Enzymatic Modification of Saponins from *Platycodon grandiflorum* with *Aspergillus niger*

**Hye Jeong Wie,**†‡ Hai Lin Zhao,**†§ Jeong Hwa Chang,**§ Yeong Shik Kim,**§ In Kyeong Hwang,**‡ and Geun Eog Ji*,‡

Department of Food and Nutrition, Graduate school, and Natural Product Research Institute, Seoul National University, Shillim-dong, Kwanak-ku, Seoul 151, Korea

*Platycodon grandiflorum* A. DC (Campanulaceae) is a traditional medicinal plant. Its root, Platycodi Radix, contains an abundant amount of saponin glycosides, platycodins, of which platycodin D is one of the major components. The chemical structures of platycodins can be modified by various types of chemical processing, but a modification mediated with microorganisms has been not reported yet. In this study, platycodin D was modified to several partially degraded platycodon glycosides after treatment with a crude enzyme extract from *Aspergillus niger* (*A. niger*). The modified platycodin D possessed a shorter sugar side-chain, and presented a remarkably reduced V79–4 cell (Chinese hamster lung fibroblasts) cytotoxicity and erythrocyte hemolytic toxicity, whereas the nitrite-scavenging activity was increased in the modified platycodin D. Sensory scores for pungency, bitterness and after-taste were improved as well in the modified platycodin D. Results suggest that *A. niger* mediated modification yielded a novel partially degraded platycodon glycoside which possesses increased bioactivities and improved sensory values, yet with reduced toxic profiles.

**KEYWORDS:** Platycodi Radix; platycodin D; *Aspergillus niger*; modification; hemolysis; sensory score

**INTRODUCTION**

Platycodi Radix is the root of *Platycodon grandiflorum* A. DC (Campanulaceae). Traditionally, it has been consumed as a food stuff and as a folk remedy for diseases such as bronchitis, asthma, pulmonary tuberculosis, and inflammatory conditions (1). Recent studies show that platycodins are one of the most essential functional components in Platycodi Radix in terms of the inhibition of pancreatic lipase (2), cholesterol lowering, and antiobesity effects (3–5). Platycodins also have anti-inflammatory activity mediated by suppression of nitric oxide (NO) production (6). Intravenous injection of purified platycodins in rats increased the number of lymphocytes, similar to the effects of ginsenoside-Rb1, ginsenoside-Re, senegen-III, and senegen-IV (7). To date, more than 20 platycodins have been separated from Platycodi Radix. Among them, platycodin D is one of the major saponin components which contains a triterpenoid backbone linked with two side chains. One side chain is a 3-O-glucose linked by a glycosidic bond, and the other is a 28-O-apiose-xylose-rhamnose-arabinose linked by an ester bond (Figure 1). We have previously noticed the importance of the 3-O-glucose in mediating the bioactive functions of platycodin D, but the bioactive role of the 28-O-side chain in platycodin D is yet to be clarified (6). It could be informative to completely or partially degrade the side glycoside chain of platycodin D to obtain its derivatives containing a shortened side chain and compare the subsequent changes in its biological activities. Moreover, platycodin D possesses a distinctive pungent flavor and bitter taste, as well as a certain degree of hemolytic toxicity when injected intravenously or intraperitoneally (8). A complete hydrolysis of ester bond in platycodin D by H+ or OH− will remove the entire 28-O-side chain and release prosapogenin D. Prosaponenin D is free of hemolytic toxicity and is readily available by chemical methods (4). However, it is still nevertheless challenging to partially cleave the 28-O-glycoside to obtain a side-chain-shortened platycodin D. The present study managed a microbiological transformation of platycodin D to generate a novel saponin entity with a shorter 28-O-sugar chain. The modified platycodin D demonstrated some favorable properties in bioactivity, sensory values, and toxicity profiles.

**MATERIALS AND METHODS**

Preparation of Crude Platycodins and Purification of Platycodin D. Platycodin D used in this study were isolated from Platycodi Radix according to the previous method (2).

Thin Layer Chromatography (TLC) of Platycodins. Two micro-liters of platycodon sapinon samples were loaded on the TLC (silica gel 60 F254, Merck Co., Darmstadt, Germany) plate and were air-dried. The samples were developed against chloroform/methanol/water (13:10:2). After development, the plate was stained by 10% sulfuric acid and dried in air.

Production of Crude Enzymes from *Aspergillus niger*. *Aspergillus niger* (*A. niger*), van Tieghem KCTC 6906 (Korean Collection for Type

82 2 8840305. E-mail: geji@snu.ac.kr.

