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RESEARCH PAPER

Enrichment of Ginsenoside Rd in *Panax ginseng* Extract with Combination of Enzyme Treatment and High Hydrostatic Pressure

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Abstract Ginsenoside Rd, a minor ginseng saponin, has several pharmacological activities. Traditionally, saponins are extracted using organic solvents or hot water extraction. However, both of these methods have disadvantages such as formation of artefacts and compound decomposition. Additionally, the use of organic solvents for extraction is hazardous to the environment. Therefore, we aimed to produce ginsenoside Rd without using organic solvents or hot extraction. We developed a simultaneous extraction and transformation process for higher yields of ginsenoside Rd using a combination of high hydrostatic pressure (HHP) and enzymes. Several commercial glycosidases in various combinations were studied for the enrichment of ginsenoside Rd from major ginsenosides by enzymatic transformation and HHP. We found that treatment with a combination of cellulase (2 U/mL), cellobiase (4 U/mL) and HHP of 100 Mpa at pH 4.8 and 45°C for 24 h resulted in a ginsenoside Rd content of 3.47 ± 0.35 mg/g of fresh ginseng. This yield is 2.1-fold higher than that of the corresponding enzyme treatment at atmospheric pressure (AP, 0.1 Mpa) at pH 4.8, 45°C and for 24 h. This simultaneous extraction and transformation process can be used for the preparation of Rd enriched ginseng beverage without using hazardous organic solvents.

Keywords: *Panax ginseng*, high hydrostatic pressure, enzymatic, extraction, ginsenoside Rd

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1. Introduction

Ginsenosides are the principle bioactive compounds in ginseng (*Panax ginseng* Meyer). More than 40 ginsenosides have been identified from ginseng roots that fall under three major chemical categories such as protopanaxadiol (PPD), protopanaxatriol (PPT), and oleanane-type ginsenosides. Among the various ginsenosides, ginsenoside Rd has emerged as an important compound because of accumulating evidence on its safety and bioactivity. Ginsenoside Rd is reported to protect against focal cerebral ischemia [1] and myocardial ischemia [2]. Ginsenoside Rd had also undergone a clinical trial in humans with acute ischemic stroke, which concluded that the compound is somewhat beneficial for acute ischemic stroke [3]. Apart from its protective effect on ischemia, ginsenoside Rd has several activities such as immunosuppressive activity [4], anti-inflammatory activity [5], immunological adjuvant [6], anti-cancer activity [7] and wound-healing activity [8]. A pharmacokinetic and safety study in healthy Chinese volunteers concluded that ginsenoside Rd was well tolerated with no pattern of dose-related adverse events with a favourable pharmacokinetic and safety profile [9].

Traditionally, the extraction of saponins such as ginsenosides involved hot extraction of the plant material with aqueous

alcoholic solutions and evaporation of the alcohol, dissolution in water, and then extraction of saponins into butanol by liquid–liquid extraction . Hot extraction leads to compound decomposition and formation of artefacts rather than genuine saponins [10]. Specifically, extraction of steroidal saponins such as ginsenosides with methanol (MeOH) may result in the formation of methyl derivatives not originally found in the plant [10]. Preparation of minor ginsenosides like Rd from ginseng involves extraction of total ginsenosides using organic solvents and then acid hydrolysis of the total extract at high temperature [11]. Acid hydrolysis and other chemical treatment methods lack specificity and produce side reactions such as epimerization, hydration, and hydroxylation, and are hazardous to the environment [11].

The use of enzymes for extraction of bioactive compounds from plant materials without the use of solvents is an attractive option [12]. The plant cell wall is made up of cellulose, hemicellulose, lignin and in some cases pectin. Cell wall degrading enzymes are commercially available, and can be used for the extraction of bioactive compounds from plant materials. Disruption of the plant cell wall with cell wall-degrading enzymes can enhance the release of the bioactive compounds from plant cells. This release process can be optimized using several enzyme preparations such as cellulases, hemicellulases, and pectinases either alone or in combinations [12].

