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

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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 platycodin 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 platycodin 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-Rb_1, ginsenoside-Rc, senegin-III, and senegin-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 microliters of platycodin saponin samples were loaded on the TLC (silica gel 60 F_{254} 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 as introduced previously (*9*).

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

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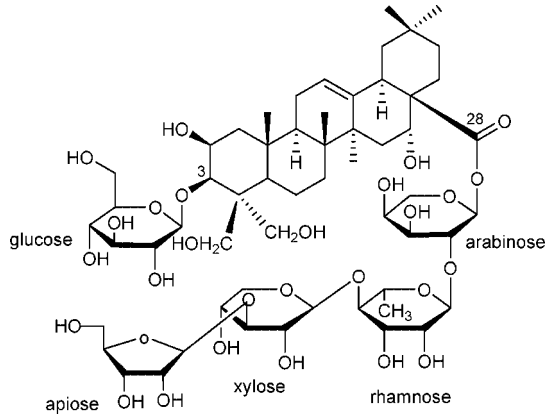

Figure 1. Structure of platycodin D, a 3-*O*-β-D-glucopyranosyl-2β,3β,16α,23,24-pentahydroxy-12-oleanen-28-oic acid-28-*O*-[β-D-apiofuranosyl(1→3)-β-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside] ester.

Cultures, Daejeon, Korea) was purchased from KCTC and subcultured in potato dextrose broth (Difco, Detroit, MI). To produce the crude enzymes, *A. niger* was grown in cultivation medium containing the following components (w/v): $(NH_4)_2SO_4$ 0.8%, $(NH_4)_2HPO_4$ 0.3%, KH_2PO_4 0.1%, and $MgC1_2 \cdot H_2O$ 0.1%. The spores were harvested from the potato dextrose agar cultures and suspended in the spore suspension solution (0.005% Tween 80, 0.9% NaCl). An inoculum corresponding to 10^7 spores per 1 mL of medium was added to the 20 mL cultivation medium under aseptic conditions. Incubation was carried out at 30 °C on a shaking incubator at 140 rpm. At the culture growth intervals of 24, 48, 72, 96, 120, 144, and 168 h, sample aliquots were withdrawn and mycelium was removed by centrifugation (4000*g*, 20 min, 4 °C). The supernatant was concentrated (4000*g*, 30 min, 4 °C) using centrifugal filter (Amicon Ultra-15, 10,000 MWCO, Millipore, Ireland) equipped with centrifugal separator (Combi-514R, Hanil, Korea). The concentrated supernatant was resuspended in 50 mM phosphate buffer (pH 6) to give the10-fold concentrated crude enzymes of the initial supernatant. The enzyme activity was calculated according to the method introduced previously (*10*).

During the screening period, platycodin alone or platycodin in combination with one of the monosaccharide such as β-D-(+)-glucose, α-L-rhamnose, D-(+)-xylose, or L-(+)-arabinose (Sigma, St. Louis, MO) were used as carbon sources to investigate the optimum carbon source that allows the microorganisms to most efficiently induce the enzymes capable of digesting the platycodin glycoside chains. Similarly, the optimum conditions of the incubation time and pH were also investigated to establish the overall optimal fermentation conditions.

The specific glycosidase activity was measured by the degradation of specific substrates by the crude enzymes to produce free pNP (*p*-nitrophenol). The concentration of free pNP is determined by microplate reader (Bio-Rad Model Benchmark, Tokyo, Japan) at 450 nm. The *p*-nitrophenyl-β-D-glucopyrannoside and *p*-nitrophenyl-α-D-rhamnopyranoside (Sigma, St. Louis, MO) were used as the substrates for β-glucosidase and α-rhamnosidase, respectively. The specific activity to digest the platycodin side chain was semiquantitatively estimated by the disappearance of the platycodin D spot in TLC. The preheated enzyme extract was used as a negative control throughout the assay.

