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Analysis of metabolites produced by enzymatic transformation of *Platycodon* saponins and their sensory evaluations

2019년 2월

서울대학교 대학원

약학과 천연물과학 전공

백 우 현
Abstract

Analysis of metabolites produced by enzymatic transformation of *Platycodon* saponins and their sensory evaluations

Woo Hyun Baek
Natural Products Science
College of Pharmacy
Master Course in the Graduate School
Seoul National University

*Platycodon grandiflorum* belongs to the Campanulaceae family. *Platycodon* saponins are the major and marker constituents of Platycodi radix (i.e., *Platycodon grandiflorum* root). *Platycodon* saponins were investigated to explore their hypolipidemic, cholesterol-lowering, and anti-obesity properties through a series of *in vivo* and *in vitro* experiments. Platycodi radix has been used as a food, as an herbal supplement, and as traditional oriental medicine in East Asia. However, *Platycodon* saponins have a bitter taste, which has had a negative impact on consumers' preferences. In order to improve the bitter taste for consumers when commercializing Platycodi radix as a health-functional food, it has either been processed with a food additive having a sweet taste or has had the content of the raw material extract decreased.
This study was conducted to improve the bitter taste by simplifying the side chains of saponins through enzymatic transformation. It was also found that the efficacy was improved by increasing platycodin D, the main ingredient of Platycodon saponins. In previous studies, we reported a method of efficiently producing platycodin D through reaction with an enzyme cellulase–enriched saponin fraction, that is, Viscozyme L (a food additive with cellulase activity), which cleaves the glycosidic bond at the C-3 position. Lecitase Ultra (a food additive with lipase activity), which was expected to cleave the ester bond of position C-28, was also used in the experiment. However, Lecitase Ultra did not cleave the ester bond of the sugar at that position. Based on the results of this study, both enzymes Lecitase Ultra and Viscozyme L commonly cleaved the glycosidic bond at the C-3 position of all saponins, resulting in the disappearance of about 18 saponins. Viscozyme L also cleaved the ester bond at the C-28 position of Platycodon saponins and then converted D-forms into deapiose forms of Platycodon saponins, while Lecitase Ultra did not show this capability.

The sensory evaluation showed that the bitterness of the Platycodon saponin decreased with the simplification of the side chain. The difference between the two enzymes was that with one of them, the apiose at the end of C-28 was cleaved to convert the saponins of D-forms into deapiose forms. When both enzymes were used, the bitter taste was improved, but when Viscozyme L was used alone, the pH was lowered, and the sour taste reappeared. Reviewing previous studies, we note that after treatment with platycodin D, platycodin D₃, and platycoside E 5 uM in mast cells treated with adipocytes, the fat suppression effect was significantly reduced. The results of Oil Red O-staining show that the activity of C-3 increases as the sugar of C-3 drops. This study sought to identify the structure–bitterness relationship of Platycodi radix by cleaving the glycosidic bond at C-3 via enzyme by using Lecitase Ultra and Viscozyme L.

**Keywords:** Platycodon grandiflorum; Platycodon saponins; Platycodin D; High-performance-liquid chromatography (HPLC); Enzymatic transformation; Lecitase Ultra; Viscozyme L; Sensory evaluation; Bitter taste

**Student Number:** 2017-25538
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I. Introduction
Platycodon grandiflorum is a perennial herb that belongs to the Campanulaceae family; the Platycodi radix is part of the root, used as an herbal medicine. It has been used to treat tonsillitis, sore throat, bronchitis, and other respiratory ailments in East Asian countries. (Its Chinese name is Jiegeng, its Korean name is Doraji, and its Japanese name is Kikyo.) Platycodon saponins, which are primary ingredients of Platycodi radix, have been reported to be effective antitumor, anti-allergy, anti-inflammatory, immune response augmentation, hyperlipidemia, and anti-obesity agents [1-7]. Platycodon saponins contain an oleanane backbone with two side chains, one a glucose unit attached through an ether linkage at C-3 of a triterpene and the other containing arabinose, rhamnose, and xylose in sequence, attached through an ester linkage between C-28 and arabinose [8-9]. Specifically, many pharmacological activities of platycodin D, the major component of Platycodon saponins, have been reported, including anti-inflammation, immunogenicity, anti-adipogenesis, a cholesterol-lowering effect, and apoptosis of several cancer cell lines [10-12]. Many previous studies have reported that the bioactivities of platycodin D are superior to those of other saponins [13-17]. But although platycodin D is a major component of Platycodon saponins, the total Platycodon saponin content in Platycodi radix is only about 2%.

Enzymatic transformation has been widely used to modify natural compounds or produce synthetic ones [18, 19]. It provides benefits such as mild stereospecific reaction, low cost, and easy reaction control [20]. In previous studies, we reported the first enzymatic transformation and hydrolytic pathway conversion of platycoside E and platycodin D3 to platycodin D. We have successfully achieved enzymatic conversion of Platycodon saponins to platycodin D within 24 h, utilizing cellulase from T. reesei [21].

