

Chiral Separation of Catechin by Capillary Electrophoresis with α -Cyclosophorooctadecaose Isolated from *Rhodobacter sphaeroides* as Chiral Selectors

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α -Cyclosophorooctadecaose (α -C18), produced by *Rhodobacter sphaeroides*, is mainly composed of homogeneous in size, and 18 glucose units per ring as a predominant form. α -C18 is cyclic, and is linked by β -1,2 linkages and one α -1,6 linkage. This backbone is substituted by acetyl (0-2 per mol) and/or succinyl groups (1-7 per mol) as a highly anionic character.¹ Bacterial genetic analysis is highly developed, as it is a remarkable model for the study of bacterial photosynthesis. *R. sphaeroides* is a facultative photosynthetic member of the alpha-proteobacteria. The strain can heterotrophically grow not only on aerobic respiration but also on anaerobic condition, as it has diverse metabolic pathways.² Although α -C18 of *R. sphaeroides* is believed to play a role in osmotic adaptation,¹ the exact biological function and roles of α -C18 remains obscure.

Catechin discussed in this work is the major component of tea flavonoids, commonly known as a class of flavan-3-ols. The flavan-3-ols are widespread compounds in the plant kingdom and are found in various natural sources including tea leaves, grape seeds, and the wood and bark of trees such as acacia and mahogany. The flavan-3-ols are reported to possess anti-oxidation, antibiotic, anti-virus, and anti-tumor pharmacological activities and also improve the immune system, reduce blood lipids and steroids, etc.³⁻⁸ It was reported that (+)-(2R;3S)-catechin and (-)-(2S;3R)-catechin as chiral drugs showed opposite effects on glycogen metabolism and in membrane fluidity which are responsible for their pharmacological and toxicological mechanisms.⁹ It was also reported that (-)-(2S;3R)-catechin has allelopathic activity, but no antibacterial activity. However, (+)-(2R;3S)-catechin showed antibacterial activity but no allelopathic activity.¹⁰ Therefore, it is important to consider powerful analytical techniques not only for the evaluation of the total amount of catechin, but also for quantitation of each enantiomer by a rapid and reproducible procedure without tedious and time-consuming pretreatments. The chiral separation of catechin has been performed by high-performance liquid chromatography (HPLC)¹¹⁻¹³ and more recently by capillary electrophoresis (CE)¹⁴⁻¹⁷ with native or modified cyclodextrins as a chiral selector. Recently, a microbial cyclic or noncyclic oligosaccharide, produced by sinorhizobial bacteria, was successfully used as a chiral additive for chiral separation of (\pm)-catechin in CE.¹⁸⁻²¹

In the present study, we purified α -C18 from *R. sphaeroides*

and used it as a novel chiral additive for chiral catechin in CE. For optimization of the various parameters, α -C18 was investigated as a chiral additive for chiral separation of catechin under various background electrolyte (BGE) conditions such as additive concentration, buffer pH value, buffer concentration, and temperature. Moreover, through removal of succinyl and acetyl substituents by alkaline treatment, the role of substituents was investigated for chiral separation of catechin.

The α -cyclosophorooctadecaoses (α -C18) were extracted from cells of *R. sphaeroides*, which were grown in a LB broth medium by trichloroacetic extraction and purified through chromatographic techniques.¹ ¹³C-NMR spectrum analysis confirmed that the α -C18 purified from *R. sphaeroides* contains high levels of succinate and acetate (Figs. 2(a) and (b)). In the NMR spectrum, the multiple resonance of C1 to C5 carbons of α -C18 was assigned at 105.3, 85.8, 79.1, 76.4 and 72.3 ppm, respectively. As the presence of the α -1,6

