

Chiral Separation and Discrimination of Catechin by Microbial Cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans Isolated from *Bradyrhizobium japonicum*

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Cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans are branched cyclooligosaccharides produced as intra- or extraoligosaccharides by *Bradyrhizobium* species. The cyclooligosaccharides have neutral or anionic form with various size ranging from 10 to 13 in degree of polymerization (DP).¹ The cyclooligosaccharides are required for *Bradyrhizobium* species's growth under hypo-osmotic conditions.^{2,3} The oligosaccharides have also been reported to be involved in the symbiotic interaction between this bacterial species and legume hosts.^{4,5} Throughout this interact property, cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans are suspected of being involved in complexation with various plant flavonoids.

Catechin is a polyphenolic compound which has been isolated from a variety of natural sources including tea leaves, grape seeds, and the wood and bark of trees such as acacia and mahogany. The catechin shows strong antioxidant activity both *in vivo* and *in vitro* and has various health-related activities, such as anticarcinogenic, anti-atherogenic, antibacterial, and antiviral activities.⁶⁻¹¹ Catechin can be used as chiral compounds. For example, it was reported that (+)- and (-)-catechin showed opposite effects on glycogen metabolism in isolated rat hepatocytes.¹² Recently Bais *et al.* reported that (-)-catechin, but not (+)-isomer, had allechemical activity and that (+)-catechin, but not (-)-isomer, showed antibacterial activity.¹³ Therefore, chiral separation and discrimination of catechin is very important. Separation of enantiomeric catechin and epicatechin has been performed by HPLC¹⁴⁻²⁰ and more recently by capillary electrophoresis (CE) with various cyclodextrins as chiral additive.²¹⁻²⁷ Cyclosophoraoses, microbial cyclooligosaccharides, have also been used for investigation of enantiomeric separation as chiral additives in CE^{33,34} and in the study of enantioselective interaction as a NMR shift agent in NMR spectroscopy.^{34,35}

In the present study, we investigated the chiral discrimination of catechin in presence of the bradyrhizobial cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans as chiral shifting reagent with ¹³C NMR spectroscopy. NMR signal splittings were observed on the interactions of cyclooligosaccharides with the enantiomers of catechin. The chiral separation of catechin also has been performed with cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans as chiral

additives in CE. The chiral separation of the catechin took place when cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans were added to the background electrolyte (BGE) in CE.

The isolation and purification of microbial cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans were carried out by hot-ethanol extraction method and chromatographic techniques with size exclusion chromatography and anion exchange chromatography.¹ The structure of cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans isolated from *Bradyrhizobium japonicum* USDA 110 was confirmed to be neutral cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans through ¹H- and ¹³C-NMR spectroscopy as reported previously.^{2,3} The molecular weight distribution and the degree of polymerization (DP) of cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans were also confirmed on matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (Figure 1C). Based on this analysis, the number-average molecular weight^{28,29} (M_n) of neutral cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans was determined as 1920.3.

NMR spectroscopic analysis enables the discrimination on the differential interactions of enantiomers of catechin with a chiral solvating agent in liquid phases. The NMR signals observed in binary selector-solute solutions are time-averaged signals of both the complexed and uncomplexed coupled with shift nonequivalence. These phenomena were observed on the ¹³C NMR spectra when cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans were mixed with the enantiomers of catechin (Figure 2). Due to the different interaction of each enantiomer with cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans, the resonance of the carbon singal (δ 29.3, 69.5 and 158.1, respectively) in positions 4, 3 and 5 of the catechin enantiomers was split (Figure 2) in D₂O containing 30% CD₃OD at pD 6.5. A significant chemical shift ($\Delta\delta$ 1.11) was observed at position 3 of the catechin enantiomers (Figure 2). The signal of the split carbon was also broadened in the presence of cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans, which suggested that the enantiomers were bound to the cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans, and the interaction induced to enantioselective.

Various CE conditions such as voltage, pH, temperature and concentration of chiral additives were studied to achieve optimum chiral separation of catechin. The values of the applied voltage ranged from 10 to 30 kV. The maximal

