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Carbohydrate Research 342 (2007) 762–766
Carbohydrate RESEARCH

Note

Enantioseparation of some chiral flavanones using microbial cyclic β-(1→3),(1→6)-glucans as novel chiral additives in capillary electrophoresis

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Received 5 September 2006; received in revised form 15 December 2006; accepted 19 December 2006
Available online 27 December 2006

Abstract—Cyclic β-(1→3),(1→6)-glucans, microbial cyclooligosaccharides produced by *Bradyrhizobium japonicum* USDA 110, were used as novel chiral additives for the enantiomeric separation of some flavanones such as eriodictyol, homoeriodictyol, hesperetin, naringenin, and isosakuranetin in capillary electrophoresis (CE). Among the flavanones, eriodictyol was separated with the highest resolution (R_s 5.66) and selectivity factor (α 1.18) when 20 mM cyclic β-(1→3),(1→6)-glucans were added to the background electrolyte (BGE) at pH 8.3.

Keywords: Cyclic β-(1→3),(1→6)-glucans; Chiral additive; Enantioseparation; Flavanones; Capillary electrophoresis

Cyclic β-(1→3),(1→6)-glucans, microbial cyclooligosaccharides produced by *Bradyrhizobium*, consist of between 10 and 13 glucose residues linked by both β-(1→3) and β-(1→6) glycosidic bonds (Fig. 1a).[1] Cyclic β-(1→3),(1→6)-glucans can be found in neutral or anionic forms. Rolin et al. described the detailed structural features of a phosphocholine (PC) substituted cyclic β-(1→3),(1→6)-glucan found in *Bradyrhizobium japonicum* USDA 110.[2] These cyclooligosaccharides generally function in the periplasmic space as osmoprotectant against osmotic pressure. The synthesis of cyclooligosaccharides was diminished when cells were grown under high osmotic potential.[3,4] The cyclooligosaccharides are also involved in the symbiotic interaction between *Bradyrhizobium* species and its specific symbiotic plant.[5,6] Throughout this interaction, cyclic β-(1→3),(1→6)-glucans are suspected of being involved in complexation with various plant flavonoids.

Flavonoids are an important class of phenolic plant constituents offering beneficial health-related properties and are commonly distributed in fruits and vegetables. Flavonoids can be subdivided into six classes: the flavanones, the flavones, the flavonols, the catechines, the anthocyanidines, and the isoflavonoids. Flavanones exist in two enantiomeric forms due to the stereogenic center at C-2. Also, the flavanone-7-*O*-glycosides possess an additional D-configured mono- or disaccharide moiety and accordingly exist as pairs of epimers. Chirality remains an important consideration not only for many compounds such as pharmaceuticals, food additives, and agrochemicals but also flavonoids. Separation of enantiomeric flavanones and epimeric flavanones-7-*O*-glycosides has been performed by HPLC[7–14] and more recently by capillary electrophoresis (CE).[15–21] The separation of some stereoisomeric flavanones and their 7-*O*-glycosides by micellar electrokinetic chromatography (MEKC) was described by Asztemborska and Mioekiewicz.[19] Enantiomeric separation of homoeriodictyol, hesperetin, naringenin, isosakuranetin, and pinostrobin was found to be feasible using a buffer

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doi:10.1016/j.carres.2006.12.018