Producing a particular ginsenoside in high yield requires selectivity, which can be achieved through the use of enzymes. In addition to extraction, enzymes can be used for transformation of the major ginsenosides into more pharmacologically active minor ginsenosides. Enzymes are highly specific to the substrate and do not produce side reactions. Several microbial glycosidases were shown to transform one ginsenoside to another by selective hydrolysis of sugar moieties attached to the aglycone component of the ginsenosides [11]. Glycosidases such as β-D-glucosidase, β-D-xylosidase, α-L-arabinofuranosidase and α-L-rhamnosidase were shown to hydrolyse specific ginsenosides [11]. Preparation of minor ginsenosides by transformation from major ginsenosides by specific microbial glycosidases also requires extraction of total ginsenosides from the ginseng tissue using organic solvents and subsequent transformation using enzymes [11]. Furthermore, currently available enzyme preparations cannot completely hydrolyse plant cell walls, which limits the extraction yields of compounds [12].

High hydrostatic pressure (HHP, 100 ~ 1,000 Mpa), a food processing method, has previously been used for the extraction of bioactive components from plant materials [13]. HHP from 100 ~ 600 Mpa has different effects on biological structures such as cell walls, cell membranes, and proteins, and damages the plant cell and internal cell structures and aids in the efficient extraction of bioactive components from the plant tissue [13]. HHP extraction of bioactive components is a non-thermal extraction process; therefore, heat labile components such as vitamins, pigments, volatile compounds, flavouring substances, alkaloids, saponins, and flavonoids are extracted without decomposition [13]. Several bioactive components such as caffeine from coffee, carotenoids from tomato, anthocyanins from grape skin, polyphenols from green tea leaves, flavonoids and phenolic compounds from Litchi fruits, and ginsenosides from ginseng has been extracted with HHP processing [13]. Few processes have developed a combination of HHP and enzymes to extract total ginsenosides without focusing on specific enrichment of a particular ginsenoside [14-16].

In the present study, we describe a process for simultaneous extraction and transformation of major ginsenosides into ginsenoside Rd by combining HHP and enzyme treatment (HHP-E). We used a combination of several commercial microbial glycosidases and cell wall degrading enzyme preparations for the maximum recovery of ginsenoside Rd under HHP-E extraction.

2. Materials and Methods

2.1. Commercial enzymes and ginseng roots

Commercially available enzymes such as *Trichoderma reesei* ATCC26921 cellulase, *Bacillus licheniformis* amylase, Viscozyme L (mixture of cell wall degrading enzymes), *Aspergillus niger* cellobiase, and *Aspergillus aculeatus* Pectinase were purchased from Sigma (Seoul, Korea). *A. niger* α-L-arabinofuranosidase was from Megazyme, Ireland. Four-year-old fresh ginseng roots (Geumsan, South Korea) were used for ginsenoside extraction and transformation.

2.2. Screening for enzyme combinations

Fresh ginseng roots were chopped and homogenized in sterile distilled (10 g in 50 mL (w/v)) water using a mixer grinder. The homogenate was adjusted to pH 4.8 with 2 M citric acid. Various enzyme (E) combinations were added to the suspension (Table 1) and subjected to 100 Mpa pressure treatments for 12 h at 45°C in a HHP machine (TFS-2L, Toyo-Koatsu Innoway Co. Ltd., Hiroshima, Japan). Simultaneously, the ginseng slurry was added with enzyme combinations and incubated at atmospheric pressure (AP, 0.1 Mpa) for 12 h at 45°C to compare the yield of ginsenoside Rd at AP and HHP. After treatment, enzymes were inactivated by subjecting the reaction mixture to 80°C for 30 min. Samples from various treatments were centrifuged and the supernatant was collected for analysis of ginsenoside Rd content.

