Journal of Chromatography, 265 (1983) 89–96
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,820

SEPARATION OF CYCLIC (1→2)-β-D-GLUCANS (CYCLOSOPHORAOSES) PRODUCED BY *AGROBACTERIUM* AND *RHIZOBIUM*, AND DETERMINATION OF THEIR DEGREE OF POLYMERIZATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received March 1st, 1983)

SUMMARY

Conditions for the separation of the eight components contained in cyclic (1→2)-β-D-glucans (cyclosophoraoses) from *Agrobacterium* and *Rhizobium* were investigated by high-performance liquid chromatography on a chemically modified amine column (μBondapak CH) with mixtures of acetonitrile and water as eluent, and on a reversed-phase column (Dextro-Pak cartridge) with methanol–water (4:96) as eluent. Although retention on the amine column was related to molecular mass, that on the reversed-phase column differed from it, and was probably related to the solubility in water.

The degree of polymerization of each cyclosophoraose was determined by high-performance liquid chromatography of its partial hydrolysate. Sophoro-oligomers having degrees of polymerization up to 24 were well resolved on a different amine column (Finepak SIL NH_2, 10 μm) within 27 min by using a simple isocratic elution of acetonitrile–water (55:45). On hydrolysis under the appropriate conditions the longest straight chain sophoro-oligomer was present in sufficient amount to be able to recognize it in the partial hydrolysate; the distinguishing last peaks in the chromatograms of the eight cyclosophoraose hydrolysates could be detected clearly as the 17th–24th peaks.

INTRODUCTION

Recently, Hisamatsu *et al.*[1] and Amemura *et al.*[2] have found that many strains of *Agrobacterium* and *Rhizobium* produce cyclosophoraoses. Hisamatsu *et al.*[3] have isolated eight components of homogeneous molecular sizes from a mixture of these by paper chromatography, followed by high-performance liquid chromatography (HPLC), and have examined their properties.

0021-9673/83/$03.00

In this present work, we have studied the simultaneous isolation of the eight pure cyclosophoraoses produced by *A. radiobacter* IFO 12664 by HPLC on a chemically amine-bonded phase and on a reversed phase, and have estimated the degree of polymerization (DP) of each cyclosophoraose by HPLC of the partial hydrolysate on another amine column. In addition, the percentage compositions of the cyclosophoraoses from four strains of *Agrobacterium* and *Rhizobium* were determined by HPLC.

EXPERIMENTAL

Apparatus

An SE-31 refractive index monitor (Showa Denko, Tokyo, Japan) was used in conjunction with a Tri Rotar SR-1 pump and a VL-614 variable-loop injector (both Japan Spectroscopy, Tokyo, Japan) for analyses of cyclosophoraoses and their partial hydrolysates. Preparative chromatography was carried out using a Twincle pump–VL-611 variable-loop injector (Japan Spectroscopy) with an SE-11 refractive index monitor (Showa Denko). The columns used were a μBondapak CH (300 × 3.9 mm I.D.) column, a Dextro-Pak cartridge (100 × 8 mm I.D.) employed in a Waters Z-Module radial compression separation system (both Waters Assoc., Milford, MA, U.S.A.), and a Finepak SIL NH_2 10 μm (250 × 4.6 mm I.D., Japan Spectroscopy) column. A Chromatopac C-RIA digital integrator (Shimadzu, Kyoto, Japan) was used for quantitative analysis.

Materials

Mixtures of cyclosophoraoses were prepared according to the method of Hisamatsu *et al.*[1]. Organisms used were *A. radiobacter* IFO 12664, *R. phaseoli* AHU 1133, *R. japonicum* IFO 13338, and *R. meliloti* J7017. Reagent-grade acetonitrile and methanol were obtained from Wako (Osaka, Japan) and were dried and freshly distilled before use. Water used in solvent preparations was deionized and distilled. Eluents were filtered through a 0.45-μm membrane filter and degassed. Cyclodextrins were purchased from Nakarai (Kyoto, Japan). Trifluoroacetic acid (TFA) and Dowex WGR, which were used in the partial hydrolyses of cyclosophoraoses, were commercially available.