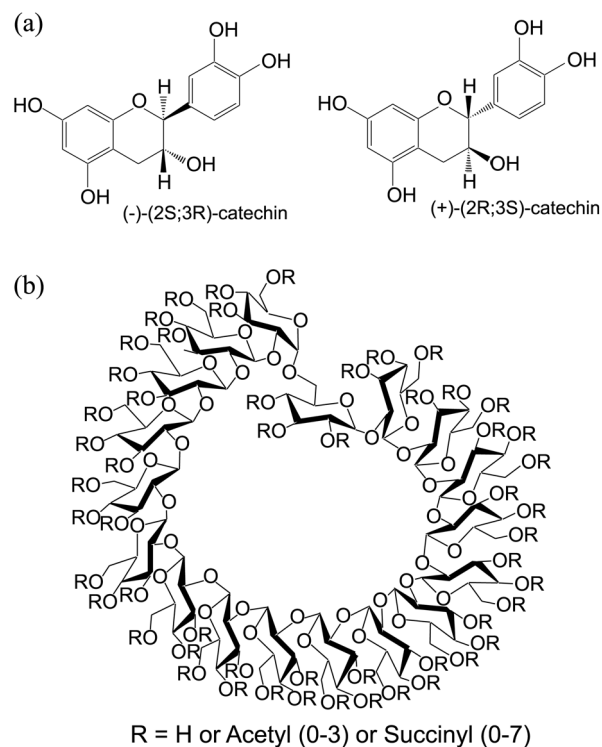


Figure 1. Chemical structures of five flavanones (a), three flavanone-7-O-glycosides and α -Cyclosophorooctadecaose (α -C18) (b) involved in this work.

linkage in the α -C18 produces several distinct chemical shift effects, the resonance of C6(α) (δ = 62 ppm) in the α -1,6 linkage is shifted upfield relative to the resonances of C6 (δ = 63–65 ppm) in the other β -1,2 linkages of glucose residues. Each of the C6 resonance linked with succinate and acetate was also shifted significantly downfield to 65.7 ppm and 66.7 ppm, respectively. Moreover, ^{13}C -NMR spectra by the distortionless enhancement polarization transfer (DEPT) technique showed that α -C18 contained high levels of succinate and acetate, where signals appeared between 27.3 and 41.4 ppm (methylene carbons ($-\text{CH}_2-$) of succinate) and between 19.2 and 23.2 ppm (methyl carbons ($-\text{CH}_3$) of acetate) (Fig. 2(a)).

The MALDI-TOF mass spectrum of α -C18 shows the glucans purified from *R. sphaeroides* (Fig 2(c)). Detailed analysis of the spectrum revealed the presence of seven sodiated molecular ions, $[\text{M} + \text{Na}]^+$, at m/z 3041.5, 3142.7, 3241.7, 3341.7, 3441.7, 3541.7, and 3641.7. It was reported that α -C18 of *R. sphaeroides* has acetyl substitution by one to two acetyl residues.¹ However, in this study, the obtained molecular signals indicated that α -C18 has one to even seven succinyl residues and/or one to three acetyl residues as substituents. Based on MALDI-TOF mass spectrometric data,²⁵ we calculated the degree of substitution on average and their standard deviations for succinate and acetate as 3.42 ± 0.72 and 0.91 ± 0.31 , respectively. For investigation of the effect of substituents attached to α -C18, succinates and acetates of highly substituted α -C18 were removed by alkaline treatment. The exact structure of alkaline treated α -C18 was also confirmed *via* a ^{13}C -NMR spectroscopic

analysis and MALDI-TOF mass spectrometry (Figs. 2(b) and (d)). In the DEPT spectral results, succinyl and acetyl groups were completely removed from the α -C18 backbone where the methylene carbon ($-\text{CH}_2-$) resonances between 27.3 and 41.4 ppm of succinate and methyl carbon ($-\text{CH}_3$) resonances between 19.2 and 23.2 ppm of acetate disappeared (Figs. 2(a) and (b)). The MALDI-TOF spectrum also revealed the presence of one molecular ion at m/z 2917.7, which indicates the calculated mass for an $[\text{M} + \text{H}]^+$ ion of unsubstituted α -C18 ($\text{u}\alpha$ -C18). $\text{u}\alpha$ -C18 appears to be mostly homogeneous in size, and it contains only minor amounts of α -C16, α -C17 and α -C19 (Fig. 2(d)).

We investigated the effective chiral separations of (\pm)-catechin under various CE conditions such as the buffer pH, buffer concentration, temperature, and type and concentration of chiral additives. The effect of type and concentration of α -C18 was investigated in a fused silica capillary with BGEs in 50 mM borate buffer (pH 8.3) with various concentrations of chiral additives. Figure 3 shows the partial electropherograms of the chiral separation of (\pm)-catechin at pH 8.3. Without addition of isolated α -C18, the migration time of (\pm)-catechin was 6.72 min, and no separation of (\pm)-catechin was achieved. Although the migration time rapidly increased with an increase of α -C18 concentration, enantiomeric resolution was successfully achieved in BGE containing native α -C18 at more than 4% concentration (Fig. 3).

