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Research Article

Novel enzymatic elimination method for the chromatographic purification of ginsenoside Rb_3 in an isomeric mixture

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ABSTRACT

Background: The separation of isomeric compounds from a mixture is a recurring problem in chemistry and phytochemistry research. The purification of pharmacologically active ginsenoside Rb_3 from ginseng extracts is limited by the co-existence of its isomer Rb_2. The aim of the present study was to develop an enzymatic elimination-combined purification method to obtain pure Rb_3 from a mixture of isomers.
Methods: To isolate Rb_3 from the isomeric mixture, a simple enzymatic selective elimination method was used. A ginsenoside-transforming glycoside hydrolase (Bgp2) was employed to selectively hydrolyze Rb_2 into ginsenoside Rd. Ginsenoside Rb_3 was then efficiently separated from the mixture using a traditional chromatographic method.
Results: Chromatographic purification of Rb_3 was achieved using this novel enzymatic elimination-combined method, with 58.6-times higher yield and 13.1% less time than those of the traditional chromatographic method, with a lower minimum column length for purification. The novelty of this study was the use of a recombinant glycosidase for the selective elimination of the isomer. The isolated ginsenoside Rb_3 can be used in further pharmaceutical studies.
Conclusions: Herein, we demonstrated a novel enzymatic elimination-combined purification method for the chromatographic purification of ginsenoside Rb_3. This method can also be applied to purify other isomeric glycoconjugates in mixtures.

1. Introduction

Natural products, including a vast number of glycosides, are commonly used in molecular pharmacological and medicinal chemistry studies [1]. Glycosides are composed of only a few types of monosaccharides, but the linkage stereochemistry and possible branching patterns result in diverse glycan structures and isomers [2–4]. Similar polarities and the same molecular weight make the analysis and purification of an isomeric mixture time consuming and tedious, which is also a recurring problem in chemistry [5,6].

Ginsenosides, as triterpene saponins composed of a dammarane skeleton with several glycosylation positions, are considered pharmacologically active components in plants [7,8]. Ginsenoside Rb_3 (Rb_3) is one of the major ginsenosides in ginseng and has therapeutic potential against type II diabetes, irradiation-induced skin diseases, and depression disorders [9–14]. However, Rb_3 and its isomer ginsenoside Rb_2 (Rb_2) normally co-exist in various parts of a ginseng plant [15–18]. They are protopanaxadiol-type ginsenosides, with four sugar moieties attached at C3 and C20 positions of the aglycon are shown in Fig. 1. The only structural difference between them is the sugar moiety in the outer position of C20; Rb_3 has xylopyranoside, while Rb_2 has arabinopyranoside (Fig. S1). Because of structural similarity, the quantification and isolation of these two compounds from ginseng extracts are difficult. However, Rb_3 showed higher pharmacological activity against skin aging than Rb_2 which promote the isolation of Rb_3 from the isomeric

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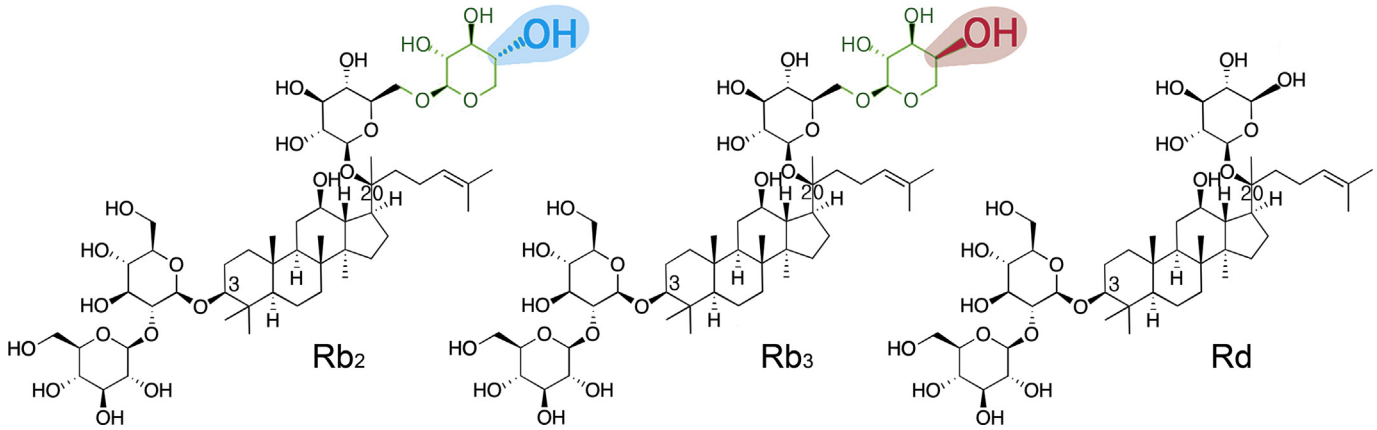