Partial hydrolysis of cyclosophoraose

A sample (2.5 mg) of cyclosophoraose was hydrolysed in 1 ml of 0.1 *M* TFA at 100°C for 60–120 min. The solution containing the hydrolysate was neutralized with Dowex WGR (OH^-), filtered, and evaporated to dryness under reduced pressure.

RESULTS AND DISCUSSION

HPLC analysis of cyclosophoraoses on μBondapak CH

The μBondapak CH column is a chemically modified amine column and is used especially for carbohydrate analysis. A mixture of acetonitrile and water is the most commonly used eluent with this type of column. In this study, the acetonitrile–water composition was varied from 56:44 to 66:34. In order to hold the mixing ratio constant, the eluent was prepared immediately before use by mixing

exact volumes of ultrasonic-degassed acetonitrile and water, and then degassing again by bubbling helium gas through for 10 min: even small changes in the mixing ratio were found to influence the chromatographic separation. However, the retention decreased slightly with time and tended to decrease more with increasing proportions of acetonitrile.

The changes in the capacity factor (k') for the eight cyclosophoraoses, designated A to H in order of increasing retention time (t_R), with changes in the acetonitrile–water composition are given in Table I. Whereas at a ratio of 56:44 or 58:42 the second peak (B) could not be separated from the third peak (C), with higher proportions of acetonitrile eight peaks were separated and an increase in the acetonitrile content increased the retention without changing the elution sequence. A typical elution pattern for the cyclosophoraoses is shown in Fig. 1. Although at an acetonitrile–water ratio of 66:34 baseline separation could be practically achieved for all the eight components within *ca.* 70 min, a considerable broadening of the later peaks, *e.g.* G and H, was unavoidable.

Separation of cyclosophoraoses on Dextro-Pak

The Dextro-Pak column is packed with a grade of C_{18}-bonded silica especially selected for carbohydrate oligomer separations[4]. Distilled water is commonly used as the eluent. However, the cyclosophoraoses could not be eluted with 100% water. By the use of an aqueous solution containing 4% methanol, excellent separations of seven peaks were achieved within an analysis time of 32 min (Fig. 2), but the elution pattern was different from that obtained on NH_2-bonded silica with acetonitrile–water as eluent. The third peak contained C and D, which could not be separated even at lower methanol contents. Moreover, the elution order of E and F reversed.

These discrepancies in the elution pattern are explained on the basis of a difference between the separation mechanisms on NH_2-bonded silica and on C_{18}-bonded silica. The elution pattern with the NH_2-bonded silica acetonitrile–water system is similar to that with the anion-exchanger ethanol–water system[5], *viz.* increased re-

TABLE I

CAPACITY FACTORS (k') OF CYCLOSOPHORAOSES (A–H) WITH CHANGES IN ACETONITRILE–WATER COMPOSITION

Chromatographic conditions: column, μBondapak CH (300 × 3.9 mm I.D.); flow-rate, 2 ml/min; detector, refractometer at 4×; temperature, ambient. Sample: cyclosophoraoses produced by *A. radiobacter* IFO 12664.

Cyclosophoraose	*Mobile phase ratio (acetonitrile–water)*					
	56:44	*58:42*	*60:40*	*62:38*	*64:36*	*66:34*
A	5.29	5.86	6.86	8.00	9.86	14.00
B	–	–	7.71	9.29	11.29	16.43
C	6.14	7.00	8.43	10.07	12.71	18.57
D	7.28	8.29	10.00	11.86	15.14	22.43
E	7.71	8.86	10.93	13.21	17.00	25.71
F	9.21	10.57	12.86	15.50	20.14	30.86
G	10.79	12.29	15.07	18.43	23.86	37.29
H	12.29	14.29	17.79	21.57	28.43	45.29