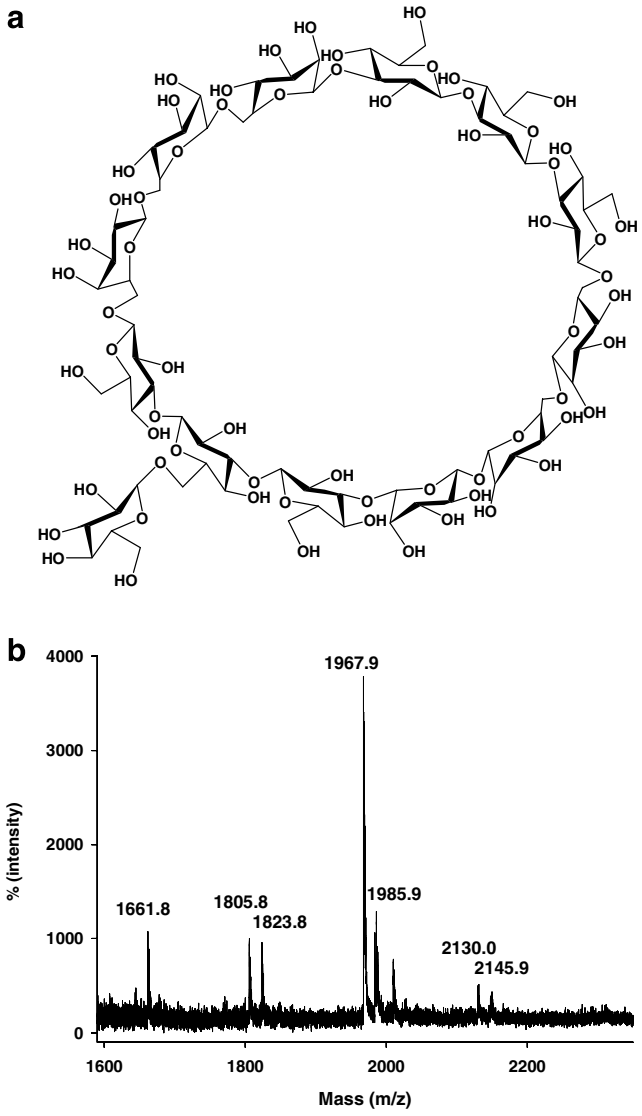

Figure 1. (a) Proposed structure for the neutral cyclic β-(1→3),(1→6)-glucans of *Bradyrhizobium japonicum* containing 13 glucose residues. (b) Mass spectrum of neutral cyclic β-(1→3),(1→6)-glucans. MALDI-TOF mass spectrometry was performed in the positive-ion mode with 2,5-dihydroxybenzoic acid (DHB) as a 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 β-(1→3),(1→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 β-(1→3),(1→6)-glucans with one sodium, respectively.

system containing sodium dodecyl sulfate (SDS) and γ-cyclodextrin. Recently, Wistuba et al. investigated chiral separation of many flavanones and flavanone-7-*O*-glycosides with native cyclodextrins and various cyclodextrin derivatives as buffer additives in CE or MEKC.[20]

In the enantioseparation of a given compound or group of compounds, it is important to use appropriate chiral selectors. As chiral selectors having universal properties do not exist, the research of new chiral selectors is an important field with regard to the separation of various stereoisomeric compounds. Cyclosophoraoses, microbial cyclooligosaccharides, have also been used as chiral additives for the investigation of enantiomeric separation in CE[21,22] and in the study of enantioselective interaction as a shift reagent in NMR spectroscopy.[23,24] Some flavanones such as isosakuranetin and neohesperidin were also enantioseparated by neutral cyclosophoraoses and their derivatives, such as highly sulfated cyclosophoraoses, in MEKC.[21]

In the present study, bradyrhizobial cyclic β-(1→3),(1→6)-glucans were used as novel chiral additives for the enantiomeric separation of some flavanones, such as eriodictyol, homoeriodictyol, hesperetin, naringenin, and isosakuranetin in CE. Their isolation and purification was carried out by hot-ethanol extraction and chromatographic techniques involving size exclusion chromatography and anion exchange chromatography.[1] Their structure was confirmed through ^{1}H and ^{13}C NMR spectroscopy as reported previously.[2,3] The molecular weight distribution and the degree of polymerization (DP) was also confirmed using MALDI-TOF mass spectrometry (Fig. 1b). The spectrum showed peaks at *m*/*z* 1661.8, 1823.8, 1985.9, and 2145.9 corresponding to DPs 10–13 cationized with potassium. Signals at *m*/*z* corresponding to the same DPs cationized with sodium at 1805.8, 1967.9, and 2130.0 were also found. Based on the MALDI-TOF mass spectrum, the average molecular weight[25,26] (M_n) was determined as 1920.3.

