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Received December 16, 2009

Revised April 12, 2010

Accepted April 18, 2010

Research Article

Enzymatic transformation of platycosides and one-step separation of platycodin D by high-speed countercurrent chromatography

Platycosides, the saponins found in the roots of *Platycodon grandiflorum* (Platycodi Radix), are typically composed of oleanane triterpenes with two side chains. In platycosides, platycodin D, a glucose unit at C-3, is a major component, which has several pharmacological activities. Because of the high demand for this compound, we attempted to enzymatically convert platycodin D₃ and platycoside E, having two and three glucose units at C-3, respectively, into platycodin D. In this study, we tested the ability of several glycosidases to transform platycosides, or more specifically, the ability to transform platycoside E and platycodin D₃ into platycodin D. To obtain pure platycodin D on a preparative scale, high-speed countercurrent chromatography with a solvent system of ethyl acetate/*n*-butanol/water (1.2:1:2, v/v/v) was used for the separation of the enzymatically transformed product. Approximately 39.4 mg of platycodin D (99.8% purity) was obtained from 200 mg of the product in a one-step separation. The results strongly support the advantage of enzymatic transformation of the platycosides for the efficient enrichment of platycodin D in the complicated extract of the medicinal plant.

Keywords: Biotransformation / High-speed countercurrent chromatography / Platycodin D / Platycodi Radix
DOI 10.1002/jssc.200900842

1 Introduction

Traditionally, the roots of *Platycodon grandiflorum* (called Platycodi Radix) have been used in oriental medicine for the treatment of cough, cold, sore throat, tonsillitis, bronchitis, and chest congestion [1]. Recently, its extract and major components such as platycodin D and D₃ have been found to have diverse pharmacological activities such as anti-inflammation [2, 3], anti-allergy [4], augmenting immune responses [5], stimulating apoptosis in skin cells [6], anti-obesity and hyperlipidemia effects [7–9] and protective effects on oxidative hepatotoxicity [10]. Platycosides are typically composed of oleanane backbones with two side chains: a glucose unit attached through an ether linkage at the C-3 position of a triterpene and an ester linkage between C-28 and arabinose [11]. Platycosides also have different substituents of either methyl, carboxyl, or hydroxymethyl groups at the C-4 position. Many previous studies have reported that the bioactivities of platycodin D, the major

component of platycosides, are superior to those of other saponins [6, 9, 12–14]. Therefore, isolation and purification of platycodin D on a large scale is needed not only to conduct pharmacological studies but also to discover new medicinal drugs. We recently developed a method for the separation of six major platycosides, including platycodin D, by high-speed countercurrent chromatography (HSCCC) [15]. However, tedious pretreatment is required for the separation of platycodin D. Although platycodin D is a major component of platycosides, the total platycoside content in Platycodi Radix is about 2%. Because of the low content of platycodin D, efficient approaches are required for better separation of platycodin D from the total saponin mixture.

Enzymatic transformation has been widely used to modify natural compounds or to produce synthetic ones [16, 17]. It provides benefits such as stereospecific and mild reaction, low cost, and easy reaction control [18]. To recover more platycodin D compound, we tested the ability of seven glycosidases to convert the saponin mixture, especially platycoside E and platycodin D₃, into platycodin D. In this study, we selected the enzyme with the highest ability to convert these compounds and optimized conditions to obtain higher amounts of platycodin D based on HPLC analysis. We also developed an efficient method for the isolation and purification of platycodin D from enzymatically transformed saponins using preparative HSCCC.