Fig. 1. Chemical structure of Rb_2, Rb_3, and Rd. Note that the only difference between Rb_2 and Rb_3 is the relative position of the hydroxyl group (Rb_2, Blue; Rb_3, Red).

mixture [14]. Two studies have reported the separation and isolation of Rb_3 by column chromatography [9,19]. However, both the studies had limitations due to the quantification level or 10 milligram-scale preparation; thus, there is difficulty in the separation of Rb_3.

Glycoside hydrolases (GHs, also known as glycosidases) are a group of enzymes that catalyzes the hydrolysis of glycosidic bonds between sugars or between a sugar moiety and an aglycon [20]. They increase the rate of hydrolysis by a maximum of 10^{17} fold compared with that by spontaneous catalysis and exhibit exquisite substrate selectivity [21]. Several attempts have been made to obtain rare minor ginsenosides from major ginsenosides using recombinant ginsenoside-transforming GHs [22–26]. Recently, a ginsenoside-transforming GH (Bgp2), which can hydrolyze Rb_2, was reported [27]. The recombinant Bgp2 selectively hydrolyzes arabinopyranoside in Rb_2 to produce ginsenoside Rd.

A mixture of Rb_2 and Rb_3 was separated previously from a PPD-type ginsenoside mixture (PPDGM) by traditional chromatography to purify Rb_3. In the present study, we aimed to develop an enzymatic elimination-combined purification (EECP) method to obtain pure Rb_3 efficiently from a mixture of isomers. After the enzymatic elimination of Rb_2, pure Rb_3 was efficiently separated from the Rb_3 and Rd mixture by traditional chromatography.

2. Materials and methods

2.1. Materials

The PPDGM from *Panax quinquefolius* (Rb_2, 2.8%; Rb_3, 4.8%) procured from Hongjiu Biotech Co. Ltd. (Dalian, China) was used as the starting material. The solvents (methanol, ethanol, butanol, and acetonitrile) used were of HPLC grade, and other chemicals were at least of analytical reagent grade. *Microbacterium esteraromaticum* GS514 was aerobically cultured on nutrient agar (R2A, BD, USA) at 37°C.

2.2. Separation of the Rb_2 and Rb_3 mixture from PPDGM

A mixture of relatively abundant major ginsenosides, PPDGM, which can be efficiently separated from crude ginseng extracts, was used for the isolation of Rb_3 [28,29]. Previously, PPDGM has been used as a substrate for the mass production of various minor ginsenosides, such as Rg_3, F_2, and $Rh_2(S)$ [23,30,31]. From 200 g of PPDGM, 10.4 g of Rb_2 and Rb_3 isomer mixture was obtained, and the yield was approximately 5.2%. These results are consistent with the findings of a previous study [14]. The ginsenosides Rb_2 and Rb_3 account for approximately 7.6% of the PPDGM, and their mixture can be efficiently separated by Octadecyl-silica (ODS) chromatography (Fig. 2A).