A review of bioactivities based on the chemical structures was also conducted in previous studies. We examined the antiproliferation and apoptosis-inducing effects of platycodin D and two prosapogenins (de-C3-platycodin D and de-C28-platycodin D) on human cancer cell lines and determined their structure–activity relationships. The prosapogenins, which are forms of platycodin D that are deglycosylated at C-3 or C-28, showed reduced cytotoxicity compared to platycodin D. The antiproliferative effect of these compounds was in the order of platycodin D > de-C3-platycodin D > de-C28-platycodin D. Based on these results, the presence of a β-D-glucopyranose moiety at the C-3 position through an O-heterosidic linkage and of a β-
Dapiofuranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranose at the C-28 position through an ester linkage might be responsible for the cytotoxic effects of platycodin D [43]. In another previous study, we investigated the effect of platycodin D, platycodin D₂, platycoside E, and deapi-platycodin D on lipid accumulation in preadipocytes [44]. The inhibitory effect of lipid accumulation in 3T3-L1 cells was measured by Oil Red O-staining. The effect of inhibiting the adipocyte was significantly increased, as the number of sugars at the C-3 position of triterpene was simplified from three to two and one. On the other hand, when apiose, the sugar moiety at the end of C-28, was eliminated, the effect of inhibiting the adipocyte was remarkably decreased [44].

However, there are no studies on the relationship between chemical structure and taste and activity. In spite of its excellent pharmacological effect and efficacy, *Platycodon grandiflorum* cannot be used as a tea or as a health drink, for example in a green tea or orange juice. This study was conducted to solve the limitations of the taste of *Platycodon grandiflorum* and to increase consumers’ preference for it. The enzymes used in this study were Lecitase Ultra and Viscozyme L. Lecitase Ultra is a food-grade enzyme, as a chimera of Thermomyces lanuginosus lipase and Fusarium oxysporum lipase, which exhibits considerable phospholipase activity as well as lipase activity. Viscozyme L is a multi-enzyme complex containing a wide range of carbohydrases. It also acts on branched pectin-like substances found in plant cell walls. Structural changes made to *Platycodon* saponins using the enzymes Lecitase Ultra and Viscozyme L were analyzed by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI/MS) and high-performance liquid chromatography using the evaporative light scattering detector (HPLC-ELSD) method.

Based on the results of this study, both enzymes Lecitase Ultra and Viscozyme L commonly cleaved the glycosidic bond at the C-3 position of all saponins, resulting in the disappearance of about 18 saponins. Viscozyme L also cleaved the ester bond at the C-28 position of *Platycodon* saponins and then converted the D-form into the Deapi-form of *Platycodon* saponins, while Lecitase Ultra did not show this capability. Platycodin D, which deglycosylated at the C-3 or C-28 positions, showed reduced cytotoxicity against cancer cells compared to the *Platycodon* saponins, and the platycoside E and platycodin D₁ that deglycosylated at the C-3 position showed an increased anti-obesity effect.
We concluded that Lecitase Ultra, an enzyme that does not affect the side chain at the C-28 position and only selectively cuts at C-3, is efficient for producing platycodin D. We also confirmed that the bitter taste was attenuated by the transformation of Platycodon saponins. And a hydrolysis pathway was established in terms of the metabolites produced by enzymatic transformation.
<table>
<thead>
<tr>
<th>Name</th>
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<th>R₂</th>
<th>R₃</th>
<th>Reference</th>
</tr>
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<td>_Ara²-Rha⁴-Xyl</td>
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<tr>
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<td>_Ara²-Rha⁴-Xyl⁴-Api</td>
<td>38</td>
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<tr>
<td>polygalacin D₂</td>
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<td>CH₃</td>
<td>_Ara²-Rha⁴-Xyl⁴-Api</td>
<td>38</td>
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<tr>
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<td>CH₃</td>
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-continued-
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<th>Name</th>
<th>Sugar 1</th>
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<th>Sugar 3</th>
<th>Sugar 4</th>
<th>Sugar 5</th>
<th>Acid</th>
<th>Rha 1</th>
<th>Rha 2</th>
<th>Ara 2</th>
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<td>39</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>polygalacin D₂</td>
<td>Glc₂-Glc</td>
<td>CH₃</td>
<td>_Ara₂-Rha₄*Xyl₃-Api</td>
<td>38</td>
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<tr>
<td>3&quot;-O-acetylplatycodin D₂</td>
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<td>CH₂OH</td>
<td>_Ara₂-Rha(3-O-Ac)₄*Xyl₃-Api</td>
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</tr>
<tr>
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<td>CH₃</td>
<td>_Ara₂-Rha₄*Xyl₃-Api</td>
<td>36</td>
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<td>Glc₂-Glc</td>
<td>CH₂OH</td>
<td>_Ara₂-Rha(3-O-Ac)₄*Xyl₂²-Api</td>
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<tr>
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<td>-Glc</td>
<td>COOH</td>
<td>_Ara₂-Rha(2-O-Ac)₄*Xyl₃-Api</td>
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</tr>
<tr>
<td>2&quot;-O-acetylplatycodin D</td>
<td>-Glc</td>
<td>CH₂OH</td>
<td>_Ara₂-Rha(2-O-Ac)₄*Xyl₃-Api</td>
<td>39</td>
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<tr>
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<td>CH₂OH</td>
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</tbody>
</table>

Figure 1. The chemical structure of the Platycodon saponins and prosapogenins (PRSs) used in the present study.

Ⅱ. Materials and Methods
1. Enzymatic transformation of *Platycodon* saponins

Several studied have reported that the removal of the glycosyl group in saponin affects the biological activity of the compound [13-15]. For example, the activity of ginsenoside increases as the number of the sugar moieties [16-17]. In contrast, the cytotoxicity of platycosides is reduced by the cleavage of glycosides [18]. Thus, it is necessary to investigate the biological activity of saponins considering the formation of sugar groups to achieve a better understanding of structure-activity relationships. Many methods for the deglycosylation of natural products have been reported, for example, heating [19], acid treatment [20], fermentation, cell reaction, and enzyme conversion [21]. Among these methods, biotransformation using enzymes is encouraged, as highly selective compounds can be obtained following the reaction [22-24]. In the present study, the enzymatic transformation of glycoside moiety was applied to improve the bitter taste of PG and confirm structural-biological activity.