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Abbreviations: ELSD, evaporative light-scattering detection; HSCCC, high-speed countercurrent chromatography

2 Materials and methods

2.1 Chemicals and reagents

β -Glucosidase from almonds (solid/liquid types), cellulase from *Trichoderma reesei* (solid/liquid types), β -galactosidase from *Aspergillus oryzae*, and β -glucuronidase from *Escherichia coli* and *Helix pomatia* were purchased from Sigma-Aldrich (St. Louis, MO, USA). Additional β -glucosidase from almonds was purchased from Fluka (Gillingham, UK). All enzymes were used without further purification. Acetonitrile, methanol, ethyl acetate, and *n*-butanol (HPLC grade) were supplied by J. T. Baker (Phillipsburg, NJ, USA) and Fisher Scientific (Pittsburg, PA, USA). Distilled water (NANO pure Diamond, Barnstead, USA) was used for all solutions and dilutions. Diaion[®]-HP20 (polystyrene adsorption resin) was purchased from Mitsubishi Chemical (Tokyo, Japan). Platycodi Radix was purchased from a local herbal market in Seoul, Korea. The pure authentic samples of six platycosides were isolated from the aqueous extract of the raw material as described previously [6, 8, 19].

2.2 Sample preparation

Saponins from Platycodi Radix (1 kg) were extracted with distilled water by refluxing for 3 h, which was repeated three times. The extracts were passed through a filter paper (Advantec Toyo, Japan), and all filtrates were combined. The filtrate was evaporated with a rotary evaporator under reduced pressure, and the dried extract was fractionated with a Diaion[®]-HP20 open column (100 cm \times 10 cm; the volume of the column was 7.8 L) and eluted with a stepwise gradient in the sequence of 100% water, methanol/water 40–60% v/v, and 100% methanol. The 100%-methanol fraction containing the enriched saponins (henceforth called the saponin-enriched fraction) separated from the crude extract of Platycodi Radix was evaporated, lyophilized, and stored in a desiccator until further use.

2.3 HPLC analysis

The HPLC analysis was carried out on a Hitachi L-6200 instrument equipped with a Sedex 75 evaporative light-scattering detection (ELSD) system and a SIL-9A auto injector (Shimadzu, Japan). All separations were carried out on a Zorbax SB-Aq C18 column (150 mm \times 4.6 mm id, 5 μ m particle size) from Agilent Technologies (Palo Alto, CA, USA). The analysis of platycosides was performed by HPLC-ELSD as described previously [19].

2.4 Selection of platycodin D-converting enzymes

The seven aforementioned enzymes were incubated with the saponin-enriched fraction in a pH 5.0 sodium acetate

buffer with agitation at 37.5°C for 24 h. The mixtures were subsequently placed in a water bath at 90°C to terminate the enzymatic reaction. The reaction mixtures were individually loaded onto an SPE[®] Vac C₁₈ column, and each column was eluted with the sequence of water, methanol/water 40–60% v/v, and 100% methanol. The 100%-methanol fraction (the saponin-enriched fraction) was evaporated, dissolved in water, and prepared for HPLC analysis. The platycosides and the saponin-enriched fraction were incubated with selected enzymes and without enzymes, and reaction products were analyzed by the retention times of their peaks on the chromatogram. The total content of saponins was measured based on a previous report [19].

2.5 Enzymatic transformation of the saponin-enriched fraction to platycodin D by cellulase

The amount of cellulase was optimized for the complete conversion of platycoside E and platycodin D₃ to platycodin D by evaluating different cellulase amounts: 0 (control), 0.1, 0.2, 0.5, 1, 3, and 5 U. Each cellulase amount was mixed with the saponin-enriched fraction (5 mg) in 0.1 M sodium acetate buffer, pH 5.0. The mixtures were incubated at 37.5°C for 24 h and subsequently heated in a water bath at 90°C for three minutes to terminate the reaction. A Waters Sep-Pak[®] Vac C₁₈ (Ireland) cartridge was used to remove polar compounds and byproducts, and a Whatman syringe filter (13 mm, 0.45 μ m) was used to clean the sample prior to HPLC analysis.

2.6 Large-scale transformation of the saponin-enriched fraction to platycodin D

Reaction mixtures containing 1 and 2 g of the saponin-enriched fraction and 200 and 400 U of cellulase, respectively in the buffer (pH 5.0) were incubated at 37.5°C for 24 h with agitation. After completion of the reaction, they were placed in a water bath at 90°C to stop the reaction. The mixture was then loaded onto a Diaion[®]-HP20 (Mitsubishi Chemical) open column (50 cm \times 3 cm, 250 mL) and was sequentially eluted with water, methanol/water 40–60% v/v, and 100% methanol. The 100%-methanol fraction was evaporated, lyophilized, and stored in the desiccator until HPLC and ESI-MS analysis.