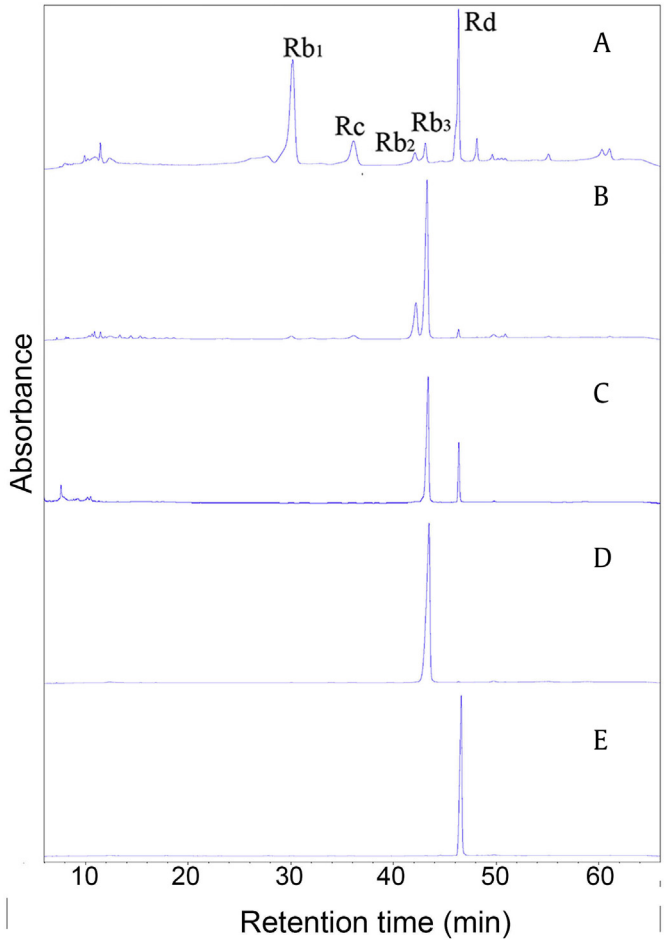

Fig. 2. HPLC analysis of purified Rb_3 from the isomeric ginsenosides mixture. (A) PPDGM. (B) Rb_2 and Rb_3 mixture purified from PPDGM. (C) Biotransformed products of the Rb_2 and Rb_3 mixture by Bgp2 treatment. (D) Isolated Rb_3 from the Bgp2-biotransformed product using RPHPLC. (E) Separated Rd from the Bgp2-biotransformed products.
PPDGM, PPD-type ginsenoside mixture; RPHPLC, recycling preparative HPLC.