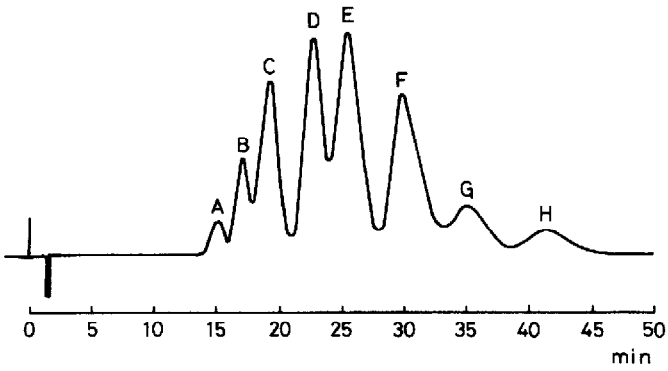

Fig. 1. Separation of cyclosophoraoses obtained from cultures of *A. radiobacter* IFO 12664 on NH_2-bonded silica. Chromatographic conditions: column, μBondapak CH (300 × 3.9 mm I.D.); eluent, acetonitrile–water (64:36); other conditions as in Table I.

tention with increasing molecular size of the sugar. On the other hand, C_{18}-bonded silica is a reversed phase and so the separation mechanism is probably an example of hydrophobic chromatography[6], *i.e.* increased retention with decreasing solubility in water. This assumption was verified by the relationship between the solubilities and the retention times of three cyclodextrins [cyclic (1→4)-α-D-gluco-oligosaccharides] (Table II). Therefore, the retention on Dextro-Pak column suggests that C and D have practically the same solubility in water and that the solubility of F is higher than that of E. Furthermore, it has become apparent that cyclosophoraoses are much more soluble than cyclodextrins, because even γ-cyclodextrin cannot be eluted with the eluent used for cyclosophoraoses, *i.e.* methanol–water (4:96).

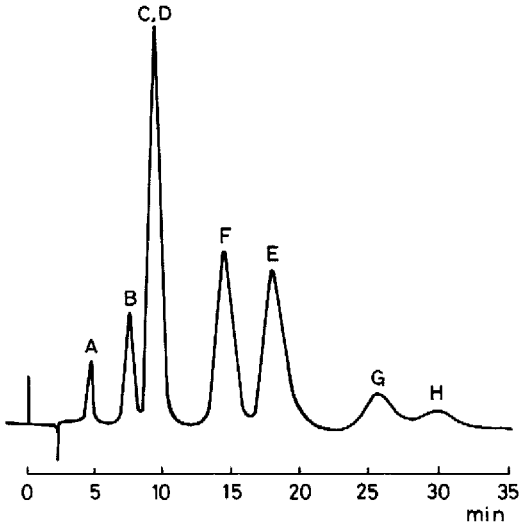

Fig. 2. Separation of cyclosophoraoses obtained from cultures of *A. radiobacter* IFO 12664 on C_{18}-bonded silica. Chromatographic conditions: column, Dextro-Pak cartridge (100 × 8 mm I.D.), eluent, methanol–water (4:96); other conditions as in Table I.

TABLE II

SOLUBILITY (*S*) IN WATER AT 25°C AND RETENTION TIME (t_R) ON A REVERSED PHASE COLUMN OF α-, β- AND γ-CYCLODEXTRIN (CyD)

Chromatographic conditions: column, Dextro-Pak cartridge (100 × 8 mm I.D.); eluent, methanol–water (12:88); other conditions as in Table I.

Cyclodextrin	*S (g/100 ml)*	t_R *(min)*
α-CyD (DP 6)	14.50	9.0
β-CyD (DP 7)	1.85	24.0
γ-CyD (DP 8)	23.20	6.8

Preparative chromatography

A Dextro-Pak column with methanol–water (4:96) as eluent is best used for preparative chromatography, as the loading capacity is relatively high (*ca.* 10 mg per column), a complete separation of all components other than C and D can be obtained within *ca.* 35 min, the eluent composition scarcely changes with time and the eluent is much cheaper than acetonitrile–water. On the other hand, the μBondapak CH column with acetonitrile–water (66:34) as eluent is inferior to the former in all the respects mentioned above except for one: C and D are separable. Further amine-bonded phases are subject to deterioration and fouling[7].

