Chiral Separation of Hesperetin and Hesperetin-*O*-glycoside in Capillary Electrophoresis Using Microbial β-1,2-Glucans

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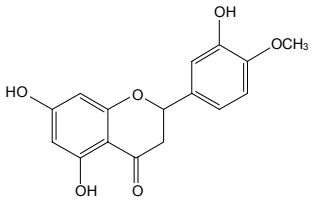
Linear β-1,2-glucans branched by β-1,6 linkages are produced from *Escherichia coli*,[1] *Erwinia chrysanthemi*,[2] and *Pseudomonas* species.[3,4] They can exist as neutral or anionic forms attached to some non-glucose molecules (succinyl, acetyl, phosphoglycerol, phosphoethanolamine moiety) with the degree of polymerization (DP) ranging from 5 to 28.[5] Unbranched cyclic β-1,2-glucans also known as cyclosophoraoses (Cys) are isolated from many strains of *Rhizobium* and *Agrobacterium* species.[6] These glucans are reportedly involved in the symbiotic interaction between *Rhizobium* species and alfalfa where the interaction could be based on the complexation ability of Cys with hydrophobic molecules like flavonoids.[7] Cyclodextrins as cyclic α-glucans have been the intensive subject of applications based on inclusion complexes with hydrophobic guests,[8] and the Cys have internal cavity diameters similar to or slightly larger than those of cyclodextrins.[9] From this point of view, several biotechnological applications such as solubility enhancer of insoluble drugs, catalyst and chiral separation have been carried out with neutral Cys.[10-14] However, there have been few reports on the function of the anionic Cys or linear β-1,2-glucans on the complexation study.[14,15] Since the previous study that showed a successful effect of anionic Cys as chiral selector on the enantioseparation of thyroxine and *N*-acetylphenylalanine,[14] there have been no further reports. Herein, in the present study, enantioseparations of some flavonoids with these linear and anionic cyclic glucans were investigated.

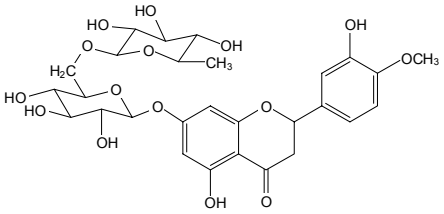
Flavonoids which are a class of plant secondary metabolites are distributed in fruits, vegetables, tea or even red wine. They fulfill many functions including pigmentation in flowers and protection from the attack by microbes and insects.[16] Also, their medicinal properties such as anticarcinogenic, antiallergic, or antiviral activities are beneficial to human health.[17-19] As a part of continuing interest in studying the natural flavonoids, analysis of enantiomers of chiral flavonoids has been carried out for over 20 years. Hesperidin (hesperetin-7-*O*-rutinoside, Figure 1B), the main flavanone glycoside in the peels of sweet orange (*Citrus sinensis*), has anti-inflammatory and anti-tumor effects and the structure of neohesperidin in bitter oranges (*Citrus aurantium*) consists of the same hesperetin backbone with another sugar moiety (neoheperinose, Figure 1C).[20] In fact, 2*S* form of hesperidin is the main constituent of *Citrus* species, and the decoction process of *Citrus* formulae partly converts 2*S*-hesperidin to the epimer. On digestion, it releases its aglycone, hesperetin (Figure 1A).[21]

To separate chiral flavonoids, high performance liquid chromatography (HPLC) or capillary electrophoresis (CE) with various chiral additives such as cyclic oligosaccharides

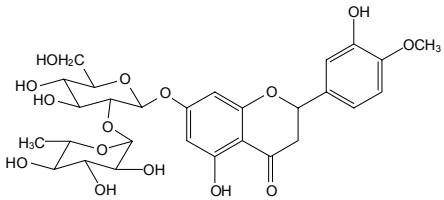
(A)



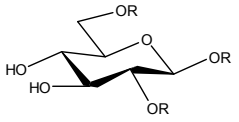
(B)



(C)

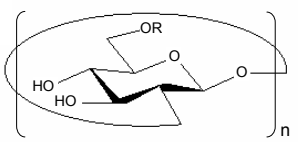


(D)



DP = 5-12
R = Glucosyl or H

(E)



n = 17-27
R = $PO_4CH_2CHOHCH_2OH$ or H

Figure 1. Chemical structure of hesperetin (A), hesperidin (B), neohesperidin (C), neutral linear β-1,2-glucans (D) and anionic Cys (E).

Table 1. Migration time (t_{m1}), separation selectivity (α), and resolution (R_s) in CE

Chiral additives / Flavonoids	Linear OPGs (70 mM)			Anionic Cys (30 mM)		
	t_{m1}	α	R_s	t_{m1}	α	R_s
Hesperetin	16.16	1.01	0.50	22.92[a]	1.00	0.00
Hesperidin	5.94	1.00	0.00	9.04	1.01	0.96
Neohesperidin	6.82	1.00	0.00	8.92	1.00	0.00

[a]15mM Anionic Cys were added to the BGE.