The effect of $\text{u}\alpha$ -C18 obtained by alkaline treatment was investigated for chiral separation of (\pm)-catechin with BGE containing various concentrations of $\text{u}\alpha$ -C18. As shown in

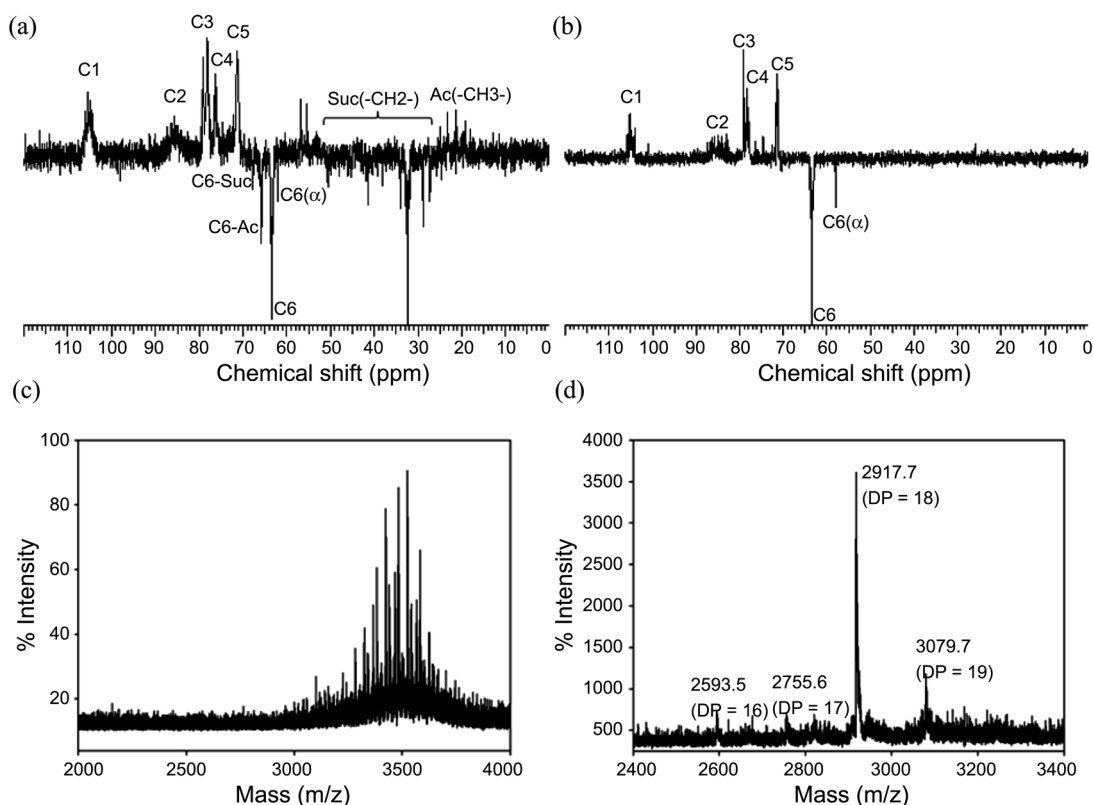


Figure 2. DEPT NMR spectra of the TCA extracted glucans of *R. sphaeroides* (a) and alkaline treated glucans (b). Positive ion MALDI-TOF mass spectra of the TCA extracted glucans of *R. sphaeroides* (c) and alkaline treated glucans (d).

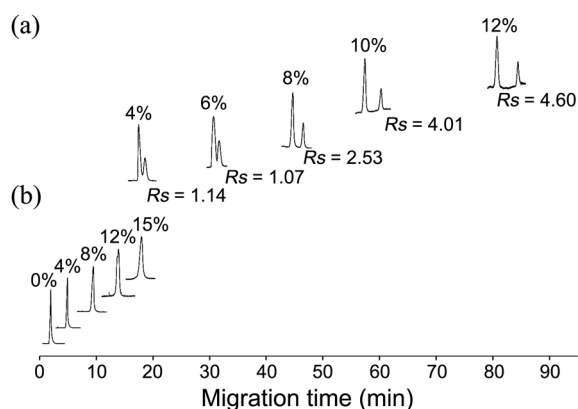


Figure 3. CE separation of (±)-catechin in 50 mM borate buffer, pH 8.3, containing α -cyclodextrin (A) and no substituted α -cyclodextrins (B) as chiral additives. Condition: capillary, 50 cm (41.5 cm effective length) \times 50 μ m i.d.; applied voltage, 25 kV; positive polarity at the inlet, 5 kPa pressure for 4 s; temperature, 20 °C; detection at 220 \pm 10 nm; analyte's composition: (+)/(-): 2/1.