*To whom correspondence should be addressed. Tel: +82 2 8808749.
Fax: 82 2 8840305. E-mail: geji@snu.ac.kr.
† Two authors contributed equally to this work.
‡ Department of Food and Nutrition.
§ Natural Product Research Institute.
Figure 1. Structure of platycodin D, a 3-0-[β-d-glucopyranosyl-2,3,6-tri-O-β-d-glucopyranosyl](1→3)-β-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside ester.

Cultures, Daejeon, Korea) was purchased from KCTC and subcultured on a shaking incubator at 140 rpm. At the culture growth intervals of 0, 5, 10, 15, 20, 25, 30, 33, and 36 h, sample aliquots were withdrawn and the supernatant was filtered by Millex LCR Filter (0.45 µm particle size, 4.6 × 250 mm) and ELSD 800 (evaporative light scattering detector, Alltech, Deerfield, IL) as previously described (5). Elution was carried out at a flow rate of 1.0 mL/min using a solvent gradient consisting water (A) and acetonitrile (B). The running conditions are as follows: 0–20 min (15–80% B), 20–25 min (80–15% B), and then equilibrated with 15% B for 10 min. From the chromatogram, the peaks were assigned according to the retention time of known compounds when available. The main peak (at retention time 13–14.5 min) corresponding to the novel entity of saponin was collected. The novel saponin entity was further analyzed by HP-1100 coupled to Triple Quadrupole Mass Spectrometer (Micromass, Manchester, UK).

Sensory Evaluation. The pungency, bitterness, and aftertastes (remaining bitterness and pungency after 10 s) of 1% platycodin before and after modification were evaluated by seven trained panelists using a 15 grade scale with an ascending order from 1 (very weak) to 15 (very strong) in the sensory score.

Nitrite-Scavenging Activity. As described previously (11), 1 mL of platycodin D or its modified derivative (at concentrations of 0.2, 1.0, and 2.0 mg/mL) and 2 mL of 1 mM NaNO2 were dissolved in buffer solution (pHs 1.2 and 3.0) to make 10 mL of the final volume. After the solution was incubated at 37 °C for 1 h, 500 µL of Griess reagent (1% sulfanilic acid/1% naphthylamine = 1:1) and 2 mL of 2% acetic acid were added to 1 mL of sample, and the solution was further incubated for 15 min. The color intensity of the reaction mixture was measured at 520 nm by spectrophotometer (DU-650, Beckman, Fullerton, CA).

Cytotoxicity. Cell viability was estimated by modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenase in viable cells using Chinese hamster lung fibroblast V79-4 (ATCC, CCL-93) cells obtained from the American Type Culture Collection (ATCC, Rockville, MD) (12, 13). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Invitrogen, CA) containing 10% fetal bovine serum (FBS, Gibco, Invitrogen, CA), 100 µg/mL of streptomycin, and 100 unit/mL of penicillin (Gibco, Invitrogen, CA) and incubated at 37 °C. V79-4 cells were then seeded at 5 × 10^4 cells/well and incubated for 24 h at 37 °C. The cells were treated with various concentrations (10, 20, 50, and 100 µg/mL) of the platycodin D before and after modification for an additional 48 h at 37 °C. During the last 4 h, the cells were incubated with 10 µM of MTT stock solution (5 mg/mL, MTT, Sigma Chemical Co., St. Louis, MO), and insoluble formazan salt was measured using a microplate reader at 570 nm (as mentioned above). The optical density of the formazan formed in the control cells was taken as 100% viability. Data are mean percentages of viable cells versus the respective controls (12, 13).

Hemolysis. Freshly prepared plasma of SD (Sprague–Dawley) rats and ICR (Institute of Cancer Research) mice was used in the experiment. HEPEX (N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid) buffer was prepared with the following composition: 135 mM NaCl, 11.9 mM NaHCO3, 0.36 mM NaH2PO4·H2O, 5 mM dextrose, 1.5 mM KCl, 0.98 mM MgCl2·6H2O, 4.6 mM HEPEX (pH 7.2). The blood samples of SD rats (male, 8 weeks old) and ICR mice (male, 12 weeks old) were withdrawn by periorbital sampling. Citric acid (3.8% w/w) was added (1:20, v/v) to each blood sample as an anticoagulant. Blood samples were centrifuged at 500g for 5 min, and the plasma was then carefully removed by aspiration. The sedimented erythrocytes were then washed five times in the HEPEX buffer. The number of erythrocytes was determined with a hemocytometer and then was adjusted to 1 × 10^10 cells/mL.

