Carbohydrate Polymers **16** (1991) 321–326

Research Note

Characterisation of Cyclosophorans Produced by an Acidic Polysaccharide-negative Mutant of *Rhizobium leguminosaram*

ABSTRACT

The cyclosophorans produced by the EPS⁻ mutant Rhizobium leguminosarum *8401 pRL1JI pss1::Tn5 have been isolated and characterised. The cyclic glucans were recovered from the clarified culture broth by absorption and subsequent elution from a charcoal column. Individual ring sizes were identified by fast atom bombardment mass spectrometry and the crude mixture fractionated by HPLC. The spectrum of ring sizes produced is typical of that produced by wild-type* R. leguminosarum.

Cyclosophorans are cyclic $\beta(1\rightarrow2)$-D-glucans secreted by several strains of *Agrobacterium* and *Rhizobium* (Hisamatsu *et al.*, 1982; Amemura *et al.*, 1983). *Xanthomonas* bacteria are reported to produce an unusual branched cyclosophoran (Amemura & Cabrera-Crespo, 1986). The culture fluids normally contain cyclosophorans of varying molecular weight, or degree of polymerisation (DP). Typical values lie in the range DP = 17–24. The distribution of DP values has been found to be characteristic of the particular bacterial strain (Hisamatsu *et al.*, 1983; Koizumi *et al.*, 1983). The largest molecular weight reported corresponds to DP = 40 (Koizumi *et al.*, 1984*a*).

These cyclic compounds are of interest for at least two reasons. First, genetic mutants of *Rhizobium meliloti* which are unable to export cyclosophorans do not fix nitrogen in their plant host (Stanfield *et al.*, 1988). These studies suggest that the cyclic glucans may play an essential role in the plant–bacterium symbiosis. Secondly, there is growing evidence (Koizumi *et al.*, 1984*b*) that cyclosophorans may resemble, and perhaps surpass, cyclodextrins in their complex-forming properties. Cyclosophorans are normally secreted into the culture medium together with large amounts of highly viscous extracellular polysaccharide (EPS). Growth conditions for optimising the yield of cyclosophorans have been described (Zevenhuizen, 1986). Production of the highly viscous EPS makes aeration and agitation of the culture broth difficult, and also

Carbohydrate Polymers 0144-8617/91/$03.50 —

hinders removal of bacterial cells from the broth. Separation of the cyclosophorans requires fractionation with alcohol followed by column chromatographic separation using ion exchange and sephadex resins. These procedures are time consuming and wasteful in the use of solvents. Higashiura *et al.* (1985) have described the production and isolation of cyclosophorans from EPS$^-$ mutants of *R. leguminosarum* bv. *phaseoli* AHU1133, obtained by chemical mutagenesis using methods described by Adelburg *et al.* (1965). This article describes the isolation and characterisation of cyclosophorans produced by EPS$^-$ mutants of *R. leguminosarum*, generated by transposon mutagenesis (Lamb *et al.*, 1982; Borthakur *et al.*, 1986).

The EPS$^-$ mutant *R. leguminosarum* 8401 pRL1JI pssl::Tn5 was grown in shake flask culture in 4Y medium (Zevenhuizen, 1986) at pH = 6·8 and 29°C. Cyclosophoran yield increased with incubation time up to 20 days when yields varied between 2·6 and 3·2 g litre^{-1}. However, a maximum yield was obtained from a 1-litre batch culture grown for 20 days in a 1-litre fermentation vessel with enforced aeration (300–400 cc min^{-1}) and mixing (400 rev min^{-1}). Culture broths were clarified by centrifugation (2×10^4 ***g***, 60 min) and then sequential filtration of the supernatant down to a pore size of 650 nm. Cyclosophorans were isolated, free from pigment, by filtration through a charcoal column. The cyclic glucans bound to the column and could be eluted separately with 30% ethanol. The collected material was evaporated to dryness and stored for analysis.