2.7 Preparation of two-phase solvent system

The two-phase solvent systems were selected according to the partition coefficient (*K*) of the target components. Mixtures of ethyl acetate/*n*-hexane/water at different proportions were prepared and thoroughly equilibrated in a separatory funnel at room temperature. The partition coefficients were determined by HPLC analysis and expressed as the peak areas of target components in the

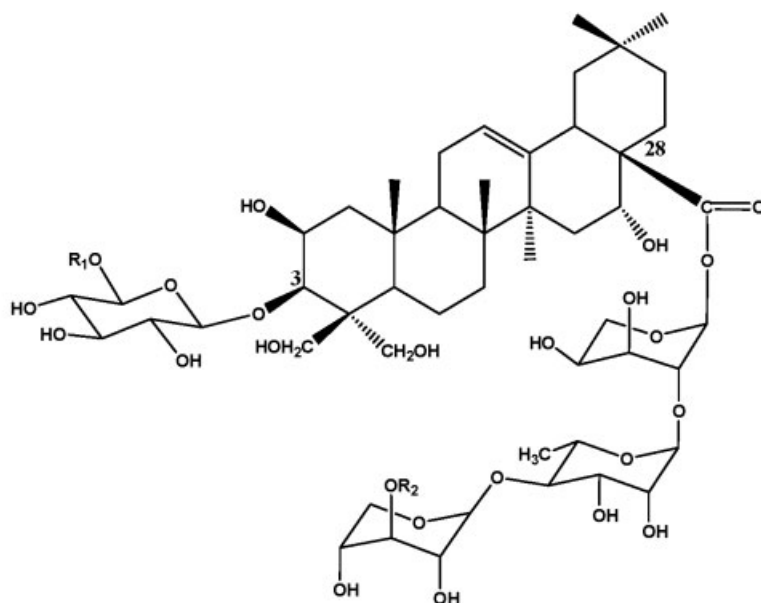
upper phase divided by those in the lower phase. The sample solution was prepared by dissolving in a 20 mL mixture of the lower and upper phase (1:1, v/v) of the solvent system used for the HSCCC separation.

2.8 Separation by high-speed countercurrent chromatography

A TBE-300A HSCCC (Tauto Biotech, Shanghai, China) with three serially connected multilayer coil separation columns (i.d. of the tubing = 1.6 mm, total volume = 260 mL) and a 20 mL sample loop was used. The speed of revolution of the apparatus was regulated in a range between 0 and 1000 rpm. A constant-flow pump (Hitachi L-6200, Japan) was used to

fill the HSCCC apparatus with the stationary phase and to push the mobile phase. The eluent was continuously monitored *via* a connection with the ELSD system through a split valve with 1:16 split ratio to the outlet of the coiled column (SEDERE, Sedex 55, France).

HSCCC was performed with a two-phase solvent system composed of ethyl acetate/*n*-butanol/water (1.2:1:2, v/v/v) for the separation of platycodin D. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, and the two phases were left partitioned at room temperature before use. Two phases were then degassed with an ultrasonicator. The multilayer-coiled column was simultaneously filled with the upper phase (stationary phase) and the lower phase (mobile phase) at a 70:30 volume ratio using a constant-flow pump. The lower



	Name	Molecular formula	Molecular weight	R ₁	R ₂
1	deapi-platycoside E	C ₆₄ H ₁₀₄ O ₃₄	1416.6	Gen	H
2	platycoside E	C ₆₉ H ₁₁₂ O ₃₈	1548.7	Gen	Api
3	deapi-platycodin D ₃	C ₅₈ H ₉₄ O ₂₉	1254.6	Glc	H
4	platycodin D ₃	C ₆₃ H ₁₀₂ O ₃₃	1386.6	Glc	Api
5	deapi-platycodin D	C ₅₂ H ₈₄ O ₂₄	1092.5	H	H
6	platycodin D	C ₅₇ H ₉₂ O ₂₈	1224.6	H	Api