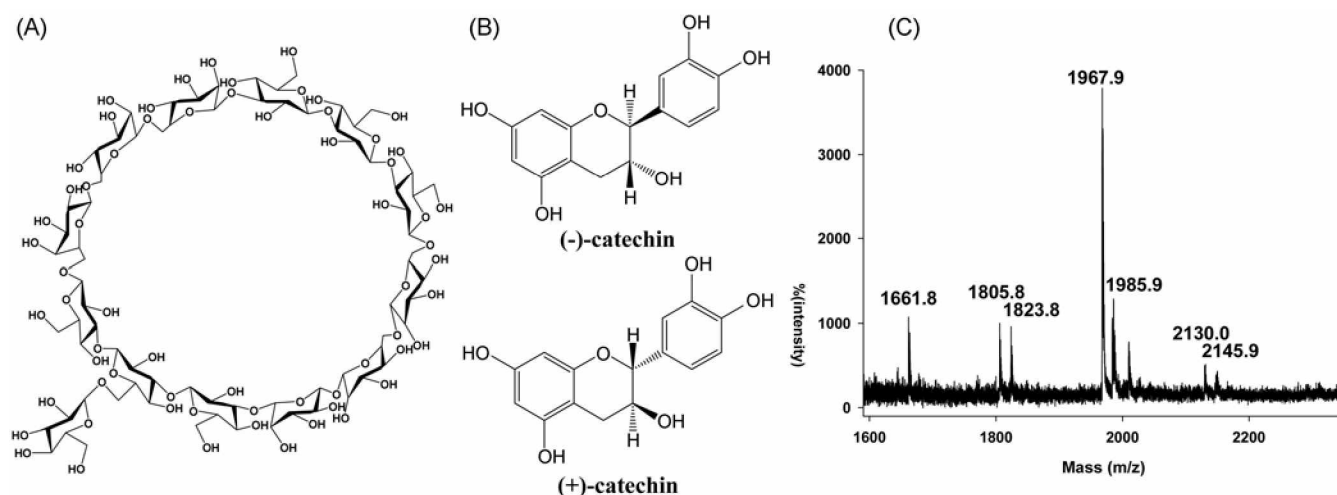


Figure 1. (A) Proposed Structure for the neutral cyclic $\beta(1\rightarrow3)$, (1 \rightarrow 6)-glucans of *Bradyrhizobium japonicum* containing 13 glucose residues.³² (B) The structure of chiral catechin ((-)-catechin and (+)-catechin) used in this study. (C) Mass spectrum of neutral cyclic $\beta(1\rightarrow3)$, (1 \rightarrow 6)-glucans. MALDI-TOF mass spectrometry was performed in the positive-ion mode with 2,5-dihydroxybenzoic acid (DHB) as matrix. Each signal at m/z 1661.8, 1823.8, 1985.9 and 2145.9 corresponds to a DP of 10, 11, 12, and 13 of neutral cyclic $\beta(1\rightarrow3)$, (1 \rightarrow 6)-glucans, cationized with one potassium, respectively. The signals at m/z 1805.8, 1967.9 and 2130.0 are the calculated mass for a DP of 10, 11, 12, and 13 cyclic $\beta(1\rightarrow3)$, (1 \rightarrow 6)-glucans with one sodium, respectively.