Figure 3, α -C18 and u α -C18 were respectively compared with respect to the chiral separation of (±)-catechin with various concentrations in the same BGE. In the case of u α -C18, no chiral separation occurred in evaluated concentration range, from 4 to 15% (Fig. 3(b)). However, α -C18 in BGEs beyond 4% concentration successfully performed chiral separation of (±)-catechin. These differences are due to the anionic structure containing the succinate moieties attached to α -C18, in other words, the succinate moieties were required for effective chiral separation of (±)-catechin. This suggests that the succinate moieties could be significantly involved in the recognition or discrimination of chiral separation of (±)-catechin. Although the exact molecular mechanism remains to be determined, the difference of the chiral selection ability of α -C18 and u α -C18 could be ascribed to their specific spatial arrangement and their capacity to facilitate hydrogen bondings due to the succinate moieties.

Various buffer solutions including 0.1 M borate (pH 8.3, 8.5, 9.0, and 10.5) and 0.1 M phosphate (pH 7.0, 7.5, and 8.0) buffer were also investigated. The effect of pH on the chiral separations and the migration times of (±)-catechin are depicted in Table 1. At pH 7.0, no chiral separation of catechin was observed even in the presence of α -C18. This was likely due to the less negative charges of (±)-catechin at this pH.²² As shown in Table 1, the chiral resolutions and the migration times of (±)-catechin increased with increasing pH, and reached a maximum at pH 8.3 and then, slightly decreased at pH 8.5 (Table 1). However, at pH 9.0 and 10.5, the migration times of (±)-catechin rapidly decreased and no chiral separation occurred even with α -C18.

To investigate the effect of buffer concentrations, 25 to 100 mM borate buffer with a 25 mM interval was tested for the chiral separation of (±)-catechin with BGE containing 8% α -C18. The chiral separations and the migration times of (±)-catechin on various buffer concentrations are also depicted in Table 1. As shown in Table 1, the chiral resolu-

Table 1. Effect of the buffer pH, concentration of buffer, and temperature on chiral separation of (±)-catechin

BGEs condition		t_{m1} (min)	t_{m2} (min)	Selectivity (α)	Resolution (R_s)
Buffer pH	7.5	39.50	42.21	1.06	2.85
	8.0	54.44	58.94	1.08	2.93
	8.3	56.209	61.72	1.08	4.01
	8.5	52.52	55.88	1.06	0.82
Buffer Concentration (mM)	25	40.93	43.37	1.05	3.48
	50	47.22	51.27	1.08	3.83
	75	49.20	51.68	1.05	3.02
	100	56.20	61.72	1.09	4.01
Temperature (°C)	15	52.47	57.01	1.08	3.40
	20	46.79	50.26	1.07	2.41
	25	38.41	40.64	1.05	2.05
	30	31.256	32.703	1.04	1.71

Condition: capillary, 50 cm (41.5 cm effective length) \times 50 μ m i.d.; applied voltage, 25 kV; positive polarity at the inlet, 5 kPa pressure for 4 s; temperature, detection at 220 \pm 10 nm.

tions and the migration times of (±)-catechin increased with an increase of borate buffer concentrations at pH 8.3, but slightly decreased at 75 mM borate buffer. The advantages of the use of a high ionic strength buffer in CE have been well documented. However, an unlimited increase in the buffer concentration is impossible due to the excessive heat generation in the separation system.²³ Although the highest resolution of (±)-catechin is achieved at 100 mM borate buffer, a buffer concentration of 50 mM was chosen for the subsequent optimizing steps.