The hemolysis test was performed according to the method described by Sakurai and others (14). In brief, an aliquot (100 µL) of platycodin D or its derivative with a series of concentrations were mixed with 100 µL of washed rat or mouse erythrocytes (1 × 10^10 cells/mL), respectively, suspended in HEPEX buffer (pH 7.2), and 300 µL of same buffer was added to give 500 µL total volume. The mixtures were incubated at 37 °C for 30 min. The unsleyed cells were then pelleted by 1650g centrifugation for 15 min. The A540 of the resulting supernatants (200 µL) was measured to determine the release of hemoglobin using the microplate reader (above mentioned). Hemolytic values were expressed as a percentage of the A540 of the supernatant from 100 µL of erythrocytes suspended in 0.4 mL of distilled water.
Statistical Analysis. There were at least three replications of all data. Values were expressed as mean ± standard deviation (SD). Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA) and the unpaired Student’s t test where appropriate. Statistical significance was accepted at p < 0.05.

RESULTS

Modification of Platycodon Glycoside by a Crude Enzyme Extract of A. niger. This study investigated the transformation of platycodon glycosides using various strains of probiotic bacteria and edible fungi. Among the experimental microorganisms such as bifidobacteria, lactobacilli, leuconostocs, yeasts, and aspergilli, A. niger KCTC 6906, a strain of aspergilli, showed the greatest cleaving capacity toward platycodon glycosides during fermentation. Specially, when A. niger was incubated in the presence of 0.05% (w/v) platycodin and 0.15% (w/v) rhamnose as the carbon source, it presented the most potent activity toward digesting the platycodon glycosides during fermentation. Specially, when A. niger was incubated in the presence of 0.05% (w/v) platycodin and 0.15% (w/v) rhamnose as the carbon source, it presented the most potent activity toward digesting the platycodon glycosides (Figure 2). The obtained enzyme extract showed greater activity in α-rhamnosidase than that of any other glycosidases such as β-glucosidase (Figure 3). Existence of the specific activities for other glycosidases such as α-glucosidase, α-xylosidase, β-xylosidase, α-galactosidase, β-galactosidase, α-mannosidase, β-mannosidase, and β-cellobiosidase were not observed (data not shown).

Transformation of platycodon D was substantially occurred after incubation with a crude enzyme extract of A. niger. After TLC development, the spot representing platycodin D vanished, instead a novel spot appeared as a major product. The newly appeared spot migrated more rapidly on TLC plates than platycodin D, but the spot was distinctly different from the chemically hydrolyzed prosapongenin D spots, which is lacking 28-O-side chain and was located at much upper position in TLC (Figure 4). It is likely that platycodin D was modified by A. niger enzyme extract to produce a novel saponin entity with a shortened side chain. Such a new spot was not observed when platycodins were treated by the heat-deactivated extract (data not shown).

The novel spot was scraped and further purified with HPLC. The ESI-MS of this compound showed a distinct high peak at 959.3 amu (Figure 5). Since the theoretical m/z of the platycodin D fragment short of the terminal apiose-xylose disaccharide is 959.0 amu, it is very likely that the linkage between rhamnose and xylose was cleaved in this modified platycodin D. Yet the fragmentations representing a triterpenoid linked only with 28-O-arabinose residue (m/z = 827.3), prosapogenin D moiety (m/z = 681.3), or even platycodigenin D (aglycon, m/z = 519.5) were clearly observed in the MS spectrum. Such fragmentation patterns seem to imply that the triterpenoid backbone of platycodin D was kept intact during the microbial modification. The molecular peak of platycodin D was not observed in ESI-MS, which generally was presented as the major peak in intact platycodin D. Yet a peak (m/z = 1091.2) representing the deapiosyl platycodin D was observed. It appears indicating that platycodin D was nearly completely digested, but it is not still clear if apiose and xyloase is cleaved one by one or directly cleaved between xylose-rhamnose. Taken together, it was suggested that the cleavage of 28-O-side chain most likely occurred between xylose and rhamnose, resulting in a modified platycodin D short of an apiose-xylose disaccharide.

Cytotoxic and Hemolytic Activities of the Modified Platycodin D. The cytotoxicity of platycodin D before and after modification toward a normal cell line of Chinese hamster lung fibroblasts was evaluated by MTT assay (Figure 6). The cells incubated with unmodified platycodin D showed an 83.7% cell survival at a dose of 10 μg/mL and only a 9.9% cell survival at 100 μg/mL, whereas cells incubated with the modified platycodin D showed a distinctly higher cell survival rate; even at 100 μg/mL, a 90.0% cell survival rate was noted. It implies that cytotoxicity was remarkably alleviated in microorganism modified platycodin D as compared to intact platycodin D.

