A Novel Ginsenosidase from an Aspergillus Strain Hydrolyzing 6-O-Multi-Glycosides of Protopanaxatriol-Type Ginsenosides, Named Ginsenosidase Type IV

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Herein, a novel ginsenosidase, named ginsenosidase type IV, hydrolyzing 6-O-multi-glycosides of protopanaxatriol-type ginsenosides (PPT), such as Re, R1, Rf, and Rg2, was isolated from the Aspergillus sp. 39g strain, purified, and characterized. Ginsenosidase type IV was able to hydrolyze the 6-O-α-L-(1→2)-rhamnoside of Re and the 6-O-β-D-(1→2)-xyloside of R1 into ginsenoside Rg1. Subsequently, it could hydrolyze the 6-O-β-D-glucoside of Rg1 into F1. Similarly, it was able to hydrolyze the 6-O-α-L-(1→2)-rhamnoside of Rg2 and the 6-O-β-D-(1→2)-glucoside of Rf into Rh1, and then further hydrolyze Rh1 into its aglycone. However, ginsenosidase type IV could not hydrolyze the 3-O- or 20-O-glycosides of protopanaxadiol-type ginsenosides (PPD), such as Rb1, Rb2, Rb3, Rc, and Rd. These exhibited properties are significantly different from those of glycosidases described in Enzyme Nomenclature by the NC-IUBMB. The optimal temperature and pH for ginsenosidase type IV were 40°C and 6.0, respectively. The activity of ginsenosidase type IV was slightly improved by the Mg²⁺ ion, and inhibited by Cu²⁺ and Fe²⁺ ions. The molecular mass of the enzyme, based on SDS–PAGE, was noted as being approximately 56 kDa.

Keywords: Ginsenosidase type IV, PPT ginsenoside, hydrolyzing multi-glycosides

Ginseng is a well-known traditional medicine that has been used for thousands of years throughout the Asian region. The most highly regarded species of ginseng plants today are Panax ginseng C. A. Meyer (Korean ginseng), Panax quinquefolium L. (American ginseng), Panax notoginseng (Sanchi or Tienci ginseng), and Panax japonicus, in addition to other species of the genus Panax.

Ginsenosides are the principal components responsible for the pharmaceutical activities of ginseng. To date, more than 60 kinds of ginsenosides have been identified. Based on the structure of their aglycones, ginsenosides have been categorized into three broad types; protopanaxadiol-type ginsenosides (PPD), such as the ginsenosides Rb1, Rb2, Rc, and Rd; protopanaxatriol-type ginsenosides (PPT), such as the ginsenosides Re, Rg1, and notoginsenoside R1; and oleanonic-acid-type saponins, such as Ro. Both PPD-type and PPT-type ginsenosides are dammarane saponins. Ginsenosides such as Ra1, Ra2, Rb1, Rb2, Rc, Rd, F2, Re, and Rg1 are 20(S)-saponins, but other ginsenosides, like Rf, Rg2, Rg3, Rh1, and Rh2, have both 20(S) and 20(R) forms.

There are a number of minor ginsenosides, such as Rg3, Rg5, Rh1, Rh2, Rh3, and F1, which are only usually found in low concentrations, and are even absent in some species such as red ginseng and wild ginseng. These minor ginsenosides have significant physiological activities, such as memory-enhancement [17], antitumor [3], antiallergic [12], and immunization [13] activities. Modern pharmacological studies suggest that the sugar moieties of ginsenosides are found to be closely associated with their biological activity [14], and that the glycosylated major ginsenosides are usually transformed into deglycosylated minor ginsenosides by intestinal bacteria [16] and/or digestive enzymes [1] in the gastrointestinal tract and then absorbed by the human body. However, it has been noted that this natural form of transformation is very limited in scope [6, 15].