The effect of temperature was investigated between 15 °C to 30 °C at 5 °C steps for chiral separation of (±)-catechin with BGEs containing 8% concentrations of α -C18. The chiral resolution and migration time of (±)-catechin rapidly decreased as the temperature increased. The migration time (t_{m2}), selectivity (α), and resolution (R_s) obtained at 15 and 30 °C are (57.0, 1.08, 3.40) and (32.7, 1.04, 1.71), respectively. These results show that increase of temperature can enhance the diffusion band broadening and deteriorate the peak efficiency and the resolution. The appropriate temperature was selected through a compromise between lower migration times and higher resolutions. As it provided reasonable run time (t_{m2} = 50.26) and resolution (R_s = 2.41), temperature of 20 °C was selected.

After optimization of the various parameters, the final conditions were determined as follows for (±)-catechin: uncoated 50 μ m ID fused-silica capillary with a total length of 50 (41.5 cm effective length) cm; BGEs, 50 mM borate buffer, pH 8.3; UV detection at 220 nm; anodic injection, 5 kPa pressure for 4 s; applied voltage, 25 kV, 20 °C. The electropherogram obtained for (±)-catechin with α -C18 in the optimal conditions is presented in Figure 3(a).

Experimental Sections

Chemicals. All chemicals were of analytical grade purity. rac-Catechin and (+)-(2R;3S)-catechin were purchased from Sigma Aldrich (St. Louis, Mo, USA).

Purification of α -Cyclosophorooctadecaose. *R. sphaeroides* (ATCC 17023) was grown at 30 °C in sistro's succinic acid minimal (SIS) medium under anaerobic condition illuminated with light.¹ To production of OPGs, aerobic chemoheterotrophic cultures were grown in a Luria-Bertani (LB) broth with shaking.² The bacteria cells were collected after 2 day by centrifugation at 8,000 rpm for 10 minute. The cell pellets were extracted with 5% trichloroacetic acid, and the extract was neutralized with ammonia water and desalted on a Sephadex G-15 column. The desalted materials was concentrated and applied to a Bio-gel P4 column. The fractions of the putative glucans detected by phenolic sulfuric acid method²⁴ were pooled and concentrated by rotary evaporation. Fractions containing oligosaccharides were pooled and lyophilized.

Structural Analyses of α -Cyclosophorooctadecaose. For NMR spectroscopic analyses, the glucans were dissolved in D₂O at room temperature. NMR spectroscopic analysis was performed on a Bruker AMX spectrometer (operated at 500 MHz for ¹H-NMR, 125 MHz for ¹³C-NMR) at 25 °C. A ¹³C-NMR distortionless enhancement polarization transfer (DEPT) spectrum was also obtained at $\theta_z = 135^\circ$ where -CH and -CH₃ signals appeared in the positive phase, with -CH₂ in the negative phase. For MALDI-TOF mass spectrometry, the glucans were dissolved in water and mixed with the matrix (2,5-dihydroxybenzoic acid). Mass spectra were recorded on a mass spectrometer (Voyager-DETM STR Bio-Spectrometry, PerSeptive Biosystems, Framingham, MA, USA) in positive-ion mode.

Deesterification of α -Cyclosophorooctadecaose. For removal of the succinyl and acetyl substituents, α -cyclosophorooctadecaose were treated in 0.1 M KOH at 37 °C for 1 h. After neutralization with AG 50W-X8 (Bio-Rad) on H1 form, the samples were desalted on a Bio-Gel P-2 column.²³

Capillary Electrophoresis, Instrumentation and Conditions. All capillary electro-phoretic experiments were performed on an Agilent 3-D CE Systems (Wilmington, DE, USA) equipped with a diode array detector. Separations were carried out on uncoated 50 μ m ID fused-silica capillary with a total length of 50.5 cm and an effective length of 42 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 BGEs consisted of an aqueous solution of 100 mM borate buffer (pH 8.3). The chiral additive buffer solutions were prepared by dissolved α -cyclosophorooctadecaose in the BGEs. The sample solutions were 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 4 s. A Voltage of 25 kV was applied with the positive polarity at the inlet. The EOF was determined with methanol as a neutral marker. Detection was done with on-column by multi UV absorbance at 220, 260 and 280 nm.

The peak resolution (R_s) and separation selectivity (α)

were calculated using the following equations, respectively:

$$R_s = 2(t_{m2} - t_{m1}) / (W_1 + W_2)$$

$$\alpha = t_{m2} / t_{m1}$$

Where t_{m1} and t_{m2} are the migration times of the first and second enantiomers, W_1 and W_2 are the corresponding widths at the peak base.

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