Modification and Isolation of Platycodins. The reaction mixture containing 1% platycodin D in 500 μL of crude microbial enzymes was incubated at 37 °C for 120 h. After the reaction, the solution was extracted with the same volume of *n*-butanol. The *n*-butanol fraction was analyzed by TLC as described above. The obtained spot corresponding to novel product was scraped, and the scraped silica gel was re-extracted with methanol. The methanol solution was centrifuged, and the supernatant was filtered by Millex LCR Filter (0.45 μm, Millipore, Bedford, MA) and condensed to an appropriated concentration. The condensed sample was loaded on high-performance liquid chromatography (HPLC). HPLC was conducted with HP-1100 (Agilent, Foster City, CA) equipped with a reverse-phased column (Apollo C18, 5 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 Tandem 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 $NaNO_2$ 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 AmericanTtype 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 μL 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. HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid) buffer was prepared with the following composition: 135 mM NaCl, 11.9 mM $NaHCO_3$, 0.36 mM $NaH_2PO_4 \cdot H_2O$, 5 mM dextrose, 1.5 mM KCl, 0.98 mM $MgCl_2 \cdot 6H_2O$, 4.6 mM HEPES (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 500*g* for 5 min, and the plasma was then carefully removed by aspiration. The sedimented erythrocytes were then washed five times in the HEPES 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} cell /mL), respectively, suspended in HEPES 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 unlysed cells were then pelleted by 1650*g* centrifugation for 15 min. The A_{540} 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 A_{540} of the supernatant from 100 μL of erythrocytes suspended in 0.4 mL of distilled water.

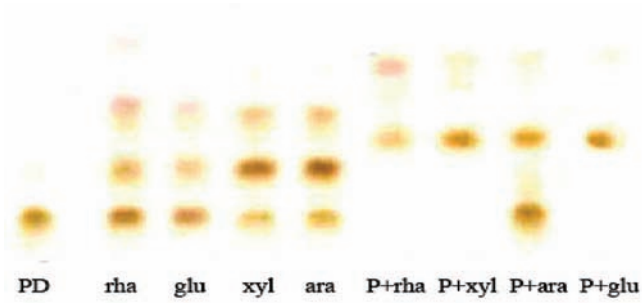

Figure 2. TLC profile of platycodin D modified by crude enzymes from *A. niger* incubated with various carbon sources (PD, platycodin D; rha, α-rhamnose; glu, β-glucose; xyl, xylose; ara, arabinose; P+rha, platycodin and α-rhamnose; P+xyl, platycodin and xylose; P+ara, platycodin and arabinose; P+glu, platycodin and β-glucose as carbon sources).

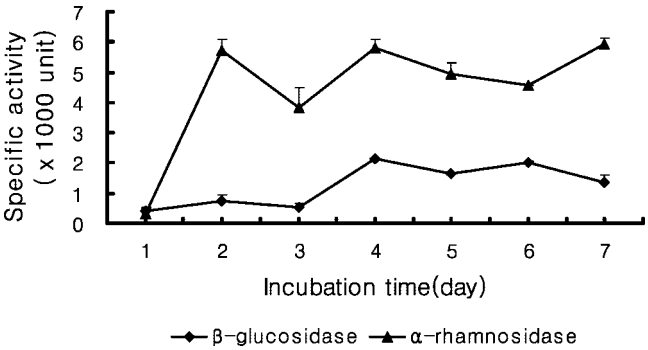

Figure 3. Specific activity of crude enzyme from *A. niger* at various incubation times. Each value is the average of triplicate measurements.