1.1 Chemicals and reagent

The enzymes used in this study are given in Table 1. In addition to their origin and color reported activities for each enzyme are also listed. All enzymes were used without any further purification. Acetonitrile, methanol (HPLC grade) were purchased from J. T. Baker (Phillipsburg, NJ, USA). Distilled water (NANO pure Diamond, Barnstead, USA) was used to prepare all solutions and dilutions. Diaion-HP20 (polystyrene adsorption resin) was purchased from Mitsubishi Chemical (Tokyo, Japan). The Platycodi radix plant was provided from Royalgreens Company in Seongnam, Korea. The pure authentic samples of six *Platycodon* saponins were isolated from the aqueous extract of the raw material as described previously [33, 41].
1.2 Sample preparation

1.2.1 Preparation of the saponin-enriched fraction

Saponins from Platycodi radix (1 kg) were extracted with methanol by refluxing for 3 h, which was repeated three times. The extracts were filtered on filter paper (Advantec, Tokyo, 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 Diaions-HP20 open column (100 cm_10 cm; the volume of the column was 10 L) and eluted with a stepwise gradient in the sequence of water/methanol at 100:0, 90:10, 80:20, 70:30, 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 concentrated to residue under reduced vacuum and stored in a desiccator until further use.
Table 1. List of enzymes and activities used in this study.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Product name</th>
<th>Color</th>
<th>Physical form</th>
<th>Side activities</th>
<th>Company (location)</th>
<th>Enzyme activity (supplier units)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus niger</em></td>
<td>Lipase from <em>Aspergillus niger</em></td>
<td>White</td>
<td>Powder</td>
<td></td>
<td>Sigma Aldrich St. Louis, USA.</td>
<td>165.4 U/g</td>
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<tr>
<td><em>Aspergillus oryae</em></td>
<td>Lecitase Ultra</td>
<td>Yellow to right brown</td>
<td>Liquid</td>
<td></td>
<td>Novozyme Co., Denmark.</td>
<td>10 KLU*</td>
</tr>
<tr>
<td><em>Aspergillus aculeatus</em></td>
<td>Viscozyme L.</td>
<td>Brown</td>
<td>Liquid</td>
<td>Xylanase, Cellulase, Hemicellulase</td>
<td>Novozyme Co., Denmark.</td>
<td>100 FBG*</td>
</tr>
</tbody>
</table>

* Enzyme activity as defined by the specific company: KLU, Kilo lipase units; FBG, Fungal Beta-Glucanase Units.
1.2.2 Preparation of the hot water extract for sensory evaluation
The dried and chipped roots of *Platycodon grandiflorum* (1 kg) were mixed with drinking water. The mixture was heated at 90 °C for 5 hours for extraction. The extracts were passed through a filter paper. The extraction solutions were concentrated into residue under reduced vacuum. Thus, 200 g of a concentrate having a sugar content of 60 brix was obtained as a hot-water extract.

1.3 HPLC-ELSD 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 Hitachi AS-2000 auto injector. All separations were carried out on a INNO Column C18 column (150mm × 4.6mm ID., 5 μm particle size) from YoungJinBiochrom Co., (Seongnal, Korea). The analysis of saponins was performed by HPLC-ELSD. HPLC conditions were as follow: eluent A, water containing 0.1% formic acid; eluent B, acetonitrile containing 0.1% formic acid; gradient, 0-5 min (20-22% B), 5-15 min (22-22% B), 15-45 min (22-31% B), 45-50 min (31-100% B), washing with 100% B, and then equilibrated with 20% B for 10 min at a flow rate of 0.9 mL/min. The ELSD system was operated with the following settings: a probe temperature of 70 °C, a gain of 12, and a nebulizer N₂ gas pressure of 2.5 bar.
1.4 Optimal enzyme concentration for enzymatic transformation

1.4.1 Enzymatic transformation of the saponin-enriched fraction

The amount of enzyme was optimized for the complete conversion of platycoside E and platycodin D₃ to platycodin D by evaluating different Lecitase Ultra amounts, 0 (control), 0.11, 0.22, and 0.33 KLU; Viscozyme L was performed by optimized amounts as previously described [33].

Each enzyme amount was mixed with the saponin-enriched fraction (5 mg) in 1 mL distilled water, pH 5.5. The mixtures were incubated at 55°C for 24 h and subsequently heated in a water bath at 80°C for five minutes to terminate the reaction. A Waters Sep-Paks Vac C18 (Ireland) cartridge was used to remove polar compounds and byproducts, and a Whatman syringe filter (13 mm, 0.45 mm) was used to clean the sample prior to HPLC analysis.