Neutral sugars present in the powdered material were released by a Saeman hydrolysis and analysed as alditol acetates by GLC (Selvendran *et al.*, 1979). Glucose was the only sugar detected. Glycosidic linkages were determined as methylated alditol acetates by GLC mass spectrometry (Ring & Selvendran, 1978; O'Neill & Selvendran, 1980). The only linkage detected was (1→2)-linked glucose indicating pure cyclosophoran. The distribution of ring sizes was investigated by fast atom bombardment mass spectrometry (FAB-MS). This technique has been used previously (Dell *et al.*, 1983) to establish the cyclic nature of these compounds and to measure their molecular weights. Small deviations of molecular mass from the expected values arise because the ions were of low intensity and it was therefore necessary to accumulate a number of spectra at low resolution in order to obtain a good peak profile. Measurements were made to an accuracy of ≈ 1–2 mass units. Figure 1 shows a FAB-MS spectrum indicating the presence of major components with ring sizes corresponding to DP = 17, 18, 19, 20 and 21. This spectrum of ring sizes is characteristic of wild-type *R. leguminosarum* and has been dubbed a type II pattern (Hisamatsu *et al.*, 1983).

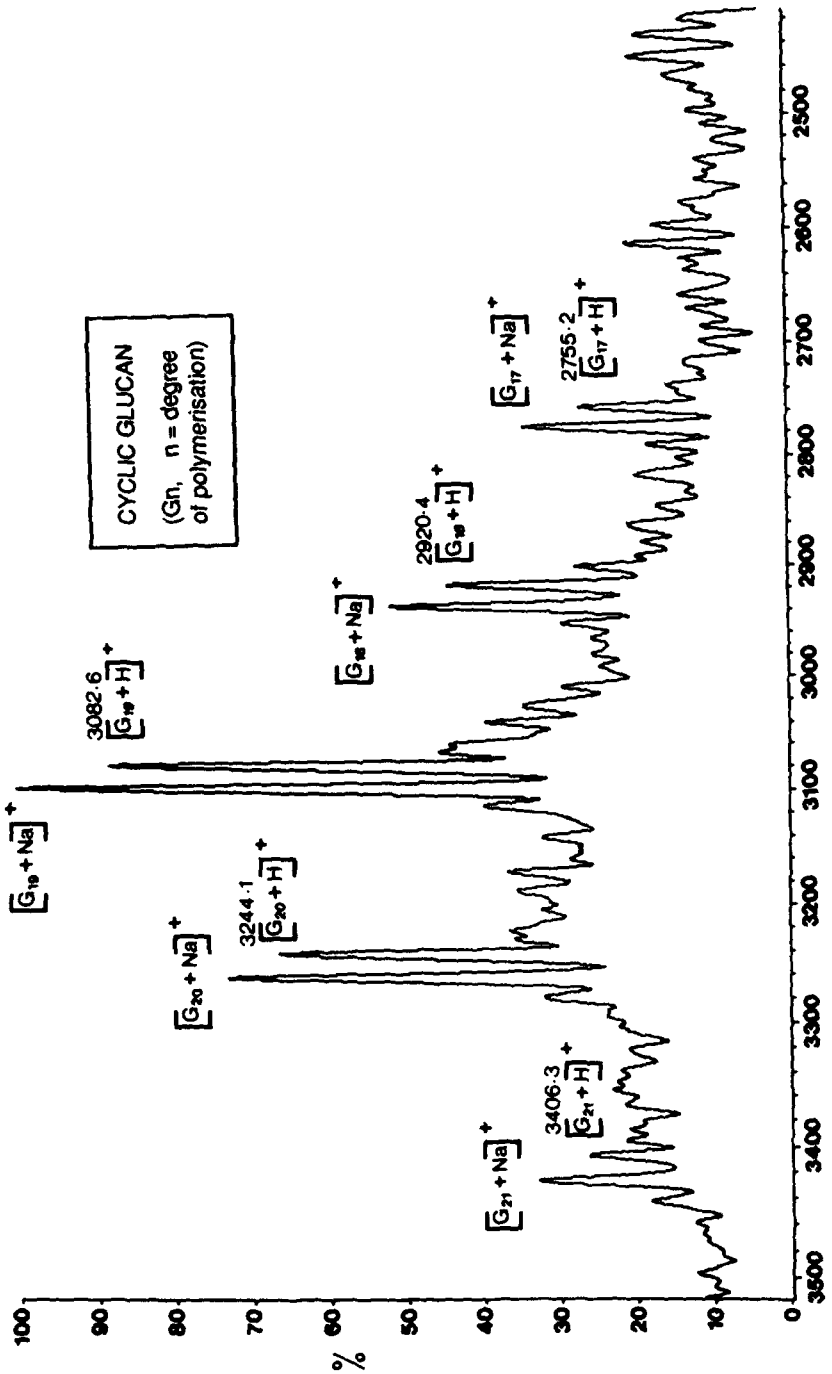

Fig. 1. Accumulated FAB-MS spectrum of cyclosophoran mixture run in a glycerol matrix.