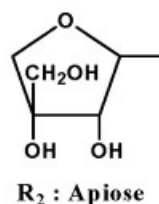
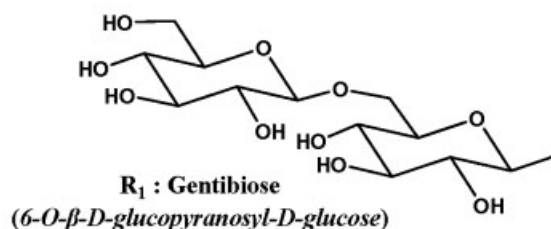


Figure 1. Structures of six platycosides from *Platycodi Radix*. Gen: gentiobiose (6-*O*-β-*D*-glucopyranosyl-*D*-glucose); Glc: β-*D*-glucose; Api: β-*D*-Apiose (furanose).

phase was pumped at a flow rate of 1 mL/min, and the apparatus was rotated at 850 rpm. After hydrodynamic equilibrium, the sample solution was injected into the separation column. The eluent was continuously monitored by the ELSD system through a split valve. The ELSD system was set to a probe temperature of 70°C, a gain of 6, and the nitrogen of the nebulizer was adjusted to 2.3 bar. The eluent was collected in glass tubes with a fraction collector, and peak fractions were combined based on a similar elution profile determined by HPLC-ELSD analysis.

2.9 MS analysis

All ESI-MSⁿ experiments were performed on a Finnigan LCQ ion trap mass spectrometer from Thermo Finnigan (San Jose, CA, USA) equipped with an ESI probe. MS conditions were as follows: the solvent was methanol, the

sheath gas flow was 80 arbitrary units, the capillary temperature was 275°C, the capillary voltage was 13 kV in positive mode and –10 kV in negative mode, and the spray voltage was 4.5 kV in positive mode and 0 kV in negative mode. For MS/MS, the collision energy with helium was set to 23–40%.

3 Results and discussion

3.1 Selection of enzymes for transformation to platycodin D

The saponin-enriched fraction including six major saponins (Fig. 1) (deapio-platycoside E, platycoside E, deapio-platycodin D₃, platycodin D₃, deapio-platycodin D, and platycodin D) was used for comparison with enzymatic metabolites. The structures of platycoside E, platycodin D₃, and platycodin D vary only by the number of glucose units at the C-3 position of a triterpene, having 3, 2, and 1 U, respectively. Additionally, three deapio-saponins – deapio-platycoside E, deapio-platycodin D₃, and deapio-platycodin D – were reacted in a similar fashion.

To select a suitable enzyme to transform platycoside E and platycodin D₃ into platycodin D, a series of experiments were conducted to evaluate the ability of various enzymes to hydrolyze glucose units at the C-3 position of a triterpene within 24 h. After incubation of aliquots of the saponin-enriched fraction with seven glycosidases for 24 h, the reactions were analyzed by HPLC-ELSD. The results show that cellulase, β-galactosidase, and β-glucosidase are able to transform platycoside E and platycodin D₃ into platycodin D but that neither glucuronidase had any effect (Table 1). Although the above three enzymes induced hydrolysis of platycoside E and platycodin D₃, their ability to cleave glycosidic bonds for transformation to platycodin D varied. Based on the experimental results, cellulase in liquid form

Table 1. The relative ratio of major platycosides after enzymatic reaction

Enzyme	Origin	Relative ratio (%) ^{a)}			
		PE	PD ₃	PD	
Con.	Control (no enzyme)	No incubation	23.8	14.9	61.3
A	Control	Incubation	24.0	14.6	61.5
B	Glucosidase	Almonds (Sigma)	14.2	15.5	70.3
C	Glucosidase	Almonds (Fluka)	21.2	14.4	64.4
D	Cellulase (solid)	<i>Trichoderma reesei</i>	0.0	0.0	100.0
E	^l Cellulase (liquid)	<i>Trichoderma reesei</i>	0.0	0.0	100.0
F	Galactosidase	<i>Aspergillus oryzae</i>	4.2	10.6	85.3
G	Glucuronidase	<i>Escherichia coli</i>	24.2	14.2	61.6
H	Glucuronidase	<i>Helix pomatia</i>	20.4	15.4	64.2

a) The numbers indicate the area percentage of each platycoside in Fig. 1. PE: platycoside E, PD₃: platycodin D₃, PD: platycodin D

