J. Microbiol. Biotechnol. (2002), **12**(3), 522–525

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Rapid Separation of Cellular Cyclosophoraoses Produced by *Rhizobium* Species

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Received: October 27, 2001
Accepted: March 12, 2002

Abstract A very rapid and efficient separation technique for cellular rhizobial cyclosophoraoses was developed based on fractional precipitation and partition chromatography. Cyclosophoraoses are known to function in the osmotic regulation and root nodule formation of legumes during the nitrogen fixation process. Cyclosophoraoses are produced as unbranched cyclic (1→2)-β-D-glucans in *Agrobacterium* or *Rhizobium* species. Recent research has shown that cyclosophoraoses can form inclusion complexation with various unstable or insoluble guest chemicals, thereby implying great potential for industrial application. Typical separation of pure cellular cyclosophoraoses has been so far carried out by several time-consuming steps, including size exclusion, anion exchange, and desalting liquid chromatographies, with a relatively poor recovery. However, the proposed method demonstrated that the successive application of fractional ethanol precipitation and one step of silica gel-based flash column chromatography was enough to simultaneously purify neutral or anionic forms of cyclosophoraoses. This novel technique is very rapid and provides a high recovery.

Key words: Cyclosophoraoses, cyclic (1→2)-β-D-glucans, *Rhizobium meliloti*, silica gel flash column chromatography, inclusion complexation

Cyclosophoraoses are unique molecules found exclusively in most members of the *Rhizobiaceae* family [5]. Cyclosophoraoses are produced as unbranched cyclic (1→2)-β-D-glucans in *Agrobacterium* or *Rhizobium* species. They are found in periplasmic space [1, 15], secreted into an extracellular medium [2, 20], and believed to play a similar role as the membrane-derived oligosaccharides from *Escherichia coli* during osmotic adaptation [15]. They are also known to be involved in the initial stage of the root-nodule formation of *Rhizobium* species during nitrogen fixation [18]. The large ring size and flexible nature of cyclosophoraoses should be advantageous compared to the relatively static β-cyclodextrin of inclusion complexation [8, 10, 11, 17]. Recent studies have also been focused on cyclosophoraoses as the effective solubilizer of various insoluble compounds, such as ergosterol, fluoroscein, indomethacin, paclitaxel, and vitamins [9, 10, 12, 13].

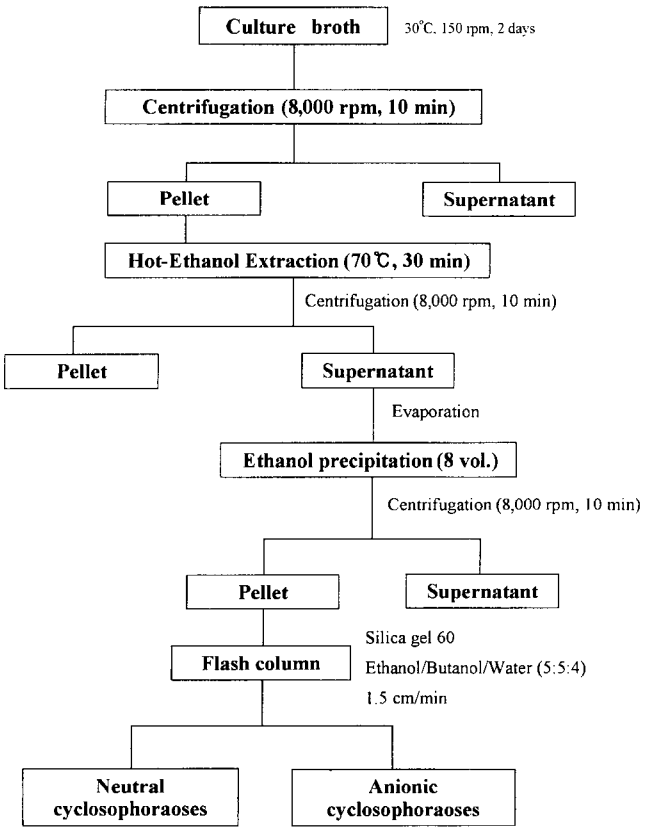

Fig. 1. Protocol of rapid separation of cellular rhizobial cyclosophoraoses.