1.4.2 Enzymatic transformation of the hot water extract

Enzyme optimization condition setting was conducted for sensory evaluation. 5 g of hot-water extract was diluted with 25 mL of drinking water. The extract was mixed with different enzyme amounts to improve the bitter taste. Lecitase Ultra amounts, 0 (control), 0.44, 0.66, 0.88 and 0.11 KLU; Viscozyme L amounts, 0 (control), 57.6, 86.4, 115.2 and 144 FBG. The mixtures were incubated at 55°C for 24 h. The reaction mixtures were subsequently heated in a water bath at 80°C for five minutes to terminate the reaction.
2. Preparative HPLC for isolation

The HPLC for the isolation of *Platycodon* saponins was performed using a preparative HPLC system consisting of a Hitachi L-6200 intelligent pump equipped with a Sedex 75 evaporative light scattering detection (ELSD). Separation was performed on a preparative column YMC-Triart C18 (YMC Co., Ltd., Kyoto, Japan; 250mm × 10.0 mm I.D., S-5 μm, 12 nm). HPLC conditions were as follow: eluent A, water containing 0.1% formic acid; eluent B, acetonitrile containing 0.1% formic acid; gradient, 0-10 min (18-21% B), 10-25 min (21-21% B), 25-30 min (21-24% B), 30-45 min (24-25% B), 45-50 min (25-100% B), washing with 100% B, and then equilibrated with 18% B for 20 min at a flow rate of 3 mL/min. The ELSD system was operated with the following settings: a probe temperature of 70 °C, a gain of 6, and a nebulizer N₂ gas pressure of 2.5 bar. 200 the sample solution was injected into the separation column.

3. HPLC-ESI/MS

The ESI-QTOF-MS was performed with an Agilent 1260 series HPLC equipped with a binary pump (Agilent Technologies, 1290 Infinity) and coupled to an Accurate-Mass Q-TOF LC/MS (Agilent Technologies, 6530). The samples were eluted with gradient, eluent A, water containing 0.1% formic acid; eluent B, acetonitrile containing 0.1% formic acid; gradient, 0-10 min (15-20% B), 10-46 min (20-28% B), 46-50 min (28-100% B), washing with 100% B, and then equilibrated with 15% B for 5 min at a flow rate of 0.3 mL/min. All of the mass data were obtained in negative ion mode [M - H]- with an ESI source and the mass range set to m/z 100–2000. The conditions of the ESI source were as follows: capillary voltage, 4000 V; nebulizing gas (N₂) pressure, 30 psig; drying gas (N₂) flow rate, 10.0 L/min; drying gas temperature, 350 °C; spectrarate, 1.03 Hz; sheath gas temperature, 350 °C; sheath gas flow 12 L/min.
Ⅲ. Results
1. Enzymatic transformation of the saponin-enriched fraction and HPLC-ESLD analysis

After enzymatic transformation to platycodin D via Lecitase Ultra was successfully performed, an 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 0.11–0.33 U of Lecitase Ultra, and the enzymatic modification of the *Platycodon* saponins was assessed by comparing the peaks on the HPLC chromatograms between different enzyme concentrations. The results from four 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 that were incubated with 0.11 KLU of Lecitase Ultra showed a remarkable decrease in the amounts of platycoside E and platycodin D3 and an increase in platycodin D. However, some platycodin D3 still remained (Fig. 2B). When the saponin-enriched fractions were incubated with Lecitase Ultra at 0.22 and 0.33 U, respectively, there were no peaks in the dotted square on the chromatogram of the HPLC-ELSD (Fig. 2) corresponding to deapi-platycoside E (1), platycoside E (2), platycodin D3 (3), or platycodin D (4). There was no remaining platycoside E or platycodin D3, and the resulting product contained significantly greater amounts of platycodin D (Figs. 2C and 2D).

According to these results, the minimal amount of Lecitase Ultra required for complete conversion of the saponin-enriched fraction of 5 mg is 0.33 KLU. The representative HPLC chromatograms for the enzyme-treated product and the remaining residues are shown in Figs. 4B and 4C. As a control, the saponin-enriched fraction was also incubated under the same conditions for 24 h without the enzymes, to determine whether additional factors affected the conversion. There was no change in the *Platycodon* saponins content in the control experiment.
2. Enzymatic transformation of the hot water extract and HPLC-ESLD analysis

After the enzymatic transformation to platycodin D by Lecitase Ultra was successfully performed, optimization of the enzyme amount was carried out to sensually improve the bitter taste. About 5 g of the hot water extract was mixed with 4.4–11 KLU of Lecitase Ultra, and the enzymatic modification of the Platycodon saponins was assessed by comparing the taste and the HPLC chromatogram peaks for each of the different enzyme concentrations. To evaluate the taste, test subjects were given the same amount to drink from each sample. The samples incubated with 0, 4.4, and 6.6 KLU of Lecitase showed little difference in bitter taste. In contrast, an improvement could be sensed in the bitter taste when the sample was treated with 8.8 and 11 KLU. The samples incubated with 4.4 and 6.6 KLU of Lecitase Ultra showed only a slight decrease in the amounts of platycoside E and platycoside D3 and an increase in platycodin D (Figs 3A, 3B, and 3C, respectively). These samples had a strong bitter taste. When the hot water extract was incubated with Lecitase Ultra at 8.8 and 11 KLU, there was no remaining deapi-plantycoside E, platycoside E, or platycodin D3, and the resulting product contained significantly greater amounts of platycodin D and demonstrated improvement in bitterness (Fig. 3E). According to these results, it is possible to form a hypothesis about the relationship between a bitter taste and high-polar Platycodon saponins, such as platycoside E, platycodin D3, and deapi-plantycoside E. These high-polar saponins have three sugar moieties at C-3.
Figure 2. HPLC chromatograms of the saponin-enriched fraction from Platycodi radix after enzymatic transformation by various amounts of Lecitase Ultra: (A) control; (B) 0.11 KLU; (C) 0.22 KLU; (D) 0.33 KLU. Column: INNO Column C18 column (150 mm × 4.6 mm ID., 5 μm), Detector: ELSD.