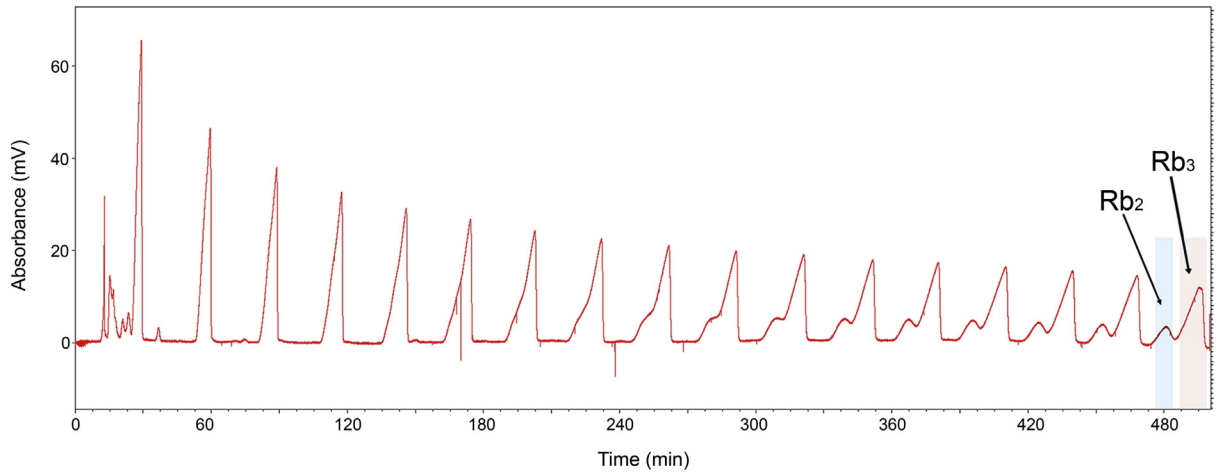

Fig. 3. RPHPLC chromatogram demonstrating the resolution of the isomeric Rb_2 and Rb_3. Conditions: column, JAIGEL-ODS-AP-L (20 mm (i.d.) × 500 mm (l)); mobile phase: H_2O (solvent A) and acetonitrile (solvent B), isocratic elution: 40% B; flow rate, 7 mL/min, detection wavelength, 203 nm; sample loading concentration of 10 mg/mL; loading volume of 1 mL.
RPHPLC, recycling preparative HPLC.

2.3. Cloning and expression

The genomic DNA of *M. esteraromaticum* GS514 was extracted using a genomic DNA extraction kit (Elpis, Daejoen, Korea). The gene encoding *bgp2* (GenBank accession number: JN852950) was amplified from the genomic DNA using *Pfu* DNA polymerase (Enzynomics, Daejoen, Korea) and the oligonucleotide primers (5′-G GTT CCG CGT GGA TCC ATG ATC CGC GAG CCC TTC CTC-3′ and 5′-G ATG CGG CCG CTC GAG CTA AGA GCC CGC GCG CAC CAA C-3′) (Macrogen Co. Ltd., Korea). The amplified DNA fragment was inserted into the linear pGEX 4T-1 vector using the EzCloning Kit (Enzynomics Co. Ltd., Korea) and transformed into *E. coli* DH5α. The resulting recombinant vector (pGEX-*bgp2*) was extracted using a plasmid extraction Kit (GeneAll Co. Ltd., Korea) and heat-shock transformed into *E. coli* BL21. The cells harboring pGEX-*bgp2* were cultured in a shaking incubator at 37°C until the OD_{600} of the culture medium reached 0.6, and then protein expression was induced by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM. After culturing for a further 18 h at 18°C, the induced bacteria cells were harvested by centrifugation at 4000 × *g* for 15 min and were suspended and disrupted by ultrasonication.

2.4. Preparation of recombinant Bgp2 using high-cell density culture

Six grams of PPDGM were dissolved in 200 mL of 10% methanol, and the undissolved precipitants were separated. The dissolved PPDGM was subjected to liquid chromatography (C18, 120 g, 39 mm × 157 mm) and was eluted with methanol–water (4:6) mixture to yield 20 fractions. The elution was fractionated every 120 mL, and the fractions containing isomers were collected and evaporated. The separated isomeric ginsenoside mixture was characterized by HPLC.

2.5. Treatment of the Rb_2 and Rb_3 mixture with Bgp2

The reaction mixture consisted of the Rb_2 and Rb_3 mixture at the final concentration of 50 mg/mL in 200 mL of crude recombinant Bgp2 (pH 7.0). After incubation for 12 h at 37°C, the mixture was centrifuged at 4000 × *g* for 15 min, and then the supernatant was loaded to a column packed with HP20 resin (340 g) (Sigma, St. Louis, MO). Two liters of water were used to remove unbound hydrophilic compounds and free sugar molecules, and the absorbed ginsenosides were eluted using three bed volumes of 95% ethanol. The eluted ethanol solution with Rb_3 and Rd was evaporated *in vacuo* to remove the ethanol.