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 Platycodin Glycoside by a Crude Enzyme Extract of *A. niger*. This study investigated the transformation of platycodin 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 platycodin 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 playcodin 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 glycosidase 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 platycodin 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

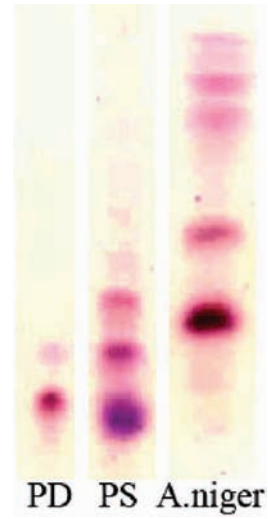

Figure 4. TLC profile of platycodin D modified by crude enzymes from *A. niger* (PD, platycodin D; PS, platycodin saponin mixtures; *A. niger*, platycodin D modified by the enzyme from *A.s niger*).

28-*O*-side chain and was located at much upper position in TLC (spot not shown) (**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 ($m/z = 827.3$), prosapongenin D moiety ($m/z = 681.3$), or even platycogigenin 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 implicates 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

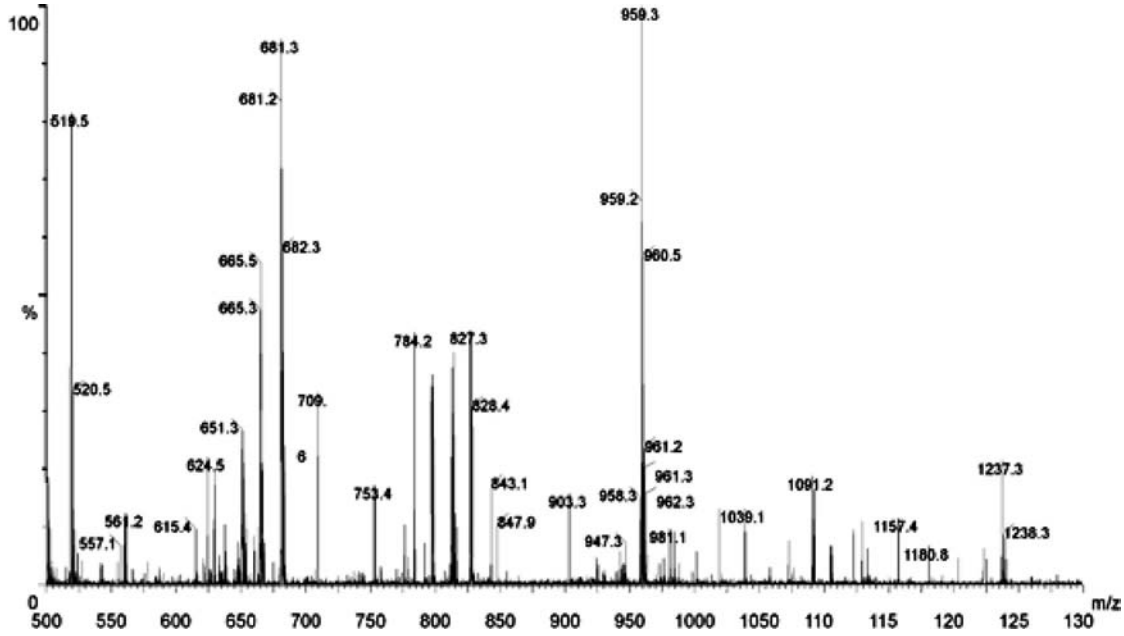

Figure 5. Mass spectrum of the main modified product of platycodin D. Sample was analyzed by direct injection on a triple-quadrupole tandem mass spectrometer.

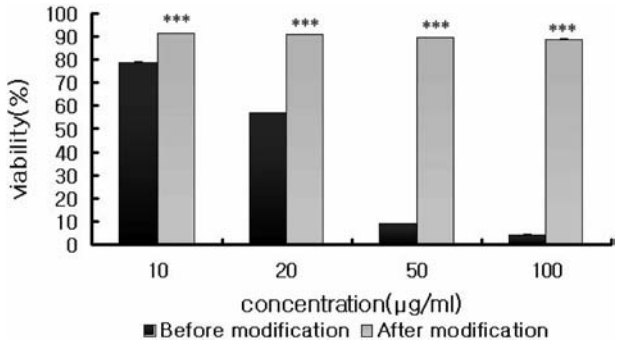

Figure 6. Cell viability treated by platycodin D before and after modification. Values are mean ± SD, significance of differences before and after modification was established at $p < 0.05$, expressed as $^{***}p < 0.001$.