1: deapi-platycoside E; 2: platycoside E; 3: platycodin D$_3$; 4: platycodin D.
Figure 3. HPLC chromatograms of the hot water extract from Platycodi radix after enzymatic transformation by various amounts of Lecitase Ultra: (A) control, (B) 0.11 KLU (C) 0.22 KLU (D) 0.33 KLU. Column: INNO Column C18 column (150mm × 4.6mm ID., 5 μm), Detector: ELSD.

1: deapi-platyicoside E; 2: platyicoside E; 3: platycodin D₃; 4: platycodin D.
Figure 4. (A) Representative HPLC chromatogram of the saponin-enriched fraction from Platycodi radix obtained by a Diaion-HP20 column with 100% methanol elution (without enzymatic reaction); (B) HPLC chromatogram of enzymatically modified saponin-enriched fraction from Platycodi radix by Lecitase Ultra; (C) HPLC chromatogram of enzymatically modified saponin-enriched fraction from Platycodi by Viscozyme L.
3. Preparative liquid chromatogram of saponin-enriched fraction and enzymatic transformation

In order to confirm the enzymatic hydrolysis pathway of the *Platycodon* saponins, Prep-LC was performed to obtain pure compounds (Fig. 5A), and then the enzyme was treated as a single component. The peak 1 fraction is deapi-platycoside E, the peak 2 fraction is platycoside E, the peak 3 fraction is deapi-platycodin D₃, and the peak 4 fraction is platycodin D₃ (Figs. 5B, 5C, 5D, and 5E). When these fractions reacted with Lecitase Ultra for 24 h, the results were as follows. Peak 1 is an unknown compound (A), peak 2 showed a molecular weight of 1006.4999, corresponding to β-Gentiobiosyl-platycodigenin (A), peak 3 showed a molecular weight of 1092.5373, corresponding to deapi-platycodin D (A) and (C), and peak 4 showed a molecular weight of 1224.5776, corresponding to platycodin D (B) and (D) (Figs. 6A, 6B, 6C, and 6D).
Figure 5. (A) Representative Prep-LC chromatogram of the saponin-enriched fraction; (B) HPLC chromatogram after separation of fraction 1; (C) after separation of fraction 2; (D) after separation of fraction 3; and (E) after separation of fraction 4.

The peak 1 fraction is deapi-platycoside E (B); the peak 2 fraction is platycoside E (C); the peak 3 fraction is deapi-platycodin D$_3$ (D); and the peak 4 fraction is platycodin D$_3$ (E).
Figure 6. HPLC chromatogram of separated fractions (Fig. 5) after reaction with Lecitase Ultra separated fraction. (A) HPLC chromatogram of deapi-platycoside E after reaction with Lecitase Ultra; (B) HPLC chromatogram of platycoside E after reaction with Lecitase Ultra; (C) HPLC chromatogram of deapi-platycodin D₃ after reaction with Lecitase Ultra; (D) HPLC chromatogram of platycodin D₃ after reaction with Lecitase Ultra.

1: unknown (A); 2: β-Gentiotriosyl-platycodigenin (A); 3: deapi-platycodin D (A) and (C); 4: platycodin D (B) and (D).
4. ESI-MS Analysis of the changes in the *Platycodon* saponins by enzymatic transformation

Extraction of ion chromatograms (EICs) were performed for specified $m/z$ ranges, based on the molecular weight of the compounds of interest. Table 2 shows the molecular weight of the *Platycodon* saponins from *Platycodon grandiflorum*. EIC scan analysis was performed to confirm changes to the contents as the *Platycodon* saponins were treated with Lecitase Ultra in a 24 h incubation. The enzymes Lecitase Ultra and Viscozyme L were treated with the saponin-enriched fraction from *Platycodon grandiflorum* for 24 h. The EIC analysis showed that the signal for the molecular weight of 1547.1000 to 1548.1000, corresponding to platycoside E, was generally found to be 0 in the enzyme-treated samples (Figs. 7A, 7B, and 7C). The signal for the molecular weight of 1223.1000 to 1224.1000, corresponding to platycodin D, also generally increased in the enzyme-treated samples (Figs. 8A, 8B, and 8C). Fig. 9, unlike the common activities of the two enzymes as shown in Figs. 7 and 8, shows the difference in the activity of the two enzymes. The signal for the molecular weight of 1091.1000 to 1092.1000, corresponding to deapiplatycodin D in the Viscozyme L-treated sample, dramatically increased like enzymes with the most glucosidase activity. However, interestingly, when the sample was treated with Lecitase Ultra, there was little change compared to the control (Figs. 9A, 9B, and 9C).

The results suggest that deapiplatycodin D and platycodin D are generally produced by Lecitase Ultra and Viscozyme L cleaving the terminal glucosyl or gentiobiosyl moieties of the linkages at C-3 of the saponins (Fig. 10). But Lecitase Ultra did not deglycosylate at C-28.

Fig. 11 shows the negative total ion chromatogram of the *Platycodon* saponins within 35 min. According to the observed molecular weight, LC-ESI/MS was conducted to obtain more information about the chemical structures. There was a significant increase in the deapiplatycodin D, platycodin D, 3”-O-acetylplatycodin D, and polygalacin D content. In contrast, during the 24 h incubation, the content decreased for many *Platycodon* saponins containing minor components, such as deapiplatycoside E, platycoside E deapiplatycodin D$_3$, platycodin
D$_3$, polygalacin D$_3$, deapi-polygalacin D$_3$, deapi-2-O-acetyl platycodin D$_2$, platycodin D$_2$, polygalacin D$_2$, 3"-O-acetylplatycodin D$_2$, and dextyl-2-O-acetyl-polygalacin D$_3$.