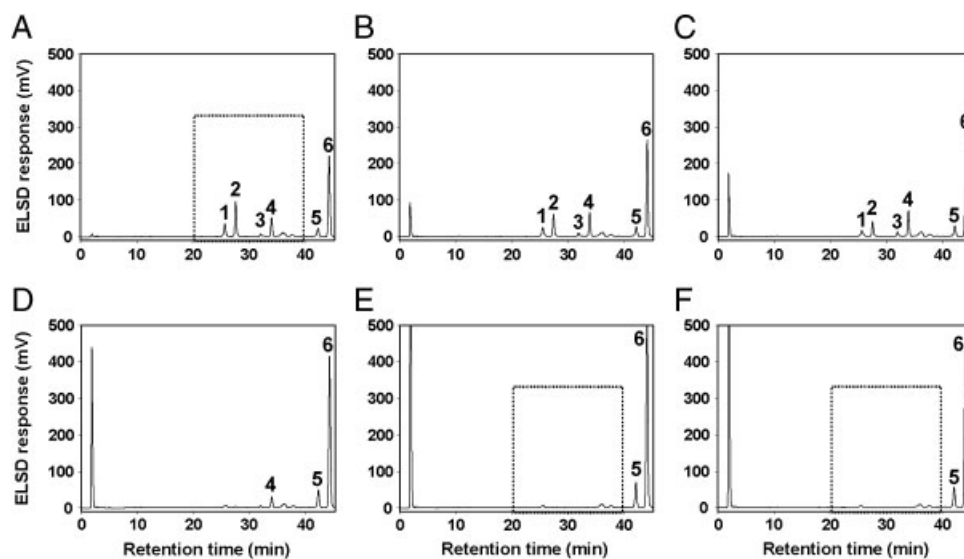


Figure 2. HPLC chromatograms of samples after biotransformation by various amounts of cellulase: (A) control, (B) 0.1 U, (C) 0.2 U, (D) 0.5 U, (E) 1 U (E), and (F) 3 U.

was selected as the most suitable enzyme for conversion to platycodin D.

3.2 Enzymatic biotransformation of the saponin-enriched fraction to platycodin D by cellulase

After enzymatic transformation to platycodin D by *T. reesei* cellulase was successfully performed, optimization of the enzyme amount was carried out to obtain the complete conversion of platycoside E and platycodin D₃ to platycodin D. About 5 mg of the saponin-enriched fraction was mixed with 0.1–3 U of cellulase, and the enzymatic modification of the platycosides was assessed by comparing the peaks on the HPLC chromatograms between different enzyme concentrations. The results of the six samples, each containing 5 mg of the saponin-enriched fraction and an amount of enzyme, are shown in Fig. 2. Compared with the control (Fig. 2A), the samples incubated with 0.1 and 0.2 U of cellulase showed only a slight decrease in the amounts of platycoside E and platycodin D₃ and an increase in platycodin D (Figs. 2B and C). When the samples were treated with 0.5 U of cellulase, there was a notable decrease of platycoside E and platycodin D₃ and increase of platycodin D; however, some platycodin D₃ still remained (Fig. 2D). When the enriched fractions were incubated with cellulase at 1 and 3 U, respectively, there were no remaining platycoside E or platycodin D₃, and the resulting product contained significantly greater amounts of platycodin D (Figs. 2E and F). According to these results, the minimal amount of cellulase required for complete conversion of the saponin-enriched fraction of 5 mg is 1 U.