Table 1. Enzyme combinations used in the present study

Enzymes/Enzyme combinations	Enzyme concentration (U/mL)	Ginsenoside Rd yield (mg/g)	
		AP 12 h	HHP 12 h
Viscozyme (V)*	20 μL	0.12 ± 0.006	0.28 ± 0.014
Pectinase (P)	5	0.16 ± 0.008	0.33 ± 0.017
Cellulase (C)	5	0.90 ± 0.045	1.06 ± 0.053
Amylase (A)	5	0.18 ± 0.009	0.33 ± 0.017
α-L-Arabinofuranosidase (Araf)	6	0.14 ± 0.007	0.33 ± 0.017
Viscozyme+pectinase (VP)	10 μL + 2.5	0.12 ± 0.006	0.28 ± 0.014
Cellulase + pectinase (CP)	2.5 + 2.5	0.78 ± 0.039	0.85 ± 0.043
Cellulase+arabinofuranosidase (CAraf)	2 + 4	0.80 ± 0.040	0.94 ± 0.047
Cellulase+cellobiase (CCb)	2 + 4	0.95 ± 0.048	1.14 ± 0.057
Cellulase+arabinofuranosidase+cellobiase (CArafCb)	2.5 + 4 + 2.5	0.85 ± 0.043	1.06 ± 0.053
Viscozyme+cellulase+cellobiase (VCCb)	7 μL + 2 + 2	0.45 ± 0.023	0.66 ± 0.033
Cellulase+pectinase+cellobiase (CPCb)	2 + 2 + 2	1.04 ± 0.052	1.19 ± 0.060
Cellulase+amylase+viscozyme (CAV)	2 + 2 + 7 μL	0.92 ± 0.046	1.06 ± 0.053
Cellulase+amylase+cellobiase (CACb)	2 + 2 + 2	0.72 ± 0.036	0.79 ± 0.040
Viscozyme + pectinase+ cellulase (VPC)	7 μL + 2 + 2	0.91 ± 0.046	0.94 ± 0.047
Amylase+cellulase+pectinase (ACP)	2 + 2 + 2	0.73 ± 0.037	0.80 ± 0.040
Aqueous extraction (95°C for 1 h)**	-	0.096 ± 0.014	-
Aqueous methanolic extract (ginseng extracted with methanol at 80°C for 1 h)†	-	0.17 ± 0.084	-

*Viscozyme is a cocktail of several cell wall degrading enzymes, hence not expressed in units.
**According to the method of Lee *et al.* [19]. †Modified method of Hyun *et al.* [17]. Higher extraction temperature causes compound decomposition, hence limited to 1 h total extraction time.

2.3. Extraction of ginseng saponins enriched with ginsenoside Rd from fresh ginseng by HHP-E

Four hundred grams of fresh ginseng roots were chopped and homogenized in 2 L of sterile distilled water using a mixer grinder. The ginseng slurry was treated with the enzyme combination that yielded higher ginsenoside Rd in the initial screening and incubated at AP and HHP (100 Mpa) condition for 12, 18, 24, and 36 h at pH 4.8 and 45°C. After treatment, enzymes were inactivated by subjecting the reaction mixture to 80°C for 30 min. The HHP-E- and AP-E-treated ginseng slurry was then pressed against a 250 μm sieve and the resultant extract was concentrated in a freeze drier for 48 ~ 72 h.