Table 2. Effect of anionic Cys concentration on the migration time (t_{m1}), separation selectivity (α), and resolution (R_s) of hesperidin in CE

Anionic Cys conc. [mM]	t_{m1}	α	R_s
0	4.12	1.00	0.00
15	7.93	1.01	0.46
20	8.51	1.01	0.77
30	9.04	1.01	0.96
40	12.19	1.01	0.89

have been utilized.[22,23] So far, there have been no reports on neutral linear glucans as chiral selectors for the enantioseparation of flavonoids. This study reports a neutral linear β-1,2-glucans as an a chiral selector for the hesperetin enantioseparation, suggesting a specific chiral interaction between linear β-1,2-glucans and hesperetin. Theses interactions of the chiral additives with the enantiomers can facilitate the formation of transient diastereomers which have different physicochemical properties.[24] These properties may distribute differentially between stationary and mobile phase in column.

The isolation of neutral linear or anionic cyclic β-1,2-glucans was performed as described in experimental sections. The structure of neutral linear β-1,2-glucans consists of some β-1,6 branched linkages and 5-12 glucose residues (Figure 1D).[3] Based on MALDI-TOF MS (data not shown), the number-average molecular weight (M_n) was calculated as 1328.3 Da. Structural analyses of the anionic Cys showed unbranched phosphoglycerol substituted Cys and M_n of 3940.2 (Figure 1E).[25]

These β-1,2-glucans were used as chiral selectors for enantioseparation of hesperetin and hesperetin-7-*O*-glycosides in CE at 15 °C. Figure 2A shows enantioseparation of hesperetin with linear β-1,2-glucans as chiral additives (α = 1.01, R_s = 0.50). However, no separation was achieved with those glucans for the epimers of hesperetin-7-*O*-glycosides (Table 1) since a sugar moiety of those (Figure 1B and 1C) might disturb the separation. No report on the chiral separation of flavanone-7-*O*-glycosides has been published with the microbial carbohydrates. However, in this study, diastereomers of hesperidin were clearly separated by using anionic Cys isolated from *R. meliloti* 2011 (α = 1.13, R_s = 1.13). Anionic Cys seem to have no preferential interaction with the neoheperidin stereoisomer, as shown in Table 1. This result suggests that anionic Cys could recognize specific glycosidic linkage irrespective of substituted glycosides, because hesperidin and neohesperidin have different glycosidic linkages (1→2 and 1→6) in the glycosides.[26]

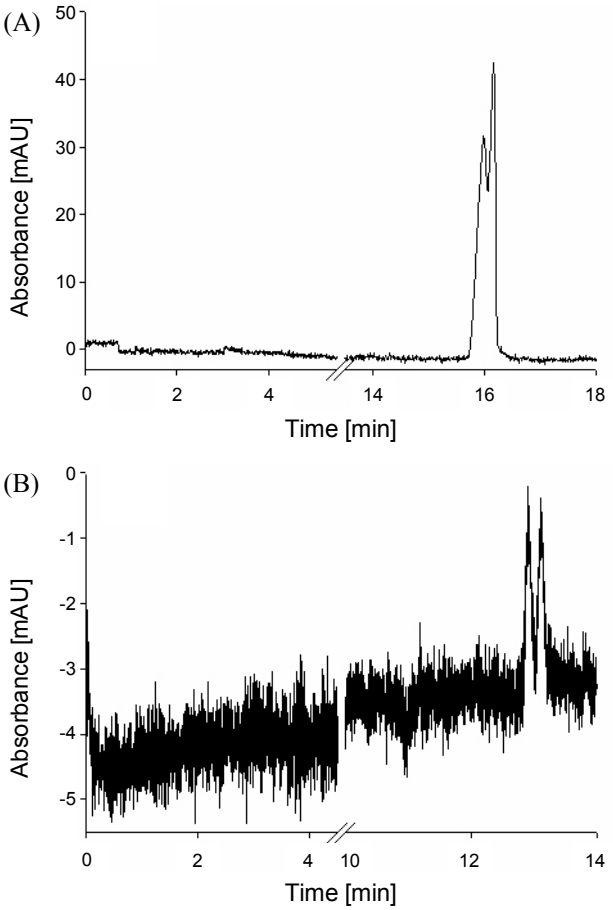


Figure 2. Electropherograms showing the enantioseparations of hesperetin with 70 mM neutral linear β-1,2-glucans (A) and hesperidin with 40 mM anionic Cys (B) as chiral selector. Separation parameters : α/R_s = 1.01/0.50 (A), α/R_s = 1.02/1.13 (B), Conditions: pH 8.3 borate buffer; 25 kV; 15 °C (A), 12 °C (B); detection at 220 nm (A) and 280 nm (B).

