.*, 10 (1972) 593.
13 R. E. Majors, *Anal. Chem.*, 44 (1972) 1722.
14 J. H. Knox and M. Saleem, *J. Chromatogr. Sci.*, 7 (1969) 614.
15 M. Hisamatau, unpublished work.
16 W. Pigman and D. Horton, *The Carbohydrates, Chemistry, Biochemistry, IIA*, Academic Press, New York, London, 1970, p. 376.