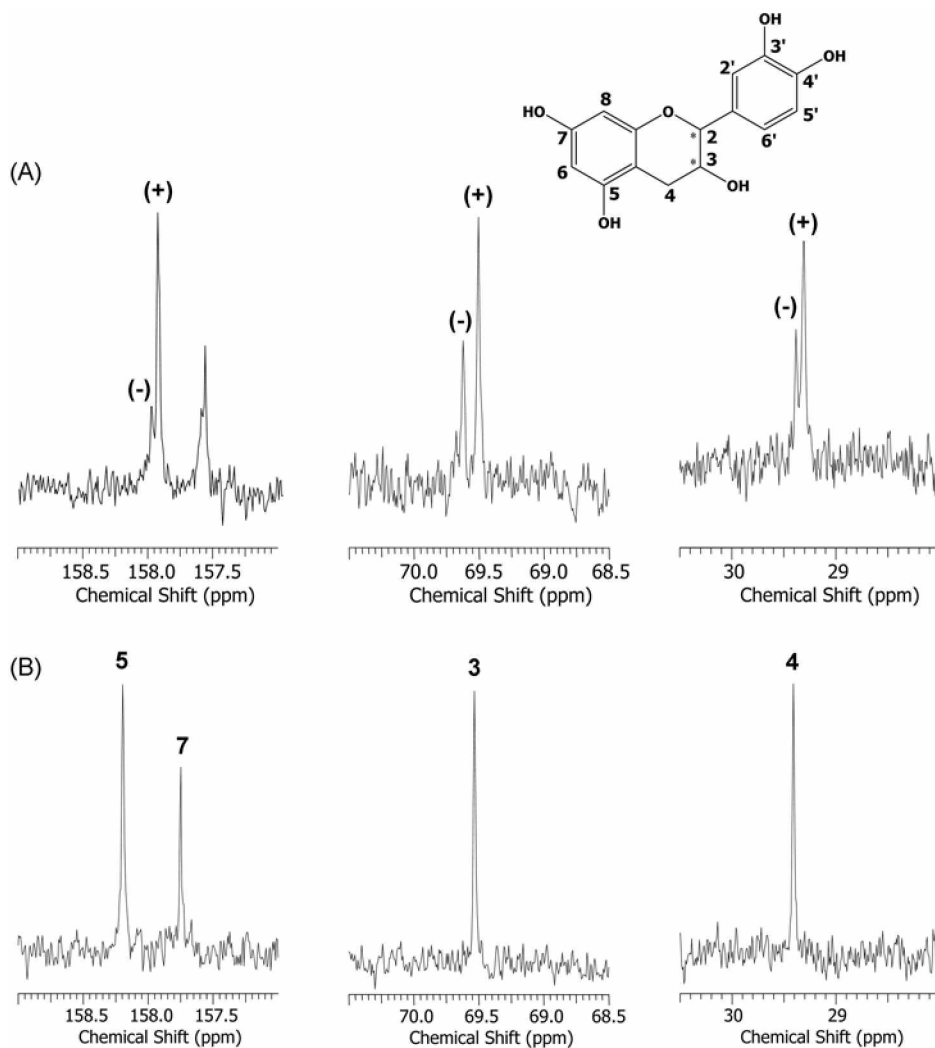


Figure 2. Partial ^{13}C NMR spectra of 20 mM nonracemic catechin ((+)/(-) = 2 : 1) enantiomers in the presence (A) and absence (B) of 20 mM cyclic $\beta(1\rightarrow3)$, (1 \rightarrow 6)-glucans.

voltage output available with a 30 kV power supply was used for a short running time. However as an optimal voltage, 20 kV was selected as it yields the best selectivity and resolution. Various buffer solutions including 100 mM borate (pH 8.3 and pH 10.0) and 50 mM phosphate (pH 7.0) buffer were also tested. The separation efficiency was achieved in basic media (pH 8.3-10.0). Good separations were accomplished at pH 8.3 using a 100 mM borate buffer. No significant change in the separation selectivity and resolution was observed when the operation temperature changed within a range between 10 and 30 °C. Various concentrations of microbial cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans ranging from 10 to 60 mM were also investigated for the optimal enantioseparation of catechin. Finally, we selected 20 mM of cyclooligosaccharides as chiral additives and 100 mM borate buffers at pH 8.3 for the optimal enantioseparation of the catechin. The chiral separation of catechin was successfully performed with the BGE, an aqueous solution of 100 mM borate buffer (pH 8.3) containing 20 mM of microbial cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans as chiral

additives in CE (Figure 3). The selectivity factor (α), and resolution (R_s) of separated catechin were showed 1.05 and 1.55, respectively.

Throughout the present investigation, we showed that microbial cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans were successfully utilized as chiral additives for the enantiomeric separation of catechin in CE and discriminated enantiomers of catechin as chiral solvating agent on ^{13}C NMR spectroscopic analysis. Although the exact molecular mechanism of the chiral separation by microbial cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans remains to be elucidated, we speculate that the chiral recognition is likely induced by stereochemical factors associated with both the β -glycosidic linkage and the ring structure of neutral cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans.

Experimental Section

Chemicals. All chemicals, including each enantiomer or racemate of catechin were purchased from Sigma Aldrich (St. Louis, Mo, USA). The structure of enantiomeric catechin is presented in Figure 1B. D_2O (99.9 atom% D) and CD_3OD (99.8 atom% D) were purchase from Aldrich Chemical Co. (Milwaukee, WI, USA).

Preparation of cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans. *Bradyrhizobium japonicum* USDA 110 was cultured in 5-L fermenter containing AG Medium to late logarithmic phase at 30 °C. The cells were harvested by centrifugation at 4 °C for 10 min at 8,000 rpm. The cell pellets were extracted with 75% (v/v) ethanol at 70 °C for 30 min. After centrifugation, the supernatant was removed and concentrated on Vacuum rotary evaporator. The sample was applied to a Bio-Gel P4 column (2 \times 60 cm) which was eluted at room temperature with 0.5% HOAc at a flow rate of 40 mL/h. The eluant fractions (3 mL) were assayed for carbohydrate by phenol-sulfuric acid method. The fractions were collected and then applied to a DEAE cellulose column (2 \times 45 cm) to separate the neutral form of cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans. Material eluting in the position expected for cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans was pooled, concentrated, and subsequently desalted using a Sephadex G-15 column (2 \times 45 cm). The Sephadex G-15 column was eluted at room temperature with 7% propanol (v/v) at a rate of 15 mL/h. Fractions (7 mL) were collected and assayed for carbohydrate content.

Structural analyses of cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans. The structure and molecular weight of cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans were confirmed through NMR spectroscopy^{2,3} and MALDI-TOF mass spectrometry analyses. For NMR spectroscopic analysis, we used a Bruker AMX spectrometer to record the ^1H -NMR spectra (at 500 MHz) and the ^{13}C -NMR spectra (at 125 MHz). The mass spectrum of the cyclooligosaccharides was obtained with a MALDI-TOF mass spectrometer (Voyager-DE STR BioSpectrometry, PerSeptive Biosystems, Framingham, MA, USA) in the positive-ion mode using 2,5-dihydroxybenzoic acid (DHB) as the matrix.