Furthermore, the separation results were dependent on the amount of glucans added to the buffer. As shown in Figure 2A, 70 mM of linear β-1,2-glucans allowed the chiral hesperetin to be separated. However, no separation of hesperetin was observed below 30 mM of linear β-1,2-glucans. As the amount of anionic Cys increase, a decrease in the velocity of EOF may lead to an increase in the migration time (Table 2). Table 2 also shows the dependence of the separation parameters on anionic Cys concentration. The peak resolution ranges from 0.46 to 0.96 according to the anionic Cys concentrations (15 - 40 mM). In the case of 30 mM concentration, the peak resolution was enhanced almost two times compared with 15 mM anionic Cys. However, the separation was not always improved with an increase of the anionic Cys concentration, but reached a maximum (R_s = 0.96) at anionic Cys concentration of about 30 mM. Additionally, optimal pH of the buffer was determined to be 8.3 while no separation was obtained at pH 7.0 or pH 9.0.

We partly studied the temperature effect, resulting that peak resolution (R_s) was changed from 0.89 at 15 °C to 1.13 at 12 °C in the hesperidin diastereomeric separation with 40 mM anionic Cys. Better resolution at lower temperature might be due to the base line separation of hesperidin though the first

migration time increased from 12.19 to 12.91 min (Table 2 and Figure 2B). When the linear β-1,2-glucans were added to the buffer as a chiral selector, hesperetin showed very weak separation (R_s < 0.2) at 20 °C rather than at 15 °C (Table 1), suggesting that lower temperature led to the better resolution result.

The biological and biotechnological functions of β-1,2-glucans have been studied in a steady manner. According to the previous report,[15] periplasmic glucans produced by *Pseudomonas* species interacted with tobramycin which was antibiotics, and the result could suggest antibiotic resistance by sequestering tobramycin with glucans in the periplasm. Through hesperetin chiral recognition of those in CE, another interaction with hesperetin could be expected. Throughout this study, β-1,2-glucans as chiral selectors achieved the enantioseparations of hesperetin and hesperidin, and the result showed the specific separation based on the chemical structures. Further studies on the exact molecular mechanism for these specific enantioseparations are in progress.

Experimental Sections

Chemicals. Hesperetin (3',5,7-trihydroxy-4'-methoxyflavanone), hesperidin (hesperetin-7-*O*-rutinoside), and neohesperidin (hesperetin-7-*O*-neohesperidoside) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Purification of linear OPGs. *P. syringae* pv. syringae (ATCC 19310) was cultured at 26 °C in TGY medium.[10] The microorganisms were collected after 1 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. The neutralized one was concentrated and then applied to a Sephadex G-25 column. The fractions of the putative glucans were pooled and concentrated by rotary evaporation. The sample was applied to a DEAE-Sephadex column to remove anionic contaminants. Finally, the neutral linear glucan was obtained by desalting on a Sephadex G-10 column.

Purification of anionic Cys. *R. meliloti* 2011 was cultured at 30 °C in GMS medium.[10] Cells were collected by centrifugation at 8,000 rpm for 10 minute. The isolation and purification of anionic Cys was carried out as described in our previous report.

Structural analyses of linear OPGs and anionic Cys. The structures of β-1,2-glucans were confirmed by ESI-MS, MALDI-TOF MS, and NMR spectroscopic analyses as described in our reports.[5,25]

Capillary electrophoresis, instrumentation and conditions. For CE experiment, Agilent 3D CE System (Wilmington, DE, USA) with a diode array detector was used. Separations were conducted on uncoated 50 μm ID fused silica capillary column with a 50.5 cm in total length and 42 cm in effective length. A new capillary was conditioned by flushing with 0.1 M NaOH and then rinsing with water for 30 min each, and subsequently equilibrating with running buffer for 10 min. Between two runs, the capillary was rinsed with 0.1 M NaOH, water, and running buffer for 3 min each. The used BGE was 50 mM borate buffer (pH 8.3 and pH 9) and 20 mM phosphate buffer (pH 7). Chiral additive solutions were prepared by dissolving microbial oligosaccharides in the BGE. Flavonoids (hesperetin, hesperidin, and neohesperidin) of 1 mg were dissolved in MeOH of 1 mL, and then the methanolic solution of 0.1 mL was mixed with BGE of 0.9 mL. The prepared samples after filtering with 0.2 μM PTFE syringe filter (Whatman Schleicher & Schuell) were injected by using a pressure of 5 kPa for 4 s. All separation was carried out at 25 kV and the analyses were monitored at either 220 or 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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