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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CYCLIC $\beta(1 \rightarrow 2)$-D-GLUCANS (CYCLOSOPHORAOSES) PRODUCED BY *RHIZOBIUM MELILOTI* AND *RHIZOBIUM TRIFOLII*

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SUMMARY

A method for the identification of cyclic glucans (cycloamyloses and cyclosophoraoses) in terms of the glycosidic linkage type and of the degree of polymerization (DP) using a reversed-phase 5-μm amine bonded silica column is proposed. The dependence of the logarithm of the capacity factors (log k') on the DP and on the structure of the cyclic oligosaccharides is established by comparing the chromatographic behaviour of $\beta(1 \rightarrow 2)$-D-glucans (cyclosophoraoses) from two different *Rhizobium* strains with that of cycloamyloses and of mono- and disaccharides. The determination of the DP of the cyclosophoraoses was achieved by high-performance liquid chromatography (HPLC) of the partial hydrolysates of three collected peaks. HPLC experiments using cyclosophoraoses from *Rhizobium meliloti* SU-47 and *Rhizobium trifolii* TA-1 demonstrate that such microbial carbohydrates are mixtures of cyclic glucans with DP ranging from 17 to more than 33, in different proportions depending upon the bacterial source.

INTRODUCTION

Bacteria of the genus *Rhizobia* are known for their ability to infect plants resulting in the formation of nodules and crown galls, and to participate in atmospheric nitrogen fixation. Related to their infective ability, there is a growing interest in the saccharidic molecules produced by these microorganisms. Three main kinds of carbohydrate molecules are produced[1–5]: ionic exocellular polysaccharides, non-ionic capsular polysaccharides and a mixture of oligomeric $\beta(1 \rightarrow 2)$ linked D-glucans (the so-called sophoraoses). The latter species have drawn the attention of a number

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of scientists because of a possible implication in the plant–*Rhizobia* recognition.

At present little is known about their biological activity. To our knowledge only two papers have been published on this topic. The first[6] simply suggests that $(1 \rightarrow 2)$-linked D-glucans may play a rôle in the attachment to plant cells. The second[7] shows a parallelism between regulation of the biosynthesis of $\beta(1 \rightarrow 2)$-D-glucans and the osmotic regulation of membrane-derived oligosaccharide biosynthesis in *Escherichia coli*, thus suggesting a general rôle for periplasmic oligosaccharides in the osmotic adaptation of soil bacteria.

Fast atom bombardment (FAB) mass spectrometry studies[8] unambiguously demonstrated that low-molecular-weight glucans are cyclic molecules, with the exception of a linear hexamer. A possible symmetrical structure for these cyclic compounds has been proposed[9] by means of conformational analysis studies. The high symmetry minimum energy conformations of glucans comprised of 17–24 monomers per cycle exhibit internal cavities characterized by diameters ranging from 8 to 13 Å. This feature resembles that exhibited by the well known α-, β- and γ-cyclodextrins [cyclic α-$(1 \rightarrow 4)$-D-glucans] whose three-dimensional structure leaves an internal cavity of 5–7 Å in diameter.

Koizumi *et al.*[10] found that the dependence of log t_R on the number of monomers for hydrolysed glucans is a function of the type of glycosidic linkage. In this paper we report our structural studies on families of glucans and demonstrate that the chromatographic performance of native glucans is strictly dependent on their structure and on the degree of polymerization. For this purpose, mixtures of $\beta(1 \rightarrow 2)$-D-glucans produced by *Rhizobium meliloti* and *Rhizobium trifolii* have been analysed. The fragments from the parent molecule were identified by means of high-performance liquid chromatography (HPLC) on partial hydrolysates of separately collected fractions.

EXPERIMENTAL

Materials

Silica with chemically bonded aminopropyl groups was used as column packing material. Spherical particles with an average diameter of 5 μm and an amine coverage of 2%, w/w (0.6 mM/g) were supplied by Phase Separation (Queensferry, U.K.). The regularity of the particles was checked by means of electron microscopy. Two stainless-steel columns (160 mm × 4.6 mm I.D.) were slurry-packed in our laboratory with amine phase suspended in carbon tetrachloride, by means of an Haskel Series 1080-357 pneumatic pump. Columns packed in this way showed an efficiency of about 30 000 plates per metre for sucrose. Peak integration for quantitative analysis was performed by an Hewlett-Packard integrator N.3385A. All solvents, of analytical grade, were filtered and degassed before use. Distilled water was filtered and passed through a Norganic Cartridge supplied by Millipore (Bedford, MA, U.S.A.), to eliminate organic substances.