2.6. Recycling prep-HPLC purification of Rb_3 from the isomeric mixture or biotransformed products

The Rb_2 and Rb_3 mixture or the biotransformed product was separated by recycling preparative HPLC (RPHPLC) (LC-9210II NEXT; Japan Analytical Industry Co., Tokyo, Japan). Rb_3 was separated from the mixture using a prepacked column (JAIGEL-ODS-AP-L, 20 mm (i.d.) × 500 mm (l), 10 μm) purchased from Japan Analytical Industry Co. (Japan). Acetonitrile (40%) was used as the mobile phase, and the flow rate of RPHPLC was set at 7.0 mL/min. The sample solution was prepared by dissolving 350 mg of the crude or Bgp2-treated Rb_2 and Rb_3 mixture in 40% acetonitrile to a final concentration of 35 mg/mL, and 1 (Rb_2 and Rb_3 mixture) or 10 mL (Bgp2-treated Rb_2 and Rb_3 mixture) of the solution was loaded for Rb_3 purification, respectively.

2.7. High performance liquid chromatography analysis

The ginsenoside samples were analyzed using an Agilent 1260 Infinity HPLC system (Agilent Co Wilmington, DE). The samples were separated on an YMC ODS C18 column (5 μm, 4.6 mm (i.d.) × 250 mm (l); YMC, Japan) at a flow rate of 0.8 mL/min. The gradient elution system consisted of water (A) and acetonitrile (B), and the following program was used: 0–5 min, 15%–30% B; 5–15 min, 30%–32% B; 15–35 min, 32%–32% B; 35–45 min, 32%–45% B; 45–60 min, 45%–50% B. The column temperature and detection wavelength were 35°C and 203 nm, respectively.

3. Results and discussion

3.1. Isolation of Rb_3 from the Rb_2 and Rb_3 mixture by RPHPLC

Rb_3 was purified from an isomeric mixture using an RPHPLC system equipped with a preparative ODS column. RPHPLC can enhance the separation of compounds by recycling the effluent sample several times over the column without increasing the length of the chromatographic column. This purification method

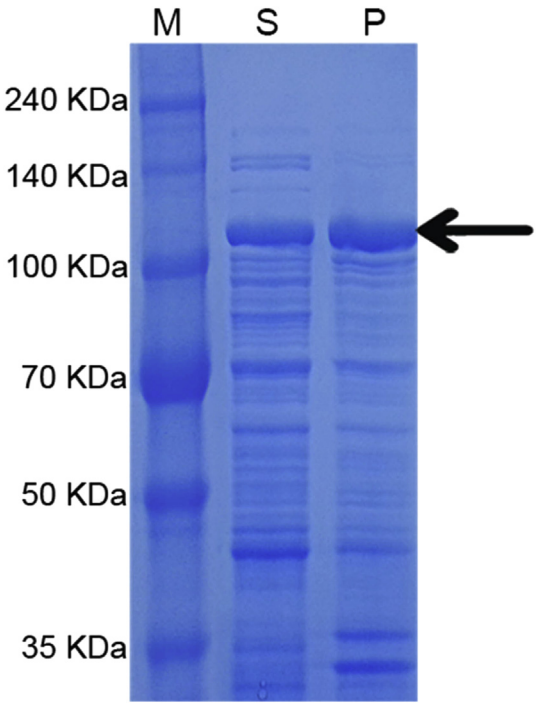

Fig. 4. SDS-PAGE analysis of the recombinant Bgp2. Lanes: M, molecular mass standard; S, supernatant of *E. coli* crude extract carrying pGEX-*bgp2*; P, precipitant of *E. coli* crude extract carrying pGEX-*bgp2*. Bgp2 is marked with an arrow.

can increase the column resolution, product purity, and yield and reduce the operation cost [32,33]. Ten micrograms of the Rb_2 and Rb_3 mixture were loaded on to the RPHPLC column for separation. Our previous experiment found that more than 10 mg of loading sample can decrease the purity of Rb_3. In the present study, the Rb_2 and Rb_3 isomers exhibited signs of resolution after 10 effective columns and were baseline-resolved after 17 effective columns (Fig. 3). The purification process required more than 8 h, yielding 2.8 mg of Rb_3 with 90.8% purity; the yield was 28%. RPHPLC is generally used to separate compounds from an isomeric mixture [34,35]. However, the same polarities of Rb_2 and Rb_3 make the separation difficult even by RPHPLC. Similar to the present study method, Liu et al [9] purified ginsenoside Rb_3 on a milligram-scale from crude extracts by chromatography.