3.3 Large-scale modification of the saponin-enriched fraction for platycodin D

We conducted further studies to scale up the reaction to a large volume. About 1 and 2 g of the enriched saponin fractions were incubated with 200 and 400 U of cellulase, respectively. We found that the enzymatic conversion of the saponin-enriched fraction on a gram scale by cellulase could be achieved (Fig. 3B) within 24 h. To remove the enzyme and byproducts such as polar compounds and sugars, Diaion[®]-HP20 column chromatography was used, and the entire process was monitored by HPLC-ELSD (Fig. 3C). The yield of the dried reaction product from 1 g of the saponin-enriched fraction was 0.72 g. The elution profiles for the conversion to the saponin-enriched fraction are shown in Fig. 3; notably, there were no peaks between 24 and 40 min in the enzyme-treated samples. The absent peaks correspond to deapio-platycoside E (1), platycoside E (2), deapio-platycodin D₃ (3), and platycodin D₃ (4). The results suggest that deapio-platycodin D and platycodin D are produced by cellulase cleaving the 1→6 glycosidic linkage of two or three glucosides at the nonreducing end of C-3 of aglycone. There was a significant increase in the contents of deapio-platycodin D

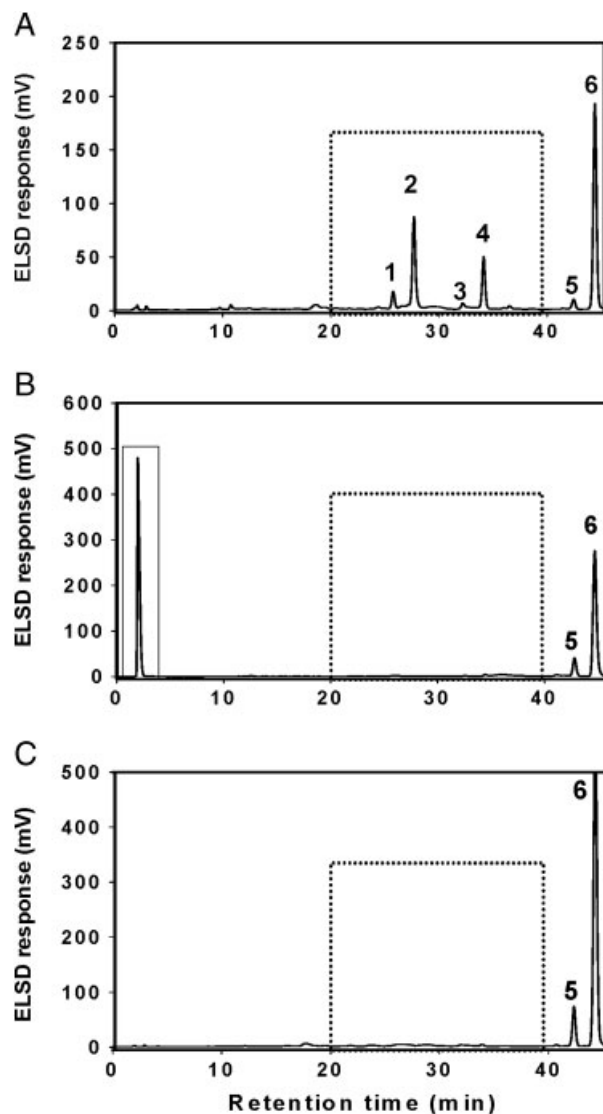


Figure 3. Representative HPLC chromatograms of enzymatic reactions. (A) Saponin-enriched fraction, (B) product of enzymatic modified platycosides by cellulase, (C) 100%-methanol fraction eluted from the Diaion[®]-HP20 column. The numbers indicate each platycoside in Fig. 1.

and platycodin D, with a corresponding decrease in the contents of deapio-platycoside E, platycoside E, deapio-platycodin D₃, and platycodin D₃ during the 24-h incubation. As a control, the enriched saponin fraction was also incubated under the same conditions for 24 h without cellulase to determine whether additional factors affected the conversion. There was no change in platycoside content in control experiment. This result indicates that *Trichoderma* cellulase cleaves β 1-6 glucose linkages of platycoside E and platycodin D₃ to produce platycodin D, as well as the linkages of deapio-platycoside E and platycodin D₃ to produce deapio-platycodin D. After enzymatic conversion, the sugar eluted at an early retention time (Fig. 3B) could be removed by Diaion[®]-HP20 column chromatography (Fig. 3C).