Consequently, preparative chromatography was performed on Dextro-Pak with methanol–water (4:96) as eluent with the third fraction containing C and D being refractionated on μBondapak CH with acetonitrile–water (66:34) as eluent. Since the eluate from the μBondapak CH column was contaminated with a slightly water-soluble material, the eluate was concentrated to dryness and the residue was dissolved in a small amount of water, centrifuged and then purified by gel-chromatography on a Sephadex G-10 column.

Determination of DP

Determination of the DP of each cyclosophoraose by HPLC of its partial hydrolysate was attempted. In order to obtain the appropriate hydrolysis conditions under which a small amount of the native cyclosophoraose would reside in the reaction product, the reagent, the reaction temperature and the reaction time were examined; the conditions described in the Experimental section were adopted. The reaction time was varied over the range 60–120 min, depending on how difficult the hydrolysis was (120 min for A, 60 min for B, 90 min for the rest). Fortunately, it seemed likely that the native cyclosophoraose coexisted at equilibrium with the corresponding straight-chain sophoro-oligomer in the reaction mixture, and therefore the hydrolysate always contained a relatively large amount of the longest straight-chain sophoro-oligomer from the beginning to the end of the reaction.

Several attempts at separating oligosaccharides on the basis of size have been made. Oligosaccharides up to DP 11 were resolved in 4.5 h on Bio-Gel P-2 (ref. 8) while ones up to DP 15 were separated in 8 h on Bio-Gel P-4 (ref. 9). Separations by HPLC on chemically bonded phases have been achieved by using μBondapak CH (up to DP 5)[10], an aminopropyl-bonded phase (up to DP 7)[11] and Partisil-10 PAC (up to DP 11)[12]. White and Corran[13] reported that refractometers can only be used