Since no research on the separation of enantiomers using cyclic β-(1→3),(1→6)-glucans as chiral additives in CE has been reported, various conditions such as voltage, pH, temperature, and concentration of the chiral additives were studied in order to achieve optimum enantiomeric separation efficiency. Various chiral flavanones such as eriodictyol, homoeriodictyol, hesperetin, naringenin, hesperidine, neohesperidine, naringin, and isosakuranetin were investigated. The values of the applied voltage ranged from 10 to 30 kV. The maximal voltage output available with a 30 kV power supply was used for a short running time. However, 20 kV was selected as an optimal voltage as it yielded the best selectivity and resolution. We tested, for optimization of the enantiomeric separation, various buffers including 100 mM borate (pH 8.3 and 10.0), 50 mM phosphate (pH 7.0), and 50 mM acetate (pH 3.0 and 5.3). In the case of phosphate and acetate buffers, no peak was detected. The enantiomerization of some flavanones such as naringenin and homoeriodictyol can be recognized by plateau formation, a characteristic phenomenon in basic media. Interconversion profiles featuring characteristic plateau formation of the elution pattern were observed at high pH.[20] We also observed the characteristic plateau for homoeriodictyol (Fig. 2e) for which the electrophoretic profiles were compared at pH 8.3 and 10. In the present experiment, plateau