Capillary electrophoresis. The capillary electrophoretic experiment was performed on an Agilent 3-D CE Systems

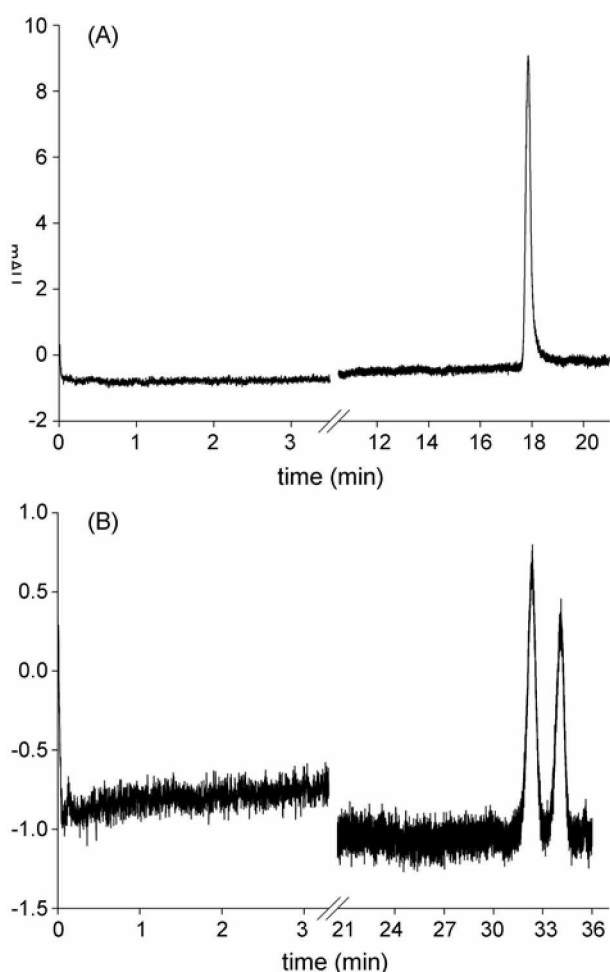


Figure 3. Electropherogram of racemic catechin in the absence (A) and presence (B) of 20 mM cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans used as novel chiral additives in CE. Condition: 25 kV; 20 °C; detection at 280 ± 10 nm. Capillary: 52 cm (43.5 cm effective length) \times 50 μm i.d.; Background electrolyte: 100 mM borate buffer, pH 8.3; Chiral selector additive: 20 mM cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans.

(Wilmington, DE, USA) equipped with a diode array detector. Separations were carried out on uncoated 50 μm ID fused-silica capillary with a total length of 52 cm and an effective length of 43.5 cm to the detector window. The capillary was conditioned by flushing with 0.1 M NaOH for 20 min and then rinsed with water for 10 min, and finally equilibrated with an appropriated running buffer for 3 min. Between two runs, the capillary was rinsed with 0.1 M NaOH, water and running buffer for 3 min each. The BGE consisted of an aqueous solution of 100 mM borate buffer (pH 8.3). The chiral additive buffer solutions were prepared by dissolving 20 mM of cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans in the BGE. The sample solution was prepared in the running buffer-methanol (9:1) mixture to a final concentration of 0.1 mg/mL and introduced into the capillary using the pressure of 5 kPa for 3 s. The separation temperature was 20 $^{\circ}\text{C}$. The applied voltage was 20 kV. The EOF was determined using methanol as neutral marker. Detection was done with on-column UV absorbance at 280 nm.

Preparation of the binary cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans-catechin solution. Catechin 20 mM, neutral cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans 20 mM, and the nonracemic mixture ((+)/(−) = 2/1) of the equimolar ratio were prepared in D_2O containing 30% CD_3OD_3 at pD 6.5. Each Mixture was stirred for 12 h under darkness, followed by degassing before NMR measurements. The pD value was obtained by adding 0.4 to the pH meter reading, in accordance with the work of Glasoe and Long.^{30,31}

NMR spectroscopic analysis. NMR spectroscopic analysis was carried out on a Bruker AVANCE 500 spectrometer for neutral cyclic β -(1 \rightarrow 3),(1 \rightarrow 6)-glucans, catechin, and their complexes. All spectra were acquired with a 5-mm probehead. Proton-decoupled ^{13}C -NMR experiments were carried out at 125.7 MHz ^{13}C frequency. Spinning tubes of 5 mm i.d. containing 0.7 mL of analytes were employed. Tetramethylsilane (TMS, Me_4Si) was used as an external reference, and chemical shifts were calibrated with an accuracy of 0.05 ppm.

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