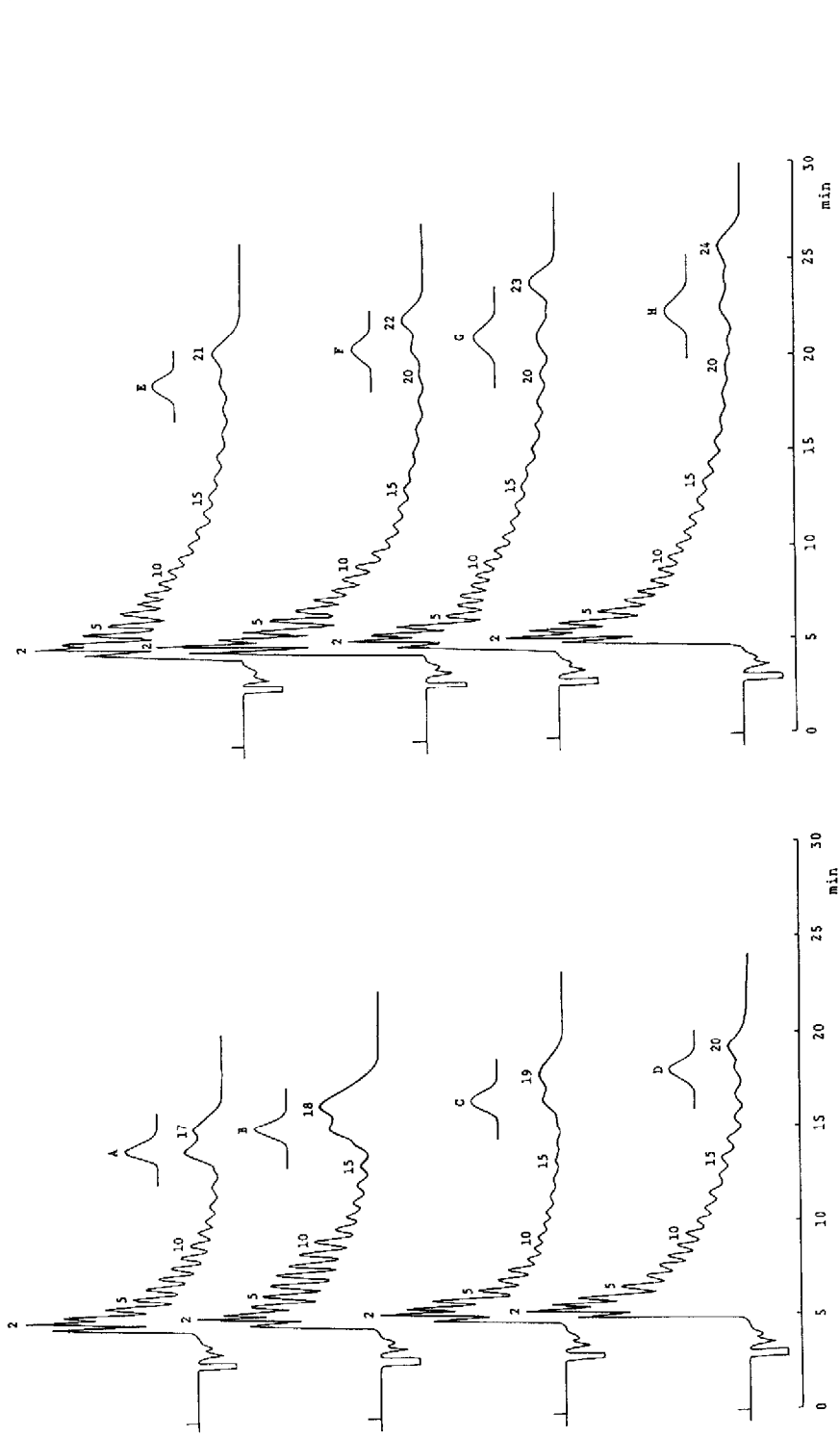

Fig. 3. Separation of D-glucose and sophoro-oligomers present in partial hydrolysate of cyclosophoraose (A–H). The number beside each peak indicates its DP. Peak 2 is sophorose. The upper trace of each chromatogram shows the chromatogram of native cyclosophoraose. Chromatographic conditions: column, Finepak SIL NH_2, 10 μm (250 × 4.6 mm I.D.); eluent, acetonitrile–water (55:45); flow-rate, 1 ml/min; detector, refractometer at 2×; temperature, ambient.

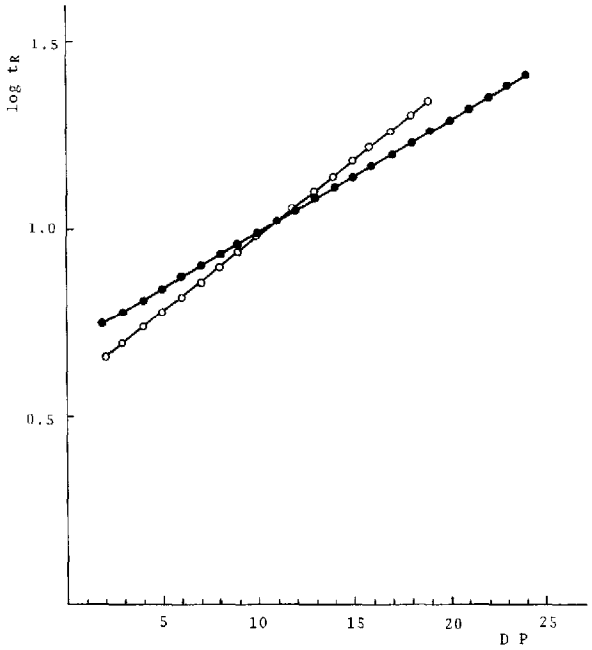

Fig. 4. Relationship between log t_R and DP of sophoro-oligomers. Chromatographic conditions: ●, as in Fig. 3; ○, column, μBondapak CH (300 × 3.9 mm I.D.); eluent, acetonitrile–water (61:39); other conditions as in Fig. 3.

satisfactorily in conjunction with isocratic elution and therefore higher oligomers cannot be analysed under these conditions, in which chemically bonded phases were used. They separated oligosaccharides up to DP 20 on silica columns dynamically modified with polyfunctional amines.

However, we have found that chemically amine-bonded silica columns provide a rapid and simple method of separating higher β-(1→2)-linked gluco-oligomers. A Finepak SIL NH_2 10-μm column with acetonitrile–water (55:45) as eluent can better be applied to the separation of sophoro-oligomers. In less than 30 min a complete separation could be obtained from DP 1 to 24. A column of μBondapak CH was also tried for this separation, but proved to be too retentive, since oligomers of DP > 20 had too high t_R values.