3.2. Elimination of Rb_2 in the isomeric mixture

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed a strong expression of ~113.4-kDa Bgp2 protein, and the expression level of Bgp2 was similar to that reported by Quan et al [27] (Fig. 4). We also confirmed that the vector without Bgp2 did not convert Rb_2 or Rb_3 (Fig. S2). The cell lysate of Bgp2 exhibited arabinopyranoside hydrolyzing activity when reacted with Rb_2 but did not react with Rb_3 (Fig. 5). The enzyme reaction was performed using crude recombinant Bgp2 cell lysate with the isomeric mixture as the substrate at a final concentration of 50 mg/mL to transform Rb_2.

As shown in Fig. 2C, Rb_2 was completely converted to Rd within 12 h after the addition of crude Bgp2 to the mixture similar with the results of Quan et al [27] reported that ginsenoside Rg_3 also exists in the conversion product of Rb_2; however, we did not detect Rg_3 in the mixture produced. This might be because the higher concentration (9.2 mg/mL) of Rb_2 reduced the conversion of Rd into Rg_3. The reaction mixture was applied to HP20 macroporous resin to remove proteins, sugars, and unbound impurities. After washing, ethanol elution of the ginsenosides from the HP20 macroporous resin was carried out. The eluant was then evaporated *in vacuo* to obtain 8.6 g of dried mixture.

The attached glycoside in the outer position of C20 forms the isomers Rb_2 and Rb_3 (Fig. 1). Bgp2 can selectively hydrolyze arabinopyranoside from Rb_2 but cannot react with xylopyranoside of Rb_3. The hydrolyzation by Bgp2 changes Rb_2 to Rd, changing the