3.4 Preparative isolation of platycodin D by high-speed countercurrent chromatography

Various two-phase solvent systems, such as chloroform/methanol/water and *n*-butanol/water solvent systems, for the separation of saponins by HSCCC have been reported and widely used [20, 21]. We previously separated platycodin D with other platycosides by HSCCC in two steps [17]. Here, we only focused on the separation of platycodin D with one-step separation by a modification of a two-phase solvent system. We optimized the separation of platycodin D by adding ethyl acetate to butanol/water systems, considering the polarity of the target compound.

Ethyl acetate/*n*-butanol/water solvent systems at different volumetric ratios were tested, and their *K*-values are listed in Table 2. Among them, the two-phase solvent systems 1–4 did not form two clear phases with samples. Thus, these solvent systems were not suitable for the separation of platycodin D. Systems 5–7 formed clear phases with samples within 30 s and had appropriate *K*-values between 0.4 and 2.5. According to the rules for selecting optimum conditions for HSCCC [22, 23], the 1:1:2 solvent system had proper *K*-values. However, the separation factor (the ratio of the two *K*-values) was not appropriate because of its partition coefficient being below 1.5. The *K*-value decreased with increasing volume of ethyl acetate. The 1.5:1:2 solvent system had a good separation factor between each *K*-value, but the *K*-value of deapio-platycodin D was insufficiently low. Although the *K*-values of the 1.2:1:2 and 1.2:1:2.2 solvent systems were appropriate, the separation factor of 1.2:1:2 was preferable for the separation of platycodin D. Therefore, the two-phase solvent system of ethyl acetate/*n*-butanol/water at a volume ratio of 1.2:1:2 was suitable for the isolation of platycodin D when the reaction product was separated by HSCCC under the above conditions. Typical HSCCC chromatograms are shown in Fig. 4A. After enzymatic transformation and one-step

Table 2. *K* (partition coefficient) values of platycodin D in different systems of ethyl acetate/*n*-butanol/water (v/v/v)^{a)}

No.	Solvent system	Settling time	1	2	3	4	5	6
1	2:7:9	> 1 min	ambiguous two-phase					
2	3:4:7	> 1 min	ambiguous two-phase					
3	1:2:2	> 1 min	ambiguous two-phase					
4	1:2:3	< 30 s	ambiguous two-phase					
5	1:1:2	< 30 s	–	–	–	0.1	2.08	1.95
6	1.2:1:2	< 30 s	–	–	–	–	2.05	1.37
7	1.2:1:2.2	< 30 s	–	–	–	–	1.67	1.2
8	1.5:1:2	< 30 s	–	–	–	–	0.26	0.99

a) The *K* value is defined as the peak area in the upper phase divided by that in the lower phase. 1: Deapio-platycoside E, 2: platycoside E, 3: deapio-platycodin D₃, 4: platycodin D₃, 5: deapio-platycodin D, 6: platycodin D.

separation, 39.4 mg of platycodin D, 3.2 mg of deapio-platycodin D, and a 3.5 mg of the mixture of two compounds could be obtained. The purities of platycodin D and deapio-platycodin D were 99.8 and 98.8%, respectively, as determined by HPLC-ELSD (Figs. 4B and C). According to a previous study, the recovery of platycodin D from 300 mg of the saponin-enriched fraction was 28.0 mg [15]. In comparison with the direct separation, about 59.1 mg of