This result indicates that Lecitase Ultra cleaves the β1-6 glucose linkages of platycoside E, platycodin D$_2$, and platycodin D$_3$ to produce platycodin D, as well as the linkages of deapi-platycoside E and deapi-platycodin D$_3$. The difference between Lecitase Ultra and Viscozyme is that Lecitase Ultra did not cleave the ester bond at C-28 of the Platycodon saponins. In contrast, Viscozyme L can convert the D-form of platycodon saponins to the Deapi-form by cleaving the ester bond at C-28 (Fig. 12). These results were confirmed by treating both Viscozyme L and Lecitase Ultra with a standard compound, such as platycodin D and polygalacin D, compounds previously isolated [21] by mass spectrometry analysis (Figs. 14A, 14B, and 14C). The fragment ions observed in the negative ions were used for identification of individual Platycodon saponins from the enzymatically transformed product. The fragment ions were labeled according to the nomenclature of Domon and Costello (ref. Domon and Costello.) Fig. 13 shows the nomenclature of platycodin D, a representative compound.

Fig. 14 shows the standard compound, platycodin D, which was isolated in previous studies [33, 41-42]. The standard compounds platycodin D and polygalacin D reacted with the enzymes Lecitase Ultra and Viscozyme L for three days. When Lecitase Ultra L reacted with the D-forms of Platycodon saponins, having one glucose moiety at the C-3 position, for example platycodin D or polygalacin, there was no change at all in the HPLC chromatogram. In contrast, when Viscozyme L reacted with D-forms of Platycodon saponins having one glucose moiety at the C-3 position, for example platycodin D and polygalacin, it could be confirmed using HPLC-ELSD that several peaks were generated (Figs. 14 and 15).

These results mean that Lecitase Ultra does not affect the sugar moieties at the C-28 position. In contrast, Viscozyme L cleaves the sugar moieties at C-28 of triterpene (Figs. 14 and 15).
Table 2. The molecular weight for major *Platycodon* sapnins [29-31].

<table>
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<th>No.</th>
<th>Name</th>
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<td>1</td>
<td>deapi-platycoside E</td>
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<td>polygalacin D₃</td>
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Figure 7. ESI-EIC (1547.1000-1548.1000) of platycoside E, Scan Frag 180.0 V.

(A) saponin-enriched fraction from Platycodi radix (without enzymatic reaction); (B) after enzyme reaction with Lecitase Ultra; (C) after enzyme reaction with Viscozyme L.
Figure 8. ESI-EIC (1223.1000-1224.1000) of platycodin D, Scan Frag 180.0 V.
(A) saponin-enriched fraction from Platycodi radix (without enzymatic reaction); (B) after enzyme reaction with Lecitase Ultra; (C) after enzyme reaction with Viscozyme L.
Figure 9. ESI-EIC (1091.1000-1092.1000) of deapi-platycodin D, Scan Frag 180.0 V.
(A) saponin-enriched fraction from Platycodi radix (without enzymatic reaction); 
(B) after enzyme reaction with Lecitase Ultra; (C) after enzyme reaction with Viscozyme L.
Figure 10. Proposed enzymatic-transformation pathway of the Platycodon saponins by Lecitase Ultra and Viscozyme L.
Figure 11. Representative LC-MS chromatograms of total ion scan in negative mode. (A) saponin-enriched fraction from Platycodi radix (without enzymatic reaction); (B) after enzyme reaction with Lecitase Ultra; (C) after enzyme reaction with Viscozyme L.
Figure 12. Proposed enzymatic-transformation pathway of platycodin D and polygalacin D by Viscozyme L.
Figure 13. Fragment ions of platycodin D, which labeled as described by Domon and Costello [45].
Figure 14. Comparison of HPLC chromatograms of platycodin D treated with Lecitase Ultra and Viscozyme L for 3 days. (A) standard compound, platycodin D (without enzymatic reaction); (B) platycodin D after enzyme reaction with Lecitase Ultra; (C) platycodin D after enzyme reaction with Viscozyme L.

Peak 1 showed a molecular weight of 1224.5, corresponding to platycodin D (A) and (B); peaks 2 and 4 were unknown compounds, of molecular weights 1006.5031 and 1106.5157 (C); peak 3 showed a molecular weight of 1092.5370, corresponding to deapi-platycodin D.
Figure 15. Comparison of HPLC chromatograms of polygalacin D treated with Lecitase Ultra and Viscozyme L for 3 days. (A) standard compound, platycodin D (without enzymatic reaction); (B) polygalacin D after enzyme reaction with Lecitase Ultra; (C) polygalacin D after enzyme reaction with Viscozyme L.
5. Sensory evaluation results of hot water extract from Platycodi radix and enzymatic transformation

The sensory evaluation of the bitter taste of the hot water extracts was performed by two groups. One group consisted of 56 people of various ages. Sample A was a Platycodi radix hot water extract, and sample B was a Platycodi radix hot water extract that had reacted with an enzyme. In the sensory evaluation, 66% of the 56 panel respondents said that the bitter taste of B was improved more than that of A. Among the respondents, 75% of those in their teens, 81% of those in their twenties, 66% of those in their thirties, 55% of those in their forties, 54% of those in their fifties, and 100% of those in their sixties replied that the bitterness had improved (Table 3.).