Hemolytic toxicity for platycodin D was shown a dose dependency in the range of 5–40 μg/mL. The concentrations that cause 50% red cell hemolysis were 18 μg/mL for SD rats.
and 37.5 µg/mL for ICR mice, respectively. However, in the modified platycodin D, such hemolytic toxicity was not seen in any of the plasma obtained from SD rats or ICR mice.

Nitrite Scavenging Activity of the Modified Platycodin D.

The nitrite scavenging activity was compared in platycodin D before and after modification. Relative to the original platycodin D, at pH 1.2, the nitrite-scavenging activity of the modified platycodin D was increased from 13.2% to 34.5% at 0.2 mg/mL, from 45.8% to 98.1% at 1 mg/mL, and from 50.2% to 99.6% at 2 mg/mL, respectively (Figure 7). Similarly, at pH 3.0, nitrite scavenging activity of the modified platycodin D was increased from 6.0% to 16.5% at 0.2 mg/mL, from 9.2% to 28.4% at 1 mg/mL, and from 19.0% to 40.3% at 2 mg/mL, respectively.

Sensory Scores of the Modified Platycodin D. Sensory scores of the original and modified platycodin D are shown in Figure 8. The pungency, bitterness, and aftertaste values were decreased by 28% (p < 0.001), 15% (p < 0.05), and 37% (p < 0.001), respectively, suggesting that the sensory values of the modified platycodin D were significantly altered to be less unfavorable than that of the untreated platycodin D.

DISCUSSION

Platycodin D belongs to the oleanane type of triterpenoid saponin with 3-O-glucose and 28-O-apiose-xylose-rhamnose-
The LD50 (50% lethal dose) of orally administered platycodins. Such a structural novelty in platycodins is unavailable rhamnose and platycodins, the enzymes with the greatest activity, selection of carbon source was proved to be critical. With fermentation in the presence of addition of rhamnose plus platycodins was proved to be more effective than the use of the individual rhamnose or platycodin. Interestingly, to induce the enzymes possessing glycosidase activity, selection of carbon source was proved to be critical. Addition of rhamnose plus platycodins was proved to be more effective than the use of the individual rhamnose or platycodin alone as carbon sources. With fermentation in the presence of rhamnose and platycodins, the enzymes with the greatest rhamnosidase activity and selectivity for platycodin D. The cleaving site of platycodin D occurred predominantly between the rhamnose and xylose with the resultant removal of terminal disaccharide unit, apiose-xylose at 28-O-xyloside chain. This deduction is consistent with the spectrum profiles obtained from TLC, HPLC, and LC-ESI-MS. In addition, it is also shown that the triterpenoid backbone is not changed during the modification.

As with our repeated experimental experience, ESI has a much softer ionization mode than electron impact (EI), chemical impact (CI) or fast-atom bombardment (FAB) ionization mode. Accordingly, the molecular ion peak of platycodins could be presented as the major peak in ESI-MS positive ionization mode as long as the compound has an adequate purity. According to MS spectrum of modified platycodin D, it is thus rationalized that enzyme extract obtained from A. niger has partly digested 28-O-side chain, whereas a control conducted with preheated enzyme extract did not show any evidence of modification.

Hemolysis is a common side effect occurring in most saponin compounds including platycodins. Hemolytic toxicity prevents platycodins from being administered intravenously. The LD50 (50% lethal dose) of orally administered platycodins in mouse and rat was 420 and 800 mg/kg, respectively, whereas the LD50 of intravenously or intraperitoneally administered platycodins would dramatically reduce to 20–40 mg/kg (8). The discovery of the elimination of the hemolytic toxicity of platycodin with A. niger enzyme extract provides with a new approach to detoxify the saponins, thus to enhance its intravenous and intraperitoneal safety. For hemolytic safety, more intensive safety test in an animal trial would be needed in the future work.

A. niger modified platycodins also reduced the cytotoxicity toward Chinese hamster cell lines, while the nitrite scavenging activity increased. The nitrite scavenging effect of platycodin extracts has been previously reported (16) and was attributed to the platycodin component. Our results imply that 28-O-side chain is important in exerting bioactive functionality of platycodins, and the bioactive properties of platycodins could be modified by enzymatic degradation of glycoside residues. In addition, the considerable reduction in bitterness and pungency should improve the potential applicability of platycodins as a dietary supplement and also as a pharmaceutical agent.

In conclusion, the chemical structures of the glycosides present in functional food or dietary supplements can be modified by various types of bioorganic processing. The enzymatic modification of platycodin D using edible microorganisms has resulted in a novel derivative component with a partly degraded glycoside chain. The resultant platycodin D derivative demonstrated improved profiles in antioxidation, toxicities and sensory values. Our finding may pilot a new microbiological approach to modify saponin glycosides to improve their bioactivities and bioavailability.

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