2.4. HPLC analysis of ginseng saponin

The ginseng extracts from HHP and AP were prepared for TLC and HPLC analysis as previously described [17]. TLC analysis of the samples was performed as previously described [17]. HPLC analysis was done in a Waters HPLC system using a Sunfire C18 column of 4.5 mm × 25 cm. HPLC-grade acetonitrile (A) and water (B) were used as mobile phase. The analysis was performed with mobile phase flow rate of 1 mL per min using a solvent gradient as follows: 0 ~ 8 min, 20 ~ 30% A; 8 ~ 12 min, 30 ~ 40% A; 12 ~ 15 min, 40 ~ 65% A; 15 ~ 20 min, 65 ~ 100% A, 20 ~ 30 min, 100% A; 30 ~ 35 min, 100 ~ 30% A; 35 ~ 40 min, 30 ~ 20% A, and column equilibration for 5 min with 20% A. The column was injected with 20 μL of samples using an automated sample injector. The elution of various ginsenosides was monitored at 203 nm. The quantity of the ginsenoside Rd was determined by comparison with the chromatogram of a standard ginsenosides Re, Rg1, Rb1, Rf, Rc, Rb2, Rb3+Rg2, Rh1, Rd, Rg3, compound K and Rh2 mixture. Ginsenosides Rb1, Rb2, Rc, Re, Rg2 and Rg3 were a kind gift from Prof. Nam-In Baek, Natural Products Chemistry Lab, Kyung Hee University, Korea. Other ginsenosides were obtained from Chromadex (USA). HPLC analysis of each sample was performed in triplicates to obtain confirmatory results.

3. Results and Discussion

3.1. Ginsenoside Rd content in HHP and enzyme combinations

Addition of enzymes to the ginseng slurry increased the level of ginsenoside Rd compared with enzyme non-treated ginseng extract using AP and HHP condition (Table 1). Treatment of ginseng slurry with HHP-E increased the level of ginsenoside Rd compared with AP-E treatment (Table 1). Among the various enzymes tested, the use of cellulase or enzyme combinations containing cellulase resulted in higher ginsenoside Rd levels (Table 1). Enzyme combinations without cellulase or cellobiase resulted in the

lowest levels of ginsenoside Rd. HHP and enzymes were previously reported to be cost effective and safe compared with organic solvents for extraction [18,19], and acid hydrolysis for transformation [11], respectively. Treatment with enzymes and HHP can destroy the cell wall and enhance the extraction of bioactive metabolites from ginseng tissue [19]. In addition, enzyme-based extraction involves low temperatures, which eliminates decomposition from heat and enables better release and more efficient extraction of bioactive compounds [12].

Enzymes plus HHP were reported to show high kinetic activity and faster reaction times than AP [20]. High ginsenoside Rd content was observed in two enzyme combinations such as HHP-cellulase/cellobiase and HHP-cellulase/cellobiase/arabinofuranosidase (Table 1). Arabinofuranosidase was studied to simultaneously use the conversion of ginsenoside Rc to Rd [21] to produce high levels of Rd. However, treatment with arabinofuranosidase did not show any significant increase in Rd levels compared with no enzyme control (Table 1). The combination of arabinofuranosidase