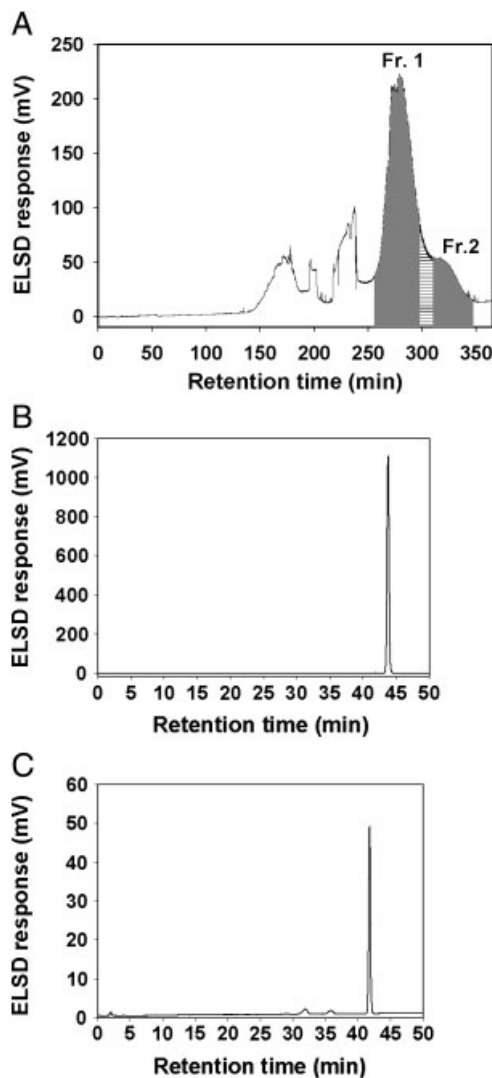


Figure 4. (A) HSCCC chromatogram of cellulase-treated platycosides, (B) HPLC chromatograms after separation of fraction 1 and (C) after separation of fraction 2. The two-phase solvent system was composed of ethyl acetate/*n*-butanol/water (1.2:1:2, v/v/v); mobile phase: the lower phase; stationary phase: the upper phase. The flow rate was 1 mL/min, the rotation speed was 850 rpm, the ELS detection was at 70 °C, and the nitrogen pressure was 2.3 bar. The collected fractions from HSCCC were analyzed by HPLC-ELSD with Agilent Zorbax SB-Aq C18 column (150 mm × 4.6 mm, 5.0 μm). Fraction 1 is mainly composed of platycodin D (99.8% pure, Rt: 44 min), and fraction 2 indicates deapio-platycodin D (98.8% pure, Rt: 42 min).

platycodin D could be obtained from 300 mg of the saponin-enriched fraction under the new separation conditions.

In summary, we were able to increase the productivity of platycodin D by 52.6% by a combination of enzymatic conversion of platycoside E and platycodin D₃ in the saponin mixture to platycodin D and of one-step separation using by HSCCC.

3.5 Structural elucidation

The isolated peaks were identified by HPLC-ELSD, and their identified structures were confirmed by ESI-MS/MS. The structures were elucidated by ESI-MS² in the negative and positive modes and compared with the previous studies [11]. The results were as follows: Peak 1 had a molecular weight of 1224 with MS² product ions in negative mode of 1205.44, 1133.38, 1091.40, 1073.29, 1061.24, 959.48, 795.12, 723.29, 681.38, 541.12, and 469.22, MS² product ions in positive mode of 1247.53, 1203.50, 1115.38, 1085.43, 1076.45, 983.27, 837.49, 705.47, 583.28, and 565.18; peak 2 had a molecular weight of 1092, MS² product ions in negative mode of 1001.4, 959.2, 723.3, 681.3, and 663.2, and MS² product ions in positive mode of 1115.73, 1071.46, 983.52, 935.42, 837.55, 705.48, 687.35, 643.50, and 433.17.

4 Concluding remarks

We report here the first enzymatic transformation and hydrolytic pathway conversion of platycoside E and platycodin D₃ to platycodin D. We have successfully achieved enzymatic conversion of PS to platycodin D within 24 h utilizing cellulase from *T. reesei*. Additionally, we successfully applied a preparative HSCCC method to isolate and purify platycodin D and deapio-platycodin D using a two-phase solvent system composed of ethyl acetate/*n*-butanol/water (1.2:1:2, v/v/v) with one-step separation and purification.

This study was supported by a grant from the Korea Healthcare Technology R&D Project, Ministry of Health, Welfare & Family Affairs, Korea (A080974) and by the National Research Foundation of Korea (NRF) grant funded by the Korea Government (MEST) (No. 20090083533).

The authors have declared no conflict of interest.

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