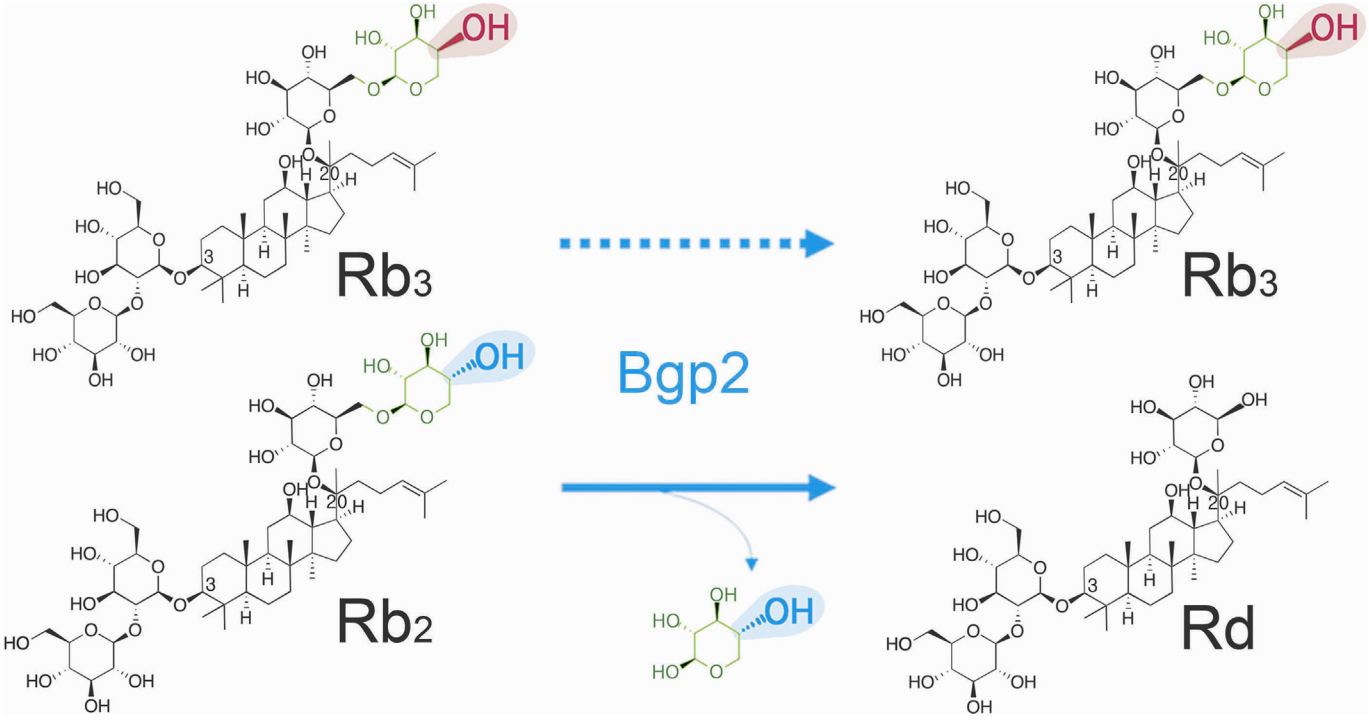

Fig. 5. Schematic view of the transformation pathways for Rb_2 conversion into ginsenoside Rd and the related structures of the ginsenosides.

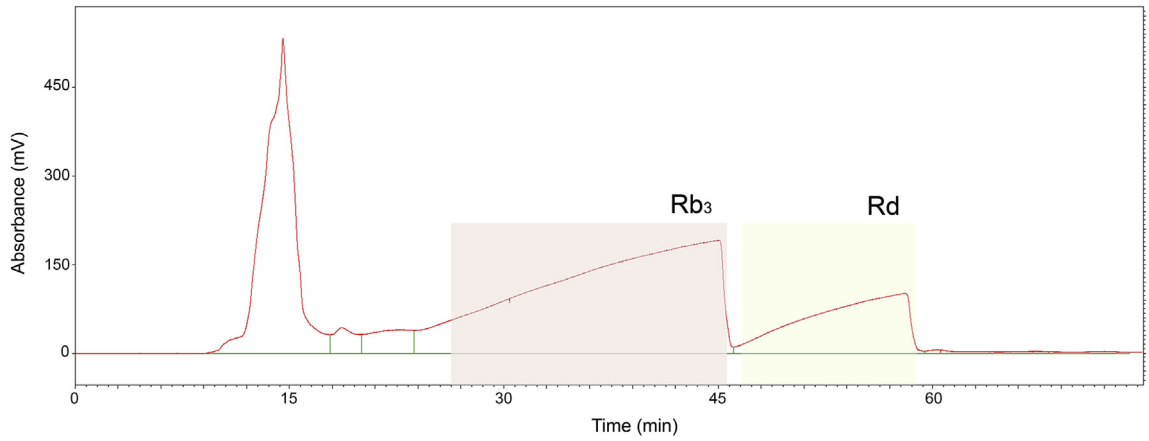

Fig. 6. RPHPLC chromatogram showing the resolution of Rb_3 and Rd. Note that these two peaks can be separated without recycling. Conditions: column, JAIGEL-ODS-AP-L (20 mm (i.d.) × 500 mm (l)); mobile phase: H_2O (solvent A) and acetonitrile (solvent B), isocratic elution: 40% B; flow rate, 7 mL/min, detection wavelength, 203 nm; sample loading concentration of 35 mg/mL; loading volume of 10 mL.
RPHPLC, recycling preparative HPLC.