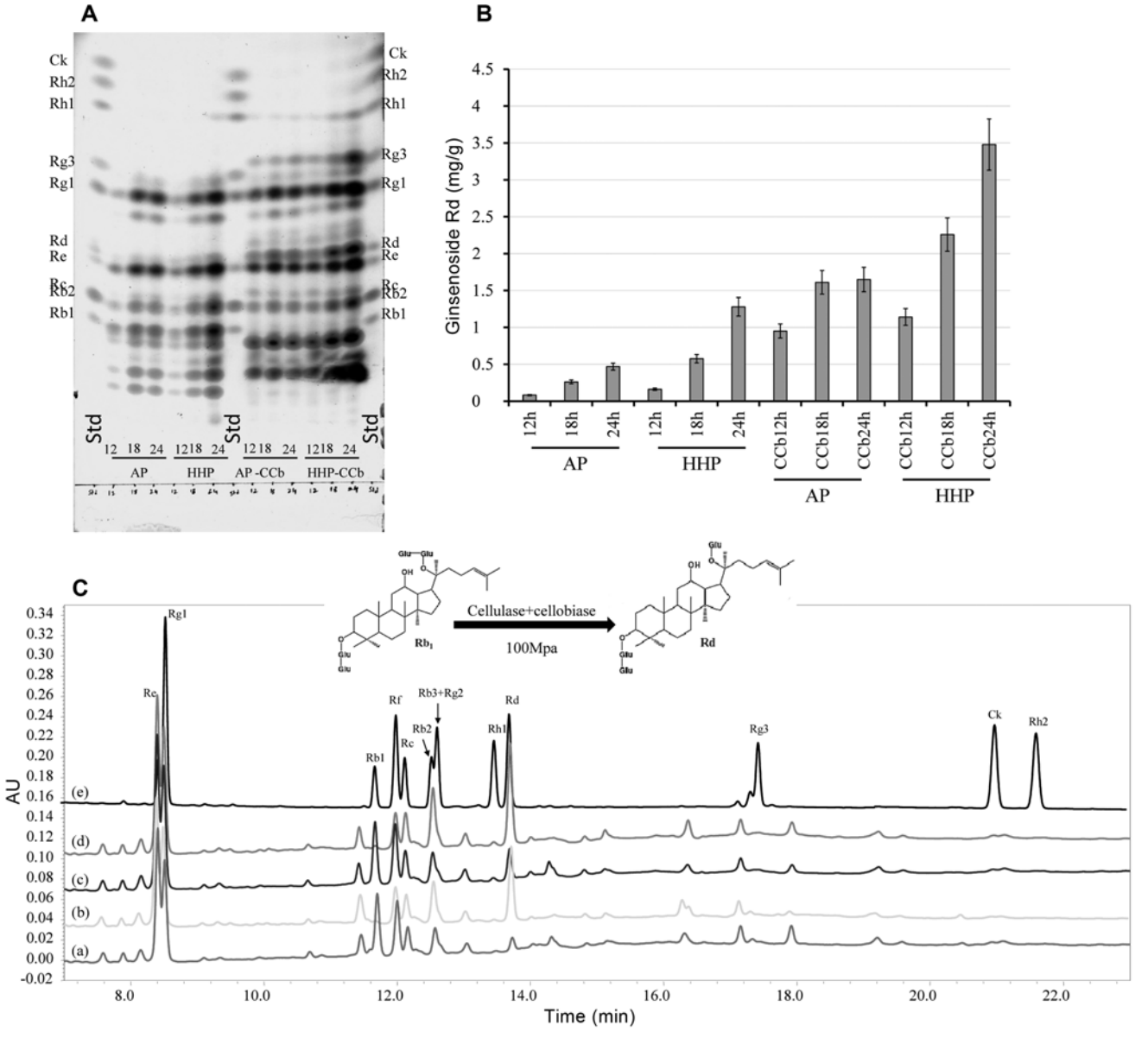

Fig. 1. Yield of ginsenoside Rd at various time points upon treatment with cellulase and cellobiase under HHP and AP. (A) TLC chromatogram showing the ginsenosides profile at AP, HHP, AP-CCb, and HHP-CCb for various time points. (B) The yield of ginsenoside Rd at various treatments at various time points. Error bars indicate standard deviation of three independent replicates. (C) HPLC chromatogram showing the ginsenoside profile of (a) ginseng extract from slurry incubated at atmospheric pressure for 24 h; (b) extract from cellulase and cellobiase treated ginseng slurry at atmospheric pressure for 24 h; (c) extract from HHP-treated ginseng slurry at 24 h; (d) extract from HHP-cellulase and cellobiase treated ginseng slurry at 24 h; and (e) standard ginsenoside mixture.