The distinguishing last peak in the chromatogram of the partial hydrolysate of cyclosophoraose A was the 17th peak, counting from the glucose peak, and consequently, the DP of A is 17. In the same way, the DP values of B, C, D, E, F, G and H were unambiguously estimated as 18, 19, 20, 21, 22, 23 and 24, respectively (Fig. 3).

Fig. 3 shows that the native cyclosophoraose is eluted slightly before the corresponding straight-chain sophoro-oligomer. Sophoro-oligomers have a linear relationship between log t_R and DP (Fig. 4). Therefore, the analysis of higher oligomers became feasible without loss of resolution of the lower oligomers.

TABLE III

PERCENTAGE COMPOSITION OF CYCLOSOPHORAOSES

Calculations were performed on a digital electronic data processor. Chromatographic conditions: column, μBondapak CH (300 × 3.9 mm I.D.); eluent, acetonitrile–water (66:34); other conditions as in Table I.

Cyclosophoraoses		*A (DP 17)*	*B (DP 18)*	*C (DP 19)*	*D (DP 20)*	*E (DP 21)*	*F (DP 22)*	*G (DP 23)*	*H (DP 24)*
Type	*Produced by*								
I	*R. phaseoli* AHU 1133	83.1	7.9	7.5	1.5	–	–	–	–
II	*R. japonicum* IFO 13338	5.6	12.2	39.8	21.5	13.0	7.9	–	–
III	*R. meliloti* J7017	1.1	4.0	4.6	15.9	25.1	33.1	11.9	4.4
IV	*A. radiobacter* IFO 12664	1.8	6.3	14.4	19.4	25.7	21.6	7.2	3.6

Determination of percentage compositions

It had been indicated by HPLC analysis on μBondapak CH that cyclosophoraoses produced by *Agrobacterium* and *Rhizobium* could be divided into four types (I–IV)[3]. Determination of the percentage composition of cyclosophoraoses representing each type was attempted: products of *R. phaseoli* AHU 1133 as type I, of *R. japonicum* IFO 13338 as type II, of *R. meliloti* J7017 as type III and of *A. radiobacter* IFO 12664 as type IV were chosen.

The resolution using μBondapak CH with acetonitrile–water (66:34) as eluent was high enough to make quantitation practicable. The quantitative assessment of the chromatogram obtained with this system was carried out by an internal normalization method, using a digital electronic data processor. Table III shows clearly the difference in composition of the four types.

REFERENCES

1 M. Hisamatsu, A. Amemura, T. Matsuo, H. Matsuda and T. Harada, *J. Gen. Microbiol.*, 128 (1982) 1873.
2 A. Amemura, M. Hisamatsu, H. Mitani and T. Harada, *Carbohydr. Res.*, 114 (1983) 277.
3 M. Hisamatsu, A. Amemura, K. Koizumi, T. Utamura and Y. Okada, *Carbohydr. Res.*, in press.
4 N. W. H. Cheetham and P. Sirimanne, *J. Chromatogr.*, 208 (1981) 100.
5 O. Samuelson, *Ion Exchange, Vol. 2*, Marcel Dekker, New York, 1969, ch. 5.
6 Cs. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
7 K. Aitzetmüller, *J. Chromatogr.*, 156 (1978) 354.
8 N. K. Sabbagh and I. S. Fagerson, *J. Chromatogr.*, 120 (1976) 55.
9 N. K. Sabbagh and I. S. Fagerson, *J. Chromatogr.*, 86 (1973) 184.
10 J. C. Linden and C. L. Lawhead, *J. Chromatogr.*, 105 (1975) 125.
11 R. Schwarzenbach, *J. Chromatogr.*, 117 (1976) 206.
12 F. M. Rabel, A. G. Caputo and E. T. Butts, *J. Chromatogr.*, 126 (1976) 731.
13 C. A. White and P. H. Corran, *Carbohydr. Res.*, 87 (1980) 165.