Table 1
Purification scheme

No.	Sample	Loading content (mg)	Purified product (mg)	Yield (%)	Purified purity (%)	Time (min)
1	Rb_2 and Rb_3 mixture	10	2.8	40.2	90.4	505
2	Enzymatic elimination	350	164	72.7	97.6	66

polarity and molecular weight, which was evident by the change in HPLC retention time from 0.9 to 3.0 min. (Rb_2 to Rd) (Fig. 2C). This makes the purification of Rb_3 more efficient.

3.3. Isolation of Rb_3 from the Bgp2-treated isomeric mixture using RPHPLC

Three hundred fifty milligrams of Bgp2-treated Rb_2 and Rb_3 mixture were used to purify Rb_3 by RPHPLC. After loading, the estimated Rb_3 peak was separated directly without recycling from Rd (Fig. 6). Ginsenoside Rb_3 has one more xylose than Rd; thus, they can be separated by the difference in polarity by traditional chromatography purification even without RPHPLC. The fractions were collected at 26.5–45.5 min (Rb_3) and 46.5–58.5 min (Rd) and evaporated separately. The content of Rb_3 and Rd produced was determined by HPLC (Fig. 2D and E). The results revealed that 164 mg of Rb_3 (97.6%) and 41 mg of Rd (98.9%) were obtained (Table 1). The recovery ratio from the isomeric mixture reached 72.7% during the process. Compared with those by the traditional chromatography method, the content of purified compound increased by 58.6 times, and the time was reduced by 13.1%.

Liu et al [9] harvested 18.5–25.2 mg of Rb_3 from crude extracts of *Panax notoginseng* using a reversed-phase semi-preparative C18 column. However, the purified content and yield were six and ten times less than those by the EECP method. Furthermore, the purification of Rb_3 can be achieved using the EECP method with less column length because of increased polarity difference because of the transformation of Rb_2 to Rd. According to the results of the present study, the column length can be reduced to 1/17 theoretically, which can significantly reduce the cost and time of isolation.

Glycosides as monosaccharides or oligosaccharides yield many isomers in plants, thus, hindering the analysis and isolation of active compounds from natural products. Several efforts have been made recently to identify and purify glycosidic isomers from mixtures. Fouque et al [6] separated and quantified an isomeric compound in a mixture by collisional excitation by multistage mass spectrometry; carbohydrate isomers were determined using the IMS-CID-IMS-MS method [36]. However, the preparative isolation of glycosidic isomers from a mixture is usually expensive, time-consuming, and laborious.

Bgp2 exhibited specificity for arabinopyranoside but showed no affinity for xylopyranoside (Fig. 5). The recognition of sugar moieties by GHs is based on the structure of active pockets of the enzymes, and most of them exhibit specificity for a few kinds of glycosides [37–39]. The GHs are widely distributed in nature and currently represented by over 241,000 sequences classified into 133 families based on amino acid sequence similarity by the Carbohydrate Active Enzyme database [40]. Because of the varied applications in the food industry and in biofuel preparation, more glycosidases are being cloned, characterized, and utilized [41]. Exploiting their selectivity, the EECP method can also be used for analytic purposes by eliminating inseparable isomeric copartners to increase peak isolation efficiency.

4. Conclusions

The analysis and purification of isomeric glycosides are considered difficult because of similar polarities and the same molecular weight of the isomers. Ginsenoside Rb_3 showed higher pharmacological potential than that of its isomer Rb2. However, these two isomers are difficult to separate, and they co-exist in ginseng. Herein, we proposed a novel EECP method to enhance chromatographic purification of Rb_3 from an isomeric mixture. Bgp2 can selectively transform Rb_2 into Rd, significantly increasing the yield and reducing the time and minimum column length for purification. The EECP method was found to be highly efficient and simple and was demonstrated to be effective to purify Rb_3 from an isomeric mixture. Moreover, the present study provides a generic concept that is promising for the purification of glycosidic isomers from crude natural products.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2019.08.003.

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