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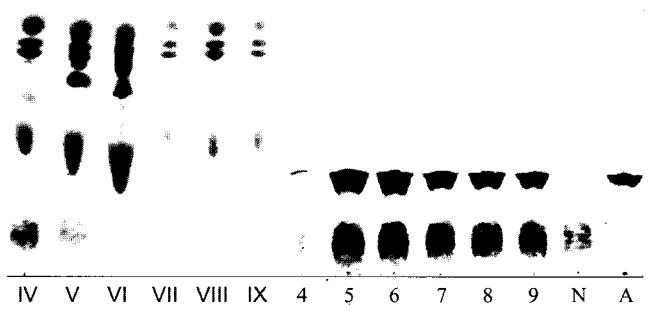

Fig. 2. Isolation of cyclosophoraoses by ethanol precipitation (fractional analysis by TLC).
IV–IX: supernatants (4–9 vol), 4–9: pellets (4–9 vol), N: standard of neutral cyclosophoraose, A: standard of anionic cyclosophoraose.

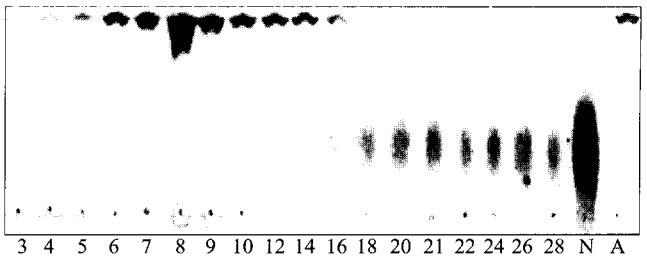

Fig. 3. Isolation of cellular cyclosophoraoses by flash column chromatography (fractional analysis by TLC).
4–18: anionic cyclosophoraoses, 16–28: neutral cyclosophoraoses, N: standard of neutral cyclosophoraose, A: standard of anionic cyclosophoraose.

In general, the separation of cellular cyclic (1→2)-β-D-glucans has been performed by four different steps: (1) hot ethanol extraction of the cells; (2) Sephadex G-50 column chromatography; (3) DEAE-cellulose chromatography; and (4) desalting chromatography [4, 16]. However, these steps are time-consuming and the recovery is poor due to many steps involved. In the present paper, we introduce a novel rapid separation technique for cellular cyclosophoraoses using silica gel flash column chromatography following the fractional ethanol precipitation. This technique is rapid and gives excellent separation efficiency compared to the current widely used method.

The bacterial strain used in this study was *Rhizobium meliloti* 2011. The overall process of purification of cyclosophoraoses is shown in Fig. 1. The cells were incubated for 2 days at 30°C and pH 7.0, shaking at 150 rpm in a 5-l jar-fermentor with 3 l culture medium. The culture medium was GMS media containing the following components per liter of distilled water: One g glutamic acid, 5 g mannitol, 1 g potassium phosphate dibasic, 0.2 g magnesium sulfate, 0.04 g calcium chloride, and vitamins [3, 14]. The cells were separated from the culture supernatant by centrifugation at 8,000 rpm at 4°C for 10 min.

Cell pellets (2 g wet wt) were extracted with 75% ethanol solution (50 ml) at 70°C for 30 min for the partial disruption of the cell membrane [2], and then the cyclosophoraoses present in the periplasmic space was extracted. The extracted solution was then centrifuged at 8,000 rpm for 10 min at 4°C. After centrifugation, the supernatant was evaporated in a rotary evaporator and concentrated to one tenth of the volume (5 ml). In order to find out the best fractional precipitation conditions for cyclosophoraoses, 4 to 9 vol of ethanol was added to each reduced supernatant solution containing various oligo- and polysaccharides, including exopolysaccharides (EPS) and lipopolysaccharides (LPS). The optimum separation efficiency was obtained with 8 vol of ethanol (Fig. 2), where the precipitates contained cyclosophoraoses as the major oligosaccharide. A final pellet of 8 vol of fractional ethanol precipitates was obtained from centrifugation with 8,000 rpm at 4°C, which was then resuspended in 2 ml of ethanol/butanol/water (5:5:4, v/v) and subjected to column chromatography [19]. The column (4×40 cm) was filled with silica gel-60 (400–230 mesh) for the solid phases using an appropriate solvent system of ethanol/butanol/water (5:5:4, v/v). After the sample loading, high air pressure was applied to make a 1.5 cm/min flow rate, which exhibited the best separation efficiency for neutral