Chromatography of $\beta(1 \rightarrow 2)$-D-glucans

HPLC analysis of cyclosophoraoses produced by *Rhizobium trifolii* TA-1 and *Rhizobium meliloti* SU-47[4,5] was performed using a Pye-Unicam LC-XPD pump equipped with a Rheodyne Type 7125 sample valve fitted with a 20-μl loop. Detection

was performed by a differential refractive index (RI) detector (Varian Assoc., Palo Alto, CA, U.S.A.). The eluent was acetonitrile–water in different percentages as reported in the figures. Aliquots of 40 mg of β(1 → 2) glucans mixtures, dissolved in 0.5 ml of distilled and filtered water, were purified, before chromatography, on an amine column (J. T. Baker Chemical, Phillipsburgh, NJ, U.S.A.), eluting with 2 ml of acetonitrile. The purified samples were then injected into the amine-silica packed column. The eluted solutions containing single peaks were collected for the determination of the number of glucose units per ring by means of partial hydrolysis, as described below.

Partial hydrolysis of β(1 → 2)-D-glucans

The collected HPLC fractions, each containing a single cyclosophoraose, were partially hydrolysed by dissolving 1 mg of sample in 0.5 ml of 0.1 M trifluoroacetic acid (TFA) at 100°C for 60–90 min[11]. After evaporation to dryness under reduced pressure, the samples containing partial hydrolysates were dissolved in 20 μl of water and neutralized with sodium bicarbonate.
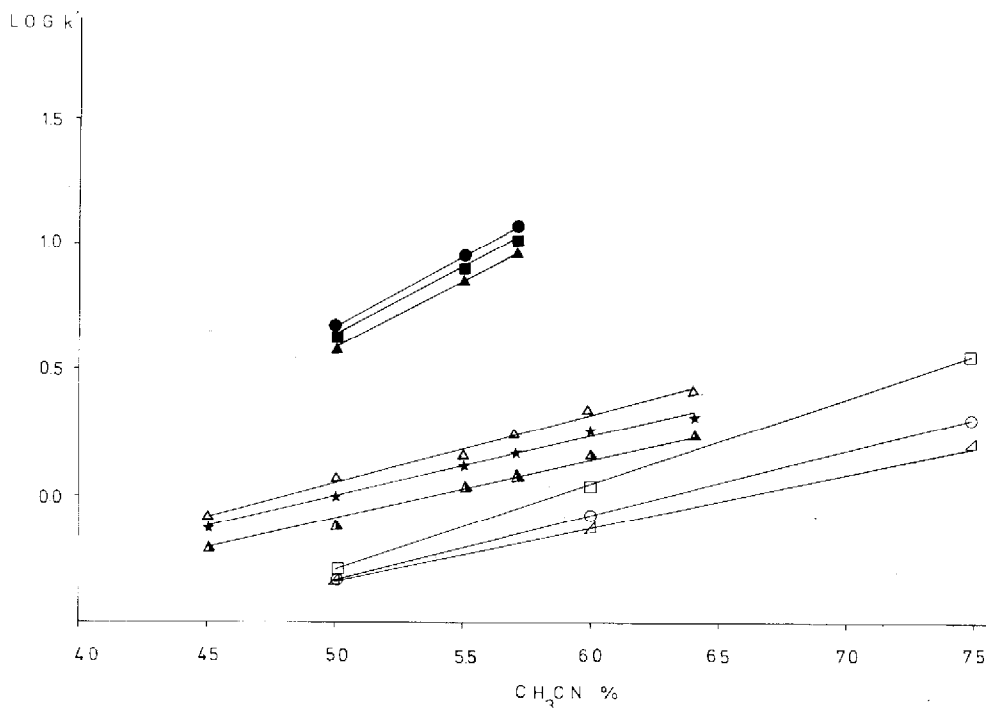