The other sensory evaluation was performed on a panel trained at the Food Environment Research Center. The panel consisted of ten women aged 40 to 50, who had been recruited by the Food and Environmental Research Center, Inc. They were educated about the evaluation method before the sensory test. The sensory test method used was a 9-point scoring method. A score of 1 meant “very weak,” a score of 5 meant “neither strong nor weak,” and 9 points meant “very strong.” During the inspection, communication between the panel members was prohibited. The objective evaluation was made, and the sample was evaluated for a sufficient time. The evaluation of the sample treated with Lecitase Ultra for 24 hours resulted in 3.8 points, and the bitter taste was evaluated to be improved over the sample without the enzyme, which was given 4.9 points (Table 4).
Table 3. Comparison of taste for Lecitase Ultra treatment of hot water extract from Platycodi radix.

A: Platycodi radix hot water extract.
B: Platycodi radix hot water extract after reaction with Lecitase Ultra.
Table 4. Sensory evaluation results of enzymatically treated samples from the Food Environmental Research Center.

<table>
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<tr>
<th>Characteristic</th>
<th>Sample 1</th>
<th>Sample 2</th>
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<td>Sweet taste</td>
<td>4.00±0.67&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.40±0.97&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bitter taste</td>
<td>4.90±0.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.80±0.92&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acrid taste</td>
<td>5.60±0.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.90±1.52&lt;sup&gt;b&lt;/sup&gt;</td>
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Significant probability: There is a significant difference when the probability p is less than 0.05.

Duncan’s post-test was performed after a one-way ANOVA, using the PASW Statistics 18 program, with a confidence level of 95% (p < 0.05).

1. Extract of Platycodi radix

2. Extract of Platycodi radix after reaction with Lecitase Ultra
Ⅳ Discussion
Platycodi radix has been conventionally used in traditional oriental medicine as well as being consumed as food. Saponins in Platycodi radix are major active components and have diverse pharmacological activities. Among these saponins, platycodin D is superior to others. However, the total content of saponins is only about 2% of the dry weight of Platycodi radix.

Another issue is that *Platycodon* saponins have a strong bitter taste. To improve this bitter taste and improve consumer preferences when commercializing Platycodi radix as a health-functional food, it has been processed with a sweet-tasting food additive or by lowering the content of the raw material extract.

This study prioritized how to increase the amount of platycodin D in Platycodi radix more than in previous methods. One previous study reported that platycodin D had been modified by a crude enzyme extract from *Aspergillus niger*, hesperidinase, and xylosidase. It also reported that this extract was effective and active in cleaving the glycosidic bond of platycodin D.

The enzymatic method has many advantages, such as high specificity, mild reaction conditions, and clean production processes. Earlier, we reported that liquid cellulose was the optimum enzyme to selectively transform platycoside E and platycodin D₃ to platycodin D and that it could similarly transform to those deapiose forms.

Lecitase Ultra, similar to cellulose, can hydrolyze one or two glucose moieties from the three glucose moieties at the C-3 of triterpene. Platycoside E, having three glucose moieties at the C-3 position, is deglycosylated to platycodin D₃ or platycodin D by Lecitase Ultra and cellulose. Also, platycodin D₁, having two glucose moieties at the C-3 position, is deglycosylated to platycodin D by Lecitase Ultra and cellulose. Cellulose can also hydrolyze xylose, present in a sugar moiety composed of arabinose, rhamnose, and xylose at the C-28 position [33]. But unlike Viscozyme L, Lecitase Ultra only focuses on changes of the C-3 sugar moieties of *Platycodon* saponins. In other words, Lecitase Ultra can hydrolyze more selectively than cellulose. Based on the results, this method is a more efficient enzymatic transformation method for converting platycodin D from other polarity saponins. The present study demonstrated for the first time the structural modification of platycoside E, platycodin D₂, platycodin D₃ and also acetylated *Platycodon* saponins by Lecitase Ultra.
With respect to taste, the bitterness and irritating taste were significantly improved, depending on the simplified side chain at the C-3 position. This finding may provide an approach to modifying saponin glycosides to improve their bioactivity and bioavailability as well as the bitter taste of the natural products.
V Conclusion
This study demonstrates an approach developed to use enzymatic transformation to increase the content of the bioactive compounds originally contained in crude extracts from Platycodi radix. Lecitase Ultra originating from *Aspergillus oryzae* was used to selectively transform saponins having three glucose moieties at the C-3 position to saponins having one glucose moiety at that position, for example D-forms such as polygalacin D and platycodin D, resulting in the disappearance of about 18 saponins. More importantly, Lecitase Ultra does not cleave the ester bond at the C-28 position of *Platycodon* saponins; in other words, it does not convert D-forms into deapiose forms of *Platycodon* saponins. However, Viscozyme L (cellulase) does demonstrate this activity. Platycodin D, one of the superior saponins, increased by almost 2.5 times compared with the crude extract. The standard compounds of *Platycodon* saponins separated by Prep-LC and countercurrent chromatography also underwent ESI-MS analysis. Through these procedures, the hydrolysis pathway of *Platycodon* saponins by enzymatic transformation was further clarified. As seen in the results of this study, both enzymes Lecitase Ultra and Viscozyme L commonly cleaved the glycosidic bond at the C-3 position of all saponins, resulting in the disappearance of about 18 saponins.

In the relationship between structure and activities, platycodin D that deglycosylated at C-3 or C-28 showed reduced cytotoxicity against cancer cells compared to the *Platycodon* saponins.