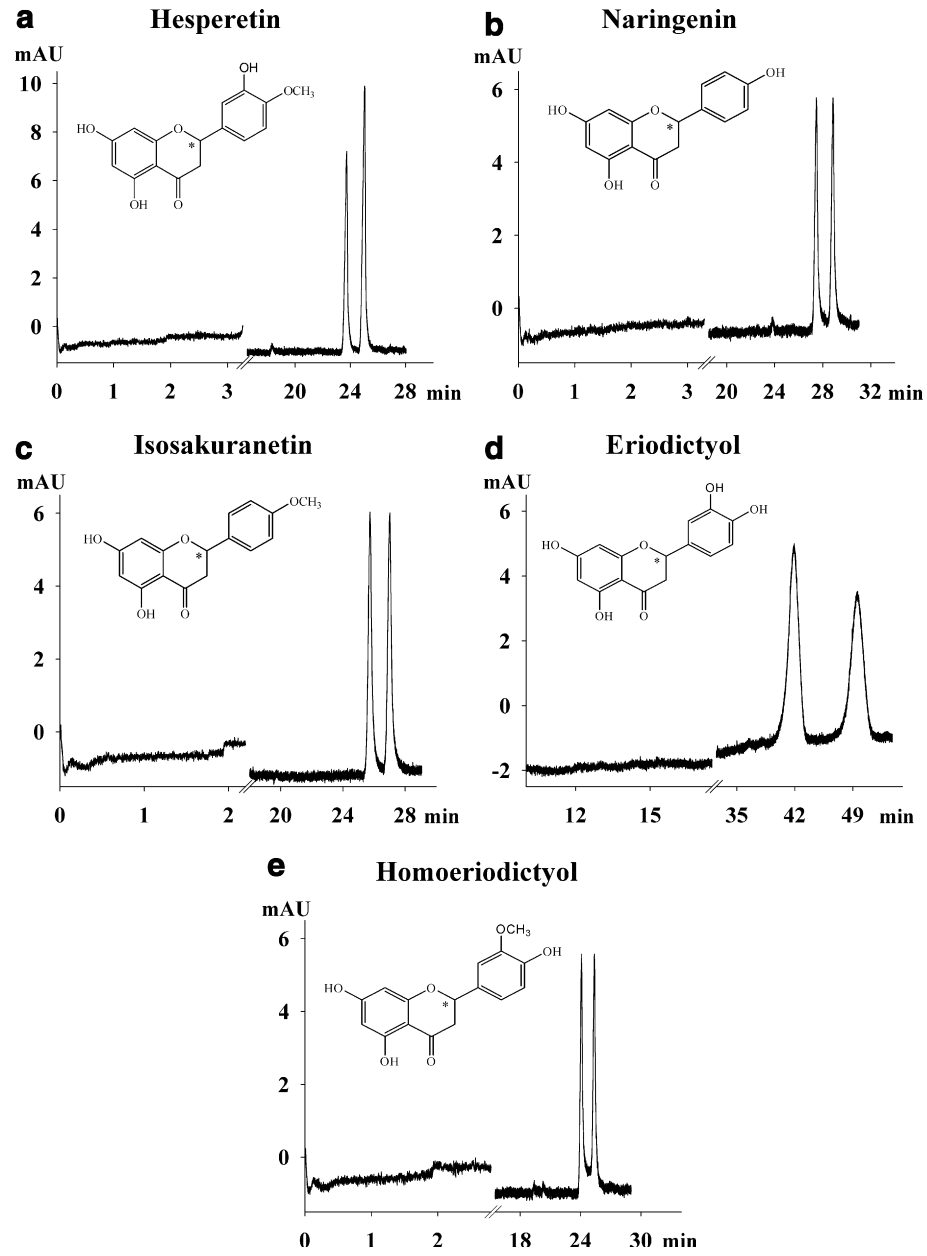

Figure 2. Enantiomeric separation of some flavanones by cyclic β-(1→3),(1→6)-glucans in CE. Condition: 25 kV; 20 °C; detection at 280 ± 10 nm. Capillary: 52 cm (43.5 cm effective length) × 50 μm i.d.; Background electrolyte: 100 mM borate buffer, pH 8.3; Chiral selector additive: 20 mM cyclic β-(1→3),(1→6)-glucans; Separated flavanones: (a) hesperetin, (b) naringenin, (c) isosakuranetin, (d) eriodictyol, and (e) homoeriodictyol.

formation was increased for higher pH value and the flavanones investigated here could enantiomerize in basic media. However, the resolution was poorer at a higher 10 pH value (data not shown). Good separations were achieved at pH 8.3 using a 100 mM borate buffer. However, 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 the cyclic glucans ranging from 10 to 60 mM were also investigated for the optimal enantioseparation of the flavanones. Finally, the optimal enantiomeric separation of selected flavanones was successfully performed with the background electrolyte (BGE), and an aqueous solution of 100 mM borate buffer (pH 8.3) containing 20 mM of the cyclic glucans as chiral additives in CE.

The apparent mobility (μ_{app}) of one enantiomer is the algebraic sum of the electrophoretic mobility (μ_e) and of the electro-osmotic flow (EOF) (μ_{EOF}). The apparent mobility (μ_{app}), the peak resolution (R_s), and the separation factor (α) were calculated using the following equations, respectively:

$$\mu_{app} = I/tE = IL/tV$$
$$R_s = 2(t_2 - t_1)/(W_1 + W_2)$$
$$\alpha = \mu_{app1}/\mu_{app2} = t_2/t_1$$

where t_2 and t_1 are the migration times of the first and second enantiomer (s), W_1 and W_2 are the corresponding widths at the peak base, L is the total length of the capillary (cm), I is the effective length to the detector (cm), V is the applied voltage (V), and E is the electric field.

Migration times, selectivity factor (α), and resolution (R_s) of the separated flavanones are shown in Table 1. Among the eight flavanones investigated, eriodictyol, homoeriodictyol, hesperetin, naringenin, and isosakuranetin were successfully separated by CE. Each of the enantiomeric flavanones was separated with resolution factors R_s between 1.41 and 5.66 in BGE containing 20 mM of the cyclic glucans (Table 1 and Fig. 2). Among the flavanones, eriodictyol was separated with the highest resolution (R_s 5.66) and selectivity factor (α 1.18) (Table 1 and Fig. 2d). As eriodictyol has four functional ionizable hydroxyl groups (Fig. 2d), which increase electrophoretic mobility in the direction opposite to that of EOF, more efficient chiral interaction between the enantiomeric complexes could be induced. Cyclic β-(1→3),(1→6)-glucans have a unique cyclic structure forming a hydrophobic cavity (Fig. 1a). This hydrophobic cavity is expected to provide a chiral environment for enantiomeric flavanones inducing a mobility difference for enantiomers. Another notable structural characteristic of the cyclic glucans is the single extra β-(1→6) glycosidic branch, which differentiates these structures from cyclodextrins and cyclosophoraoses (Fig. 1a). This extra β-(1→6) glycosidic branch might contribute to the enantiomeric separation of chiral flavanones through extra interaction with enantiomers in CE. However, no separation was obtained for some epimers of flavanone-7-*O*-glycosides. The sugar moiety of flavanone-7-*O*-glycosides, naringin, hesperidin, and neohesperidin, appears to disturb the separation of epimers by cyclic glucans.