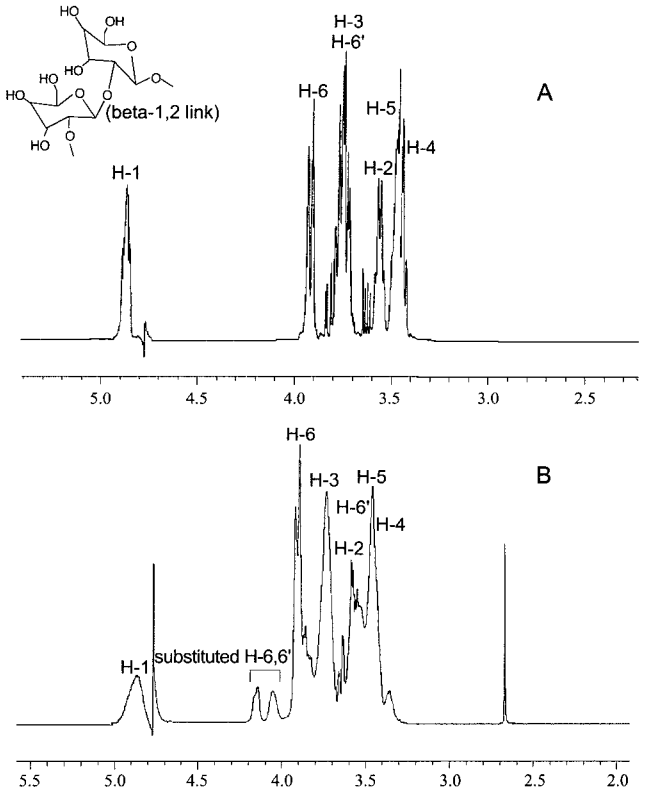

Fig. 4. ^{1}H-NMR Spectra of cyclosophoraoses.
(A) Neutral form. The resonance at 4.8 ppm corresponded to the anomeric proton (H-1) of cyclosophoraoses and the peaks of H-2, H-3, H-4, H-5, H-6, H'-6 were designated at 3.61, 3.75, 3.47, 3.52, 3.93, and 3.78 ppm respectively. (B) Anionic form. Two peaks at 4.0–4.2 ppm were identified as H-6,6' peaks of cyclosophoraoses substituted with phosphoglycerol.

Table 1. Comparison of net yields between new and typical method.

Purification steps (New/Typical)	Yield (mg/l)
Hot-ethanol extraction	60
8 vol ethanol precipitation/ Sephadex G-50 chromatography	≥50/≥39
Flash column chromatography/ DEAE-cellulose chromatography	≥45/≥28

or anionic cyclosophoraoses. The total elution time for the rhizobial cyclosophoraoses was very fast and complete separation was obtained within 20 to 30 min. Each of 20 ml eluate was collected in each test tube. The separated components were analyzed by spotting each fraction along the long side of a 7 cm × 15 cm TLC plate. The spots were visualized by spraying with 5% ethanolic sulfuric acid and heating at 250°C to characterize the organic components. Pure anionic cyclosophoraoses (R_f= 0.52) as well as neutral ones (R_f= 0.35) were separated with good resolution in one step, as shown in Fig. 3.

This method was much faster than the coventional chromatographic separation methods for rhizobial cyclosophoraoses [4, 16]. In addition, since several steps were simplified into one step, sample loss was minimized. The separated neutral and anionic cyclosophoraoses by the new method were confirmed by ^{1}H-NMR spectroscopy as shown in Figs. 4(A), 4(B).

We used flash column chromatography for the rapid and efficient separation of cyclosophoraoses produced by *R. meliloti* 2011. The important steps in the new separation method can be summarized as follows: (1) LPS and EPS were completely eliminated by fractional ethanol precipitation, where 8 volumes of ethanol exhibited the great efficiency for separation; (2) using silica gel flash column chromatography, the separation of neutral and anionic cyclosophoraoses was completed very rapidly in one step. The new separation technique described in this study was much more effective than the typical methods in respect to the time and cost required as well as the final recovery. The purification yield of cyclosophoraoses in each step was compared to the typical method (Table 1). The final net yield (≥75%) was clearly higher compared to that of the typical method (≥46.7%). It was based on the solubility difference between the polysaccharides and oligosaccharides in the presence of alcoholic solvent. This method will be very useful when the large-scale separation of cellular cyclosophoraoses is needed for further industrial applications.

Acknowledgment

This work was supported by grants from the Bioproducts and Biotechnology Research Group (01-J-BP-01-B-59) from Ministry of Science and Technology in South Korea to SDG.

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