Therefore, the transformation of major ginsenosides, using enzymes in vitro, to produce highly active and easily absorbed minor ginsenosides is very important for the development of ginseng drugs and products. The enzymatic transformation method has profound potential for the...
preparation of minor ginsenosides via the selective hydrolysis of the sugar moieties of major ginsenosides, owing to its high specificity, yield, and productivity. Researchers have found many kinds of microorganisms that can convert major protopanaxatriol-type ginsenosides into minor ginsenosides, including *Aspergillus niger* [10], *Mucor spinosus* [23], *Absidia coerulea* [2], and *Penicillium* sp. [9], amongst a number of others [4, 8, 19, 24].

Our laboratory has previously reported on several new enzymes that can hydrolyze multi-glycosides of ginsenosides [20–22] and other saponins [5], such as the ginsenosidase type I (hydrolyzing 3-O- and 20-O-multi-glycosides of PPD-type ginsenosides [20]) and ginsenosidase type II, (hydrolyzing 20-O-multi-glycosides of PPD-type ginsenosides [22]).

In this paper, a new glycosidase, named ginsenosidase type IV, hydrolyzing 6-O-multi-glycosides of PPT-type ginsenosides such as Re, Rl, Rf, and Rg2, was isolated from the *Aspergillus* sp. 39g strain, purified, and characterized.

**Materials and Methods**

**Materials**

The *Aspergillus* sp. 39g strain was isolated from Chinese traditional *koji* (*Daqu* in Chinese). The standard ginsenosides Re, Rf(S), Rg1, Rg2(S), Rβ1(S), F1, notoginsenoside Rf1, and aglycone were obtained from Dalian Bio-Chem Co. Ltd. (Dalian, China). DEAE-cellulose DE-52 was sourced from Whatman Ltd. (Maidstone, UK). The thin-layer chromatography (TLC) plates utilized were silica gel G-60 from Dalian Bio-Chem Co. Ltd. (Dalian, China). DEAE-cellulose DE-52 was sourced from Whatman Ltd. (Maidstone, UK). The thin-layer chromatography (TLC) plates utilized were silica gel G-60 F254 (Merck & Co. Inc., NJ, USA). Standard proteins (14,300–97,200) were purchased from Takara Bio Inc. (Otsu, Japan).

**Microorganism Culturing**

The *Aspergillus* sp. 39g strain was cultured by shaking in a medium, of 200 ml in a 1,000 ml conical flask, containing 1% *Sophora* flower extract and 5% wheat bran extract, at 28–30°C for 96 to 108 h. The cell growth, enzyme activity, and maltose reduction in the fermentation were determined in accordance with existing procedures [7].

**Crude Enzyme Extraction**

The culture of the *Aspergillus* sp. 39g strain was centrifuged to remove the cells, and (NH₄)_2SO₄ was slowly added to the cell-free culture with constant stirring to a 40% saturation and the mixture stored at 4°C for 4 h. After removing the protein precipitated by the centrifugation, more (NH₄)₂SO₄ was added to the reaction mixture to stop the reaction. The reaction product in the n-butanol layer was then analyzed by TLC with chloroform:methanol:water [70:30:5 (v/v/v)] as the developing solvent. The spots on the silica plate were scanned using a Shimadzu CS-930 spectrophotometer (Shimadzu Corp., Kyoto, Japan). One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 µmol of substrate per hour.

**Determination of Protein Concentration**

The concentration of protein was measured by the method of Lowry et al. [11] using bovine serum albumin as the standard protein.

**HPLC Method**

The product ginsenosides from enzyme reactions, and enzyme protein purity, were both examined by HPLC with a Waters 2695 Separations Module with Waters 2996 Photodiode Array Detector (Waters Corp., Milford, USA).

A Knauer C-18 chromatography column (5 µm, Φ3 mm×300 mm) was used to analyze the enzymatic reaction products (Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer GmbH, Berlin, Germany). The measuring wavelength was 203 nm, the injected volume was 10 µl, and the flow rate was 1.0 ml/min. The mobile phase was A (acetonitrile) and B (water): 0–35 min, A 19%; 35–55 min, A from 19% to 29%; 55–75 min, A from 29% to 40%; 75–105 min, A from 40% to 100%.