Fig. 1. Plot of logarithms of capacity factors *vs.* acetonitrile percentage. Column (160 mm × 4.6 mm) packed with 5-μm amine-bonded silica. Flow-rate: 1 ml/min. Detector: differential refractive index at 2 · 10^{-5} RI units full scale. Room temperature. ▷—▷, D-(−)-Arabinose; ○—○, D-(+)-mannose; □--□, sucrose; ▲--▲, α-cyclodextrin; ★—★, β-cyclodextrin; △—△, γ-cyclodextrin; ▲--▲, ■—■, ●—●, native glucan from *Rhizobium meliloti* SU-47 with DP = 21, 22, 23 respectively.

M. BENINCASA *et al*

RESULTS AND DISCUSSION

Chromatographic separation of carbohydrate samples has been achieved by use of different systems like ion-exchange resins, silica gel, derivatized silica and gel permeation[12–16], the latter method being mainly used for oligo- and polysaccharides. In spite of the different conditions employed for these analytical methods, all authors agree that the determining factor for separation, in ion exchange as well as in silica systems, is an effective hydrogen-bond network between the carbohydrates and the surface of the packing material and/or the eluent. Using derivatized or underivatized
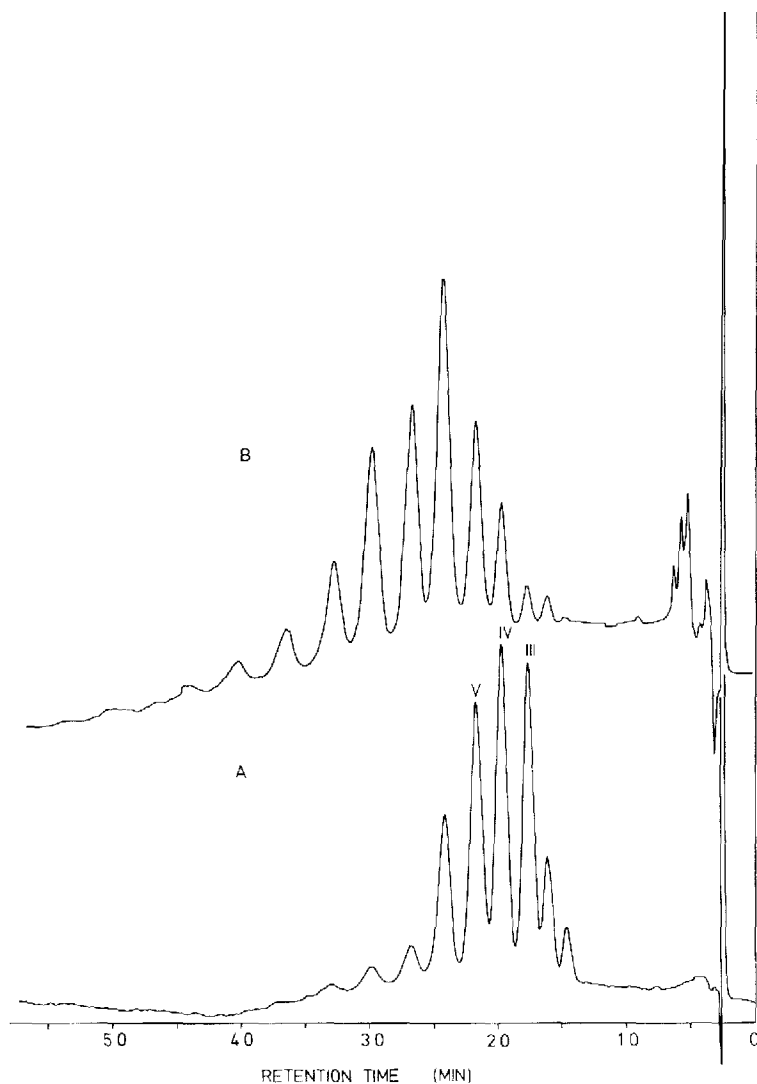