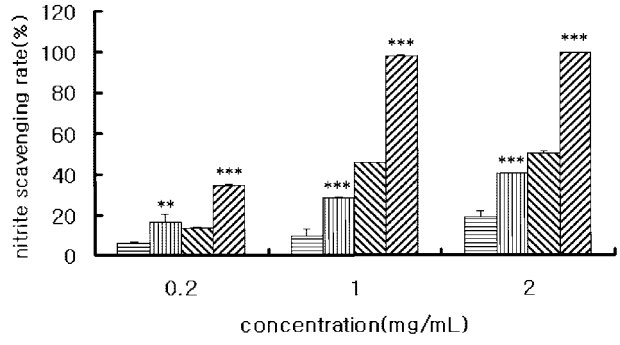

Figure 7. Nitrite-scavenging activity of platycodin D before and after modification at different pHs and concentrations. Values are mean ± SD. Significance of differences between before and after modification was established at $p < 0.05$, expressed as $^{**}p < 0.05$, $^{***}p < 0.001$.

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.

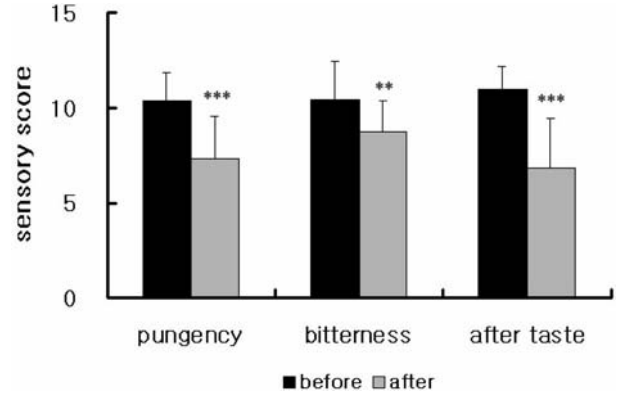

Figure 8. Sensory scores of platycodin D before and after modification with a 15 grade scale starting from 1 (very weak) to 15 (very strong). Significance of differences between before and after modification was established at $p < 0.05$, expressed as $^{**}p < 0.05$, $^{***}p < 0.001$.

DISCUSSION

Platycodin D belongs to the oleanane type of triterpenoid saponin with 3-*O*-glucose and 28-*O*-apiose-xylose-rhamnose-

arabinose sugar chains. The alteration of chemical and pharmacological properties of platycodin D through microbiological modification has been not reported previously. Prior to this study, several commercially available enzymes such as hesperidinase (including α-rhamnosidase) and β-xylosidase (both from *A. niger*, Sigma, St. Louis, MO) were screened, but none of them were effective in cleaving the glycoside covalent bond with selectivity. The current study demonstrates for the first time that the inner-glycosidic bond is selectively cleavable by crude enzymes prepared from several food microorganisms. The enzymes from *A. niger* showed the greatest activity for α-rhamnosidase and thus were able to partially digest the platycodin glycosidic bond. We further found that the presence of rhamnose and platycodins in the culture medium of *A. niger* could enhance the transforming 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*-glycoside 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.

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 were generated which was able to partially cleave platycodin D at rhamnose-xylose to eliminate of apiose-xylose. Such a structural novelty in platycodins is unavailable from the chemical modification.

Hemolysis is a common side effect occurring in most saponin compounds including platycodins. Hemolytic toxicity prevents platycodins from being administered intravenously. The LD_{50} (50% lethal dose) of orally administered platycodins in mouse and rat was 420 and 800 mg/kg, respectively, whereas the LD_{50} 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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