Platycodin D converted from platycoside E and platycodin D3 that deglycosylated at C-3 showed an increased anti-obesity effect. We concluded that Lecitase Ultra, an enzyme that does not affect the side chain at C-28 and only cleaves at C-3, is efficient in producing platycodin D.

During the sensory evaluation by 56 people, 66% of the respondents said that the bitter taste of the sample was improved after the reaction with Lecitase Ultra more than the crude extract of Platycodi radix. A second sensory evaluation was performed by panels trained at the Food Environment Research Center. These results also suggested that the bitter taste was improved by the transformation of the *Platycodon* saponins.
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국문초록

백 우 현

서울대학교 약학대학원
약학과 천연물과학전공

길경은 초롱꽃과(Campanulaceae)의 식물인 도라지(Platycodon grandiflorum A. DC.)의 뿌리로 거담, 기침, 기관지염, 인후염 등의 치료제로 동양의학에서 전통적으로 사용되어 왔다. 길경의 주요 약효성분은 올레아난 계열의 트리테르펜 사포닌으로 전체의 2%정도 함유되어 있고, 지금까지 30여종 이상이 알려져 있다. 사포닌의 약리효과로는 면역증강작용, 항염증, 항당뇨 및 항비만, 소화효소분비억제, 콜레스테롤 억제 등이 있다. 길경 사포닌 중 다양한 생물활성을 보이고 약물학적 가치가 높은 플라티코딘 D는 길경에 함유된 야이 미량이므로 상업적 이용이 어려웠다. 따라서 본 연구실의 이전연구로, 플라티코دين D를 당분해효소 셀룰라제를
첨가하여 고함량의 플라티코딘 D 강화분획물로 전환한 효소변환기술을 개발하였다. 그러나 길경 추출물의 셜룰라제를 통한 효소변환물의 맛을 보았을 때, 신맛과 불쾌한 향을 유발하여 기능성식품 등의 상업적 개발의 한계성을 가지고 있음을 발견하였다. 뿐만 아니라, 비록 플라티코시드 E와 플라티코딘 D₃를 모두 플라티코딘 D로 전환시키는 특이적인 활성을 갖고 있으나, 동시에 플라티코딘 D와 폴리갈라산 D를 포함한 길경사포닌의 28번 탄소 말단에 위치하는 아피오스를 절단하며 지방축적억제효과, 암 증식 억제효과 활성을 급격히 하락시키는 단점 또한 가지고 있다. 본 연구에서는 이러한 문제점을 보완할 수 있는 Aspergillus oryae로부터 유래한 레시타제 울트라를 이용하여 더욱 발전된 효소변환기술을 개발하였다. 이전 연구에서 고속향류크로마토그래피를 통해 분리한 순도 98% 플라티코딘 D와 폴리갈라산 D에 Lecitase Ultra를 효소반응시킴으로써, 28번 탄소에 위치한 에스테르 결합은 그대로 보존하며 3번 탄소에 위치한 글루코오스의 글리코시드결합만을 선택적으로 절단된다는 사실을 고성능 액체 크로마토그래피 및 질량분석기를 통해 확인할 수 있었다. 즉, 셜룰라제보다 길경사포닌들의 화학적인 변형을 더욱
선택적으로 유도할 수 있는 레시타제 울트라를 이용하여 플라디코딘 D의 함량을 안정적이고 효율적으로 증가시킬 수 있음을 확인하였다. 이러한 연구 과정에서, 극성이 높고 유사한 구조를 가지고 있는 사포닌들의 가수분해 전환경로를 고성능 액체 크로마토그래피 및 질량분석기를 이용하여 규명하였다.

한편, 도라지의 맛은 구조적인 식감과 향 이외에 아린맛, 쓴맛 및 단맛이 복합적으로 포함되어 있다. 오래된 도라지는 약효가 좋으나, 목과 혀에 통증처럼 아린 맛을 주기 때문에, 녹차, 굴피차 등과 같이 차나 건강음료로서 이용되지 못하고 있다. 본 연구는 앞서 언급하였던 셀룰라제를 이용한 효소전환물의 한계성 보완하는 동시에 도라지의 아린맛, 쓴맛의 개선에 대한 연구를 진행하였다.

도라지청과 도라지청에 레시타제 울트라와 적절히 반응한 효소전환물을 시료로 하여 맛을 비교하였다. 10대에서 60대의 다양한 연령층으로 구성된 56명의 일반인들을 대상으로 관능평가를 실시한 결과, 66%의 피험자가 효소전환물이 도라지청과 비교하여 상대적으로 쓴맛이 개선되었다고 응답하였다. 또한 식품환경연구센터에 의뢰하여 숙련된 패널들을 통해 쓴맛이
개선되었다는 검사결과를 얻었다. 레시타제 올트라의 3번 탄소에 위치한 당사슬의 단순화에 따른 맛의 개선되었다는 결론을 얻을 수 있었다.

결론적으로, 이러한 연구결과를 토대로 셀룰라제의 단점을 보완하는 레시타제 올트라를 이용하여 길경의 주요 사포닌인 플라티코딘 D를 효율적으로 증가시킬 수 있었고, 동시에 쓴맛과 아린맛을 약화시킴으로써 상업적 이용가능성을 제시하였다.

주요어 : 길경, 길경사포닌, 플라티코딘 D, 효소전환, 레시타제 올트라, 글리코시드 결합, HPLC-ELSD, LC-ESI/MS, 관능검사, 쓴맛

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