Fig. 2. HPLC of $\beta(1 \rightarrow 2)$-D-glucans on 5-μm amine-bonded silica column (160 mm × 4.6 mm). Eluent: acetonitrile–water (64:36). Flow-rate: 1 ml/min. RI detector at $2 \cdot 10^{-5}$ units full scale. (A) $\beta(1 \rightarrow 2)$-D-glucans from *Rhizobium trifolii* TA-1; (B) $\beta(1 \rightarrow 2)$-D-glucans from *Rhizobium meliloti* SU-47.

silica (in the latter case eluting with organic modifiers), hydrogen bonding is thought to occur directly between the carbohydrates and the surface of the packing material. The data of D'Amboise *et al.*[17] on carbohydrate separations on bonded amino-silica seem to leave little possibility for other explanations. It is expected that the greater the hydroxyl number, the longer is the retention time for carbohydrates.

This is verified for sucrose in comparison with mannose, arabinose and other monosaccharides (Fig. 1). It is clear, however, that the number of hydroxyl groups cannot be the only explanation for chromatographic separation of sugars. The considerations mentioned above cannot explain the separation of hexoses, of disaccharides and of α- and β-isomers[18]. The latter findings can be better explained by considering that the determining factor in carbohydrate separation (within each given class) on amine-modified silica is the distribution of hydroxyl groups around the molecule[19]. In particular the separation of α- and β-isomers invariably occurs with a longer retention time for the isomer exhibiting the most exposed hydroxyl group.

All the preceding considerations are verified by our studies. Fig. 1 shows the very good linear correlation between the capacity factors, log k' and the percentage of organic modifier for mono- and disaccharides as well as for higher-molecular-weight carbohydrates like α-, β- and γ-cyclodextrins and cyclic-$\beta(1 \rightarrow 2)$-D-glucans. Chromatograms showing the separation of cyclic glucan mixtures produced by the two *Rhizobia* strains are presented in Fig. 2. It is evident that a satisfactory separation of each cyclosophoraose is achieved. Peaks near to the void volume are attributed to some proteic impurity in the initial culture medium. Purified samples obtained from these peaks gave a positive ninhydrin test.

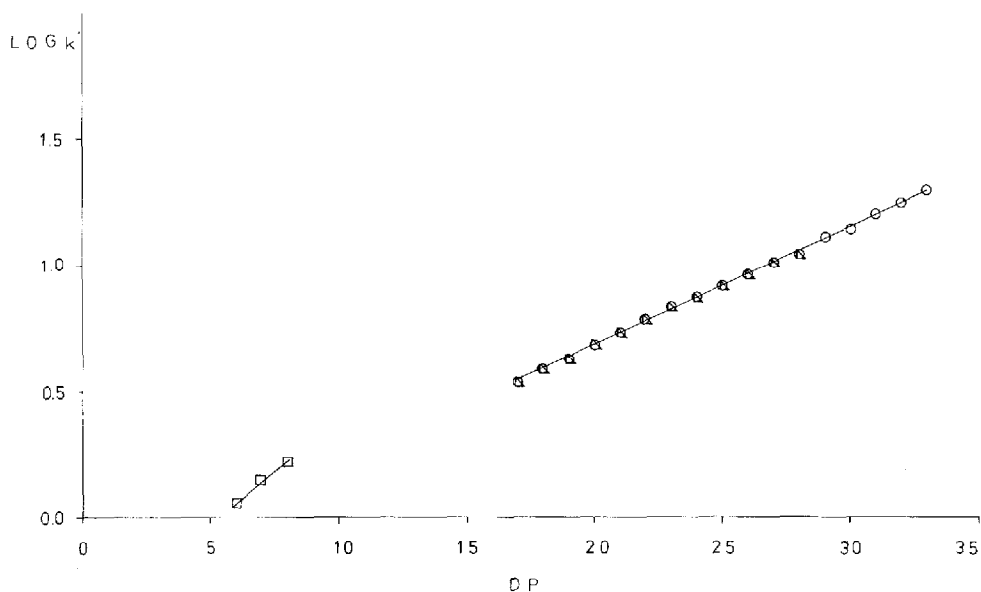The plot of log k' for all the mixture components as a function of the number