The results of the present investigation indicate that microbial cyclic β-(1→3),(1→6)-glucans can be successfully used as novel chiral additives for the enantiomeric separation of some chiral flavonones in CE. The cyclooligosaccharides appear to provide the capacity for the required differentiation in both the binding of enantiomers and the appropriate mobility. Although the exact molecular mechanism of the chiral separation by microbial cyclic β-(1→3),(1→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 glucans.

1. Experimental

1.1. Chemicals and reagents

Naringenin, naringin, hesperetin, hesperidin, and neohesperidin were purchased from Sigma Aldrich (St. Louis, MO, USA). Eriodictyol, homoeriodictyol, and isosakuranetin were purchased from Carl Roth (Karlsruhe, Germany).

1.2. Preparation of cyclic β-(1→3),(1→6)-glucans

B. japonicum USDA 110 was grown in a 5-L fermenter containing AG medium to late logarithmic phase at 30 °C.[5] The cells were harvested by centrifugation at 4 °C for 10 min at 8000 rpm. The cell pellets were extracted with 75% (v/v) EtOH at 70 °C for 30 min.[1] After centrifugation, the supernatant was removed and concentrated under diminished pressure using a rotary evaporator. The concentrated sample was chromatographed on a Bio-Gel P4 column (Bio-Rad, 2 × 60 cm). The sample was eluted at room temperature with 0.5% AcOH at a flow rate of 40 mL/h and the eluant fractions (3 mL) were assayed for carbohydrates by the phenol–sulfuric acid method. The fractions were collected and then applied to a column (2 × 45 cm) of DEAE-cellulose to separate the neutral form of cyclic β-(1→3),(1→6)-glucans. Materials eluting in the position expected for cyclooligosaccharides were pooled, concentrated, and subsequently desalted using a Sepha-

Table 1. Migration times, separation factor (α), and resolution (R_s) of investigated enantiomeric flavanones with 20 mM cyclic β-(1→3),(1→6)-glucans as chiral additives in CE

Name of flavanone	t_R[a]	t_1[b]	t_2[c]	α	R_s
Hesperetin	13.35	23.72	25.02	1.05	1.56
Naringenin	13.63	27.47	28.85	1.05	1.67
Isosakuranetin	12.87	25.74	27.00	1.04	1.41
Eriodictyol	20.45	41.90	49.54	1.18	5.66
Homoeriodictyol	13.52	24.08	25.36	1.05	1.54

[a] Migration time of the enantiomers in min from capillary column without cyclic β-(1→3),(1→6)-glucans.
[b] Migration time of the first enantiomers in min from capillary column with cyclic β-(1→3),(1→6)-glucans.
[c] Migration time of the second enantiomers in min from capillary column with cyclic β-(1→3),(1→6)-glucans.

dex G-15 column (2 × 45 cm). The pooling material was eluted at room temperature with 7% (v/v) propanol at a rate of 15 mL/h on a Sephadex G-15 column. Fractions (7 mL) were collected and assayed for carbohydrate content by the phenol–sulfuric acid method.

1.3. Structural analyses of cyclic β-(1→3),(1→6)-glucans

The structure and molecular weight of cyclic β-(1→3),(1→6)-glucans were confirmed through NMR spectroscopy[2,3] and MALDI-TOF mass spectrometry analyses. For NMR spectroscopic analysis, we used a Brucker AMX spectrometer to record the ^{1}H 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-dihydroxybenozoic acid (DHB) as the matrix.

1.4. Capillary electrophoretic conditions

All capillary electrophoretic experiments were performed on an Agilent 3-D CE System (Wilmington, DE, USA) equipped with a diode array detector. Separations were carried out on an 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 aq soln of 100 mM borate buffer (pH 8.3). The chiral additive buffer solutions were prepared by dissolving 20 mM of cyclic β-(1→3),(1→6)-glucans in the BGE. The sample solutions were prepared in the running buffer–MeOH (9:1) mixture to a final concentration of 0.1 mg/mL and introduced into the capillary using a pressure of 5 kPa for 3 s. The separation temperature was 20 °C. The applied voltage was 20 kV. The EOF was determined using MeOH as a neutral marker. Detection was performed with on-column UV absorbance at 280 nm.

Acknowledgement

This study was supported by the Korea Research Foundation (KRF-2006-005-J03402) and partly supported by the Biodiscovery project in MOST.SDG.

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