HPLC was used to fractionate these major components and it revealed the presence of additional minor components. Optimum separation was obtained using a Zorbax ODS, 3-μm, 9·4 mm × 250 mm column. The column could be loaded with up to 10 mg without too much loss of separation. The column was eluted with 3% CH_3OH/H_2O (v/v) at an elution rate of 1·5 ml min^{-1}, which was increased to 2·5 ml min^{-1} in order to elute the last two component peaks. This resulted in a separation into nine peaks (Fig. 2(a)). Rechromatography of peaks 5 and 6 using 2% CH_3OH improved their separation (Fig. 2(b)). ^{13}C-NMR was used to check the purity of the major components.

Thus it has been shown that the EPS$^-$ mutant *R. leguminosarum* 8401 pRL1JI pssl::Tn5 produces the same pattern of cyclosophorans as the parent strain. The cyclic glucans can be isolated from the clarified broth using a charcoal column and then fractionated into individual ring sizes using HPLC. Individual ring sizes can be measured by FAB-MS. Such methods facilitate the preparation of purified cyclosophorans and thus an examination of their biological role and industrial applications.

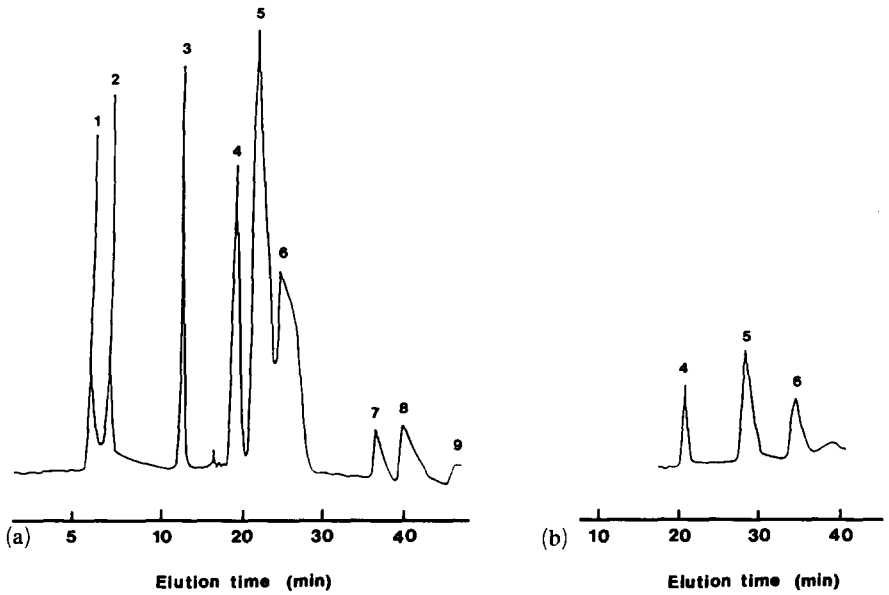

Fig. 2. Separation of crude cyclosophoran preparation on Zorbax ODS, 3000 nm, 9·4 × 250 mm column. (a) Eluant 3% methanol, 1·5 ml min^{-1} increased to 2·5 ml min^{-1}, 10 mg loading. (b) Eluant 2% methanol, 2·5–2·7 ml min^{-1}, 0·8 mg loading. Peaks 1–6 correspond to DP 15–20, respectively, peak 7 corresponds to DP 22, peak 8 to DP 21 and peak 9 to DP 23.

ACKNOWLEDGEMENTS

The authors wish to thank Dr I. Colquhoun for NMR studies and J Eagles for mass spectrometry.

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(Received 22 May 1990; revised version received 24 July 1990; accepted 28 July 1990)