Fig. 3. Plot of logarithms of capacity factors *vs.* the degree of polymerization. Chromatographic conditions as in Fig. 2. □, Cyclodextrins; ▷, *Rhizobium trifolii* TA-1 native glucans; ○, *Rhizobium meliloti* SU-47 native glucans.
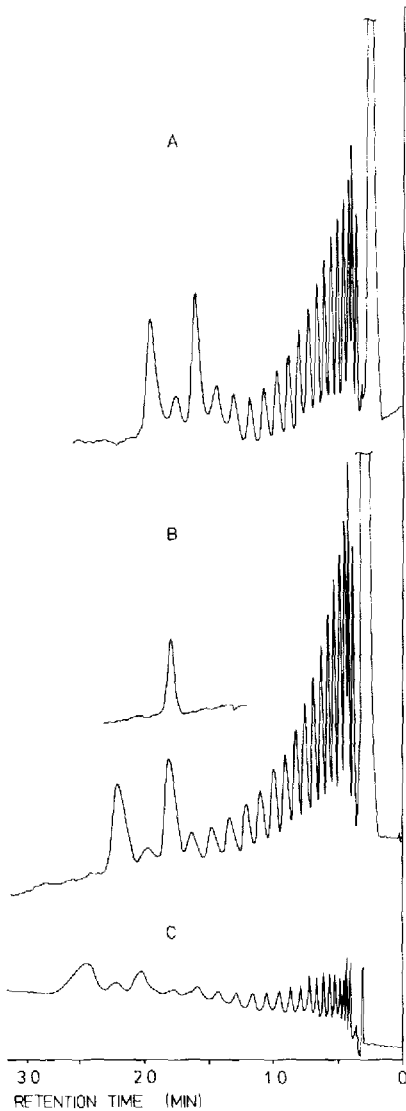
Fig. 4. HPLC of (A) the hydrolysate of peak III of Fig. 2, (B) the hydrolysate and native of peak IV of Fig. 2, (C) the hydrolysate of peak V of Fig. 2. Chromatographic conditions as in Fig. 2.

of monomers per cycle (also referred to as the degree of polymerization, DP) shows good linearity for cyclodextrins as well as for the larger sophoraoses from *Rhizobia* strains (Fig. 3). The different slopes can easily be attributed to differences in the overall three-dimensional molecular shape of the two series of compounds and, probably, to the more pronounced hydrophilic properties of the cyclodextrins external surfaces. It is to this latter property that we can ascribe the higher k' values obtained in the case of cyclodextrin samples.

The identification of each component of the mixtures of $\beta(1 \rightarrow 2)$-D-glucans

together with the determination of its number of monomers per cycle was performed on isolated samples, corresponding to peaks III, IV and V of Fig. 2, collected by semipreparative HPLC. These samples were subsequently hydrolysed as described in the Experimental, and then the hydrolysate was injected into an amine-bonded silica column (Fig. 4). The logarithms of the capacity factors as a function of the number of monomers in the fragments show an excellent coincidence for the three different cycles. In addition, fitting the log k' data with a straight line is very satisfactory, especially for the larger oligomers where the uncertainty in the retention time measurement is smaller (Fig. 5). The good linearity of log k' as a function of the number of monomeric units obtained in the case of hydrolysed samples led us unambiguously to assign the chromatographic peaks to fragments coming from the purified cyclosophoraoses. The total number of peaks in the hydrolysate chromatograms can then be considered as equal to the number of monomers per cycle. As a result of these considerations the samples isolated from peaks III–V, relative to mixtures produced by *Rhizobium trifolii* TA-1, are characterized by 19, 20 and 21 β-D-glucopyranoside units per ring, respectively.