Table 2. Comparison of ginsenoside Rd yield

Material	Ginsenoside Rd yield (mg/g of ginseng)	Fold compared to AP-E/other method	Process	Reference
4 year old fresh ginseng	3.47 ± 0.35	2.1↑	HHP (100 Mpa) -Cellulase+cellobiase	Present study
Red ginseng	3.099	1.26↑	HHP (100 Mpa)	[14]
Ginseng	0.05	3.6↓	HHP (100 Mpa) -Speczyme +Pectinase	[15]
Jangnesam (Mountain ginseng)	3.21	ND	HHP (100 Mpa) -Viscozyme+Cellulase+ Amylase	[16]
Fresh ginseng	0.241	1.009↑	HHP (80 Mpa)	[19]
Red ginseng	2.695	1.94↑	HHP (80 Mpa)	[19]

↑, indicate fold increase; ↓, indicate fold decrease; ND, not determined.

with cellulase and cellobiase did not show significant differences in Rd levels compared with that of cellulase and cellobiase (Table 1). These results suggest that the commercial α-L-arabinofuranosidase (from *A. niger*) either lacked specificity towards ginsenoside Rc or has low kinetic activity. Therefore, for the study of ginsenoside Rd yield at various time points, the combination cellulase and cellobiase was chosen.

3.2. Enrichment of ginsenoside Rd in ginseng extract using HHP-cellulase/cellobiase

The productivity of ginsenoside Rd at various time points was visualized and measured using TLC and HPLC, respectively (Fig.1). The time course analysis of ginsenoside Rd enrichment showed that maximum Rd content occurred at 24 h (Fig. 1). The TLC and HPLC profile shows the disappearance of ginsenoside Rb1in the enzyme treated category, which indicates that ginsenoside Rd is transformed from ginsenoside Rb1 (Figs. 1A and 1C). Longer incubation (36 h) of ginseng extract in HHP (100 Mpa)-cellulase/ cellobiase treatment resulted in lower ginsenoside Rd content (data not shown). This result could occur because of conversion of ginsenoside Rd to compound F2 and Rg3 [11,22,23]. The yield of ginsenoside Rd in HHP-cellulase/ cellobiase at 24 h was 3.47 ± 0.35 mg/g of fresh ginseng, which was 2.1-fold higher compared with AP-cellulase/ cellobiase treatment (Fig. 1B, Table 2). Increased ginsenoside Rd content in enzyme treatment under HHP condition compared with enzyme treatment at AP could be because of enhanced activity of cellulase (cell wall degrading enzyme) at high pressure. The stability and activity of most enzymes are not altered at pressures less than 100 MPa, while pressures greater than 300 Mpa induce denaturation of enzymes [24,25]. It was previously reported that cellulase activity is enhanced to 1.7-fold at 100 Mpa compared to activity at atmospheric pressure [26]. Murao *et al*. reported that several commercial cellulases from different microbes exhibited enhanced activity at high pressure [27]. Cellulase together with HHP will release the major ginsenosides trapped within the cells. Whereas, cellobiase (β-glucosidase catalyse the breakdown of β-1,4-linked glucose dimer cellobiose [28,29]) cleave the cellobiose unit linked to Rb1 and removes a glucose while the other glucose is still attached to the aglycone part of the ginsenoside, results in the transformation to Rd. Conversion from Rb1 to Rd is evident from the disappearance of Rb1 band and appearance of Rd in the TLC chromatogram only in the enzyme treatment category (Figs. 1A and 1C). Furthermore, there is an increasing trend in the level of ginsenoside Rd at various time points indicating a higher release of major ginsenoside Rb1 (cellobiase substrate) from ginseng slurry upon longer incubation. The yield of ginsenoside Rd achieved in this study is comparatively higher when considering the cost of raw material, enzymes and process conditions than the previously reported methods (Table 2).

4. Conclusion

Our study shows that a combination of cellulase, cellobiase, and HHP can produce higher ginsenoside Rd levels compared with extraction methods. We demonstrated that HHP plus enzyme treatment is a viable alternative to the organic solvent extraction and subsequent transformation using acid hydrolysis or microbial fermentation for the preparation of pharmacologically active ginsenosides such as Rd.

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