The validity of the latter conclusion has been tested using a γ-cyclodextrin sample. Aliquots of this compound were treated by a similar hydrolysis procedure. The chromatogram obtained from the hydrolysate showed only eight peaks (Fig. 5), *i.e.*, exactly the number of α-glucopyranoside units pertaining to the cycle. The hy-
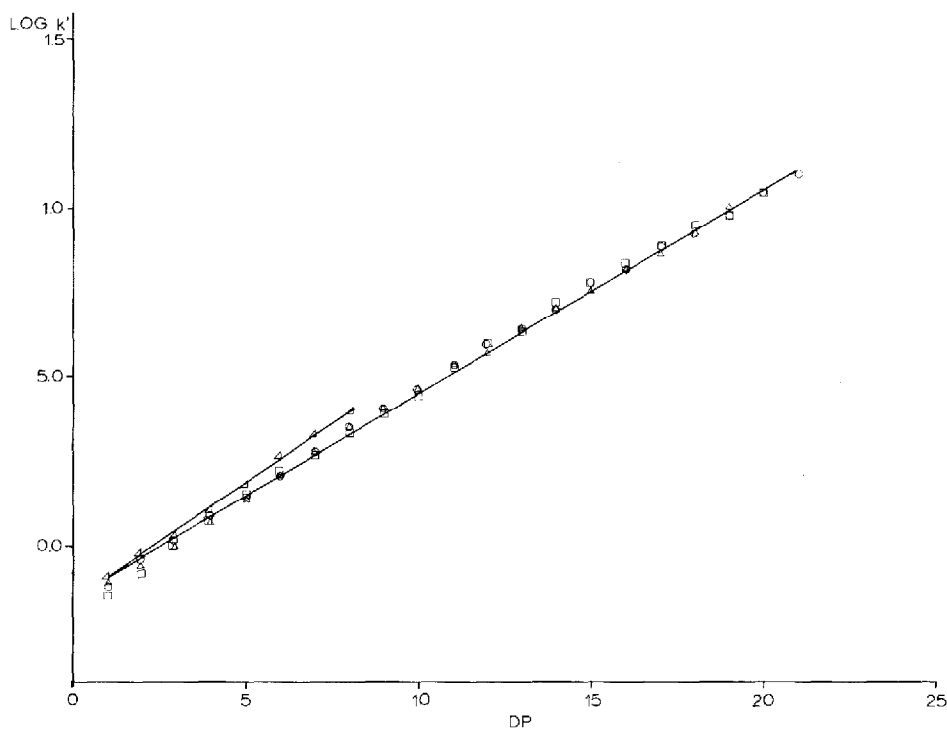

Fig. 5. Plot of logarithms of capacity factors *vs.* degree of polymerization. $\triangleright$, γ-Cyclodextrin hydrolysate; $\triangle$, hydrolysate of peak III of Fig. 2; $\square$, hydrolysate of peak IV of Fig. 2; $\bigcirc$, hydrolysate of peak V of Fig. 2. Chromatographic conditions as in Fig. 2.

TABLE I

PERCENTAGE DISTRIBUTION OF CYCLIC $\beta(1 \rightarrow 2)$-D-GLUCANS

DP of bacterial strain	R. Meliloti SU-47	R. Trifolii TA-1
17	0.7	3.4
18	1.5	10.0
19	1.7	18.9
20	6.1	24.5
21	11.3	16.5
22	20.6	11.7
23	15.4	4.2
24	15.3	2.5
25	10.1	2.1
26	8.7	3.2
27	1.3	2.0
28	4.5	1.0
29	1.3	--
30	1.0	—

drolysate chromatograms of $\beta(1 \rightarrow 2)$-D-glucans also show the relative amounts of the fragments: the abundance of low-molecular-weight fragments is rather high and there is a remarkable presence of the linear oligomer arising from the simple opening of native cyclic glucan (largest retention time in Fig. 4). The seemingly anomalous area of the last-but-two peak is caused by the overlapping of an hydrolysate peak and the native cyclic glucan. The latter is characterized by a shorter retention time with respect to the linear fragment having the same number of monomers. This difference, as well as the slightly longer retention time for the γ-cyclodextrin hydrolysate, is easily attributable to a different interaction with the amino groups of the packing material.

An evaluation of the percentage of the different cyclosophoraoses belonging to mixtures produced by the two *Rhizobia* strains has been obtained from chromatographic experiments by measuring peaks with an integrator. The results are shown in Table I. It is seen that the production of cyclosophoraoses by *Rhizobia* is characterized by the more abundant glucans, which differ for each microbial strain. *Rhizobium trifolii* TA-1 is able to synthesize larger amounts of cycles with 19–21 glucoside residues whereas *Rhizobium meliloti* SU-47 produces mainly cycles having 22–24 monomers. Both strains are able to synthesize β-glucans containing at least up to 33 monomers per cycle, but the chromatographic results show only small amounts (less than 1%) of these compounds.

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