The sample used for the HPLC was prepared as follows: a 1–2 ml enzymatic reaction mixture was eluted on a 10 ml column of AB-8 Diaion resin column (Tianjin Chemical Plant, Nankai University, China). The resin column was first washed with 80 ml of a 0.02 M phosphate buffer (pH 6.0) and 50 ml of 20% alcohol, and then eluted with 60 ml of 83% alcohol to separate and collect the reaction products. These products were dried by vacuum distillation, and dissolved in 1 ml of methanol before the HPLC analysis.
A TOSOH TSK-Gel-2000 SW chromatographic column (Φ7.8 mm × 300 mm) was used to examine the purity of the enzyme. The mobile phase was conducted with a 0.02 mol/l phosphate buffer (pH 6.7) containing 0.05% sodium azide; the measuring wavelength was 280 nm, the injected volume was 100 µl, and the flow rate was 1.0 ml/min.

**Determination of Enzyme Molecular Mass**

The purity and molecular mass of the resulting ginsenosidase was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using lysozyme (14.3 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (29.0 kDa), ovalbumin (44.3 kDa), serum albumin (66.4 kDa), and phosphorylase b (97.2 kDa) as the standard proteins under the same conditions [18]. Protein bands were stained with Coomassie brilliant blue R-250. The molecular mass of the enzyme was determined by plotting a log of the molecular masses of the standard proteins.

**Effects of pH, Temperature, and Metal Ions**

The optimal pH for ginsenosidase type IV was determined at 40°C with different buffers at 0.02 M. For the pH ranges of 2.2–3.0, 4.0–5.0, and 6.0–8.0, glycine-hydrochloric acid, citrate, acetate, and phosphate buffers were used, respectively. To determine the optimal temperature, the enzyme was incubated in a phosphate buffer (pH 6.0) from 20°C to 80°C.

The effects of various metal ions (10–50 mM) on ginsenosidase activity were determined in a phosphate buffer (pH 6.0) at 40°C. The activity assayed in the absence of metal ions was recorded as 100%.

**RESULTS**

**Enzyme Fermentation**

To examine the production behavior of ginsenosidase during the fermentation of the *Aspergillus* sp. 39g strain, cell growth, enzyme production, and maltose reduction were all measured, with the results displayed in Fig. 1.

Cell concentrations and ginsenosidase production of the *Aspergillus* sp. 39g strain increased promptly after fermentation for 72 h, with both of them a maximum peak after fermentation for 96 to 108 h. Enzyme production was still maintained at a high level after fermentation for 108 h. Meanwhile, maltose production reduced rapidly after fermentation for 72 h, becoming stable again after fermentation for 108 h. Thus, the enzyme fermentation time was defined as 96 to 108 h in the experiments.

**Enzyme Purification**

The cell-free culture of the *Aspergillus* sp. 39g strain was treated with (NH₄)₂SO₄ at 40% saturation to remove any precipitate, and further treated with (NH₄)₂SO₄ at 70% saturation to harvest the crude enzyme. The crude enzyme solution was then eluted on a DEAE-Cellulose DE-52 column (Φ2.0 cm × 10 cm) (Whatman Ltd., Maidstone, UK) and fractionated to collect different fractions, as shown in Fig. 2.

The enzyme activity of each fraction was assayed using ginsenoside Re as the substrate. The fractions 5 to 20, and 48 to 56 eluted by 0.12 M KCl could hydrolyze ginsenoside Re to produce Rg1 and F1. The enzyme protein in fractions 5 to 20 was not pure enzyme; the fractions 50 to 52 exhibited the highest enzyme activity and displayed almost a single band on the PAGE gel. As an extra precaution, further purifications of fractions 50 to 52 were performed with the same results. The enzyme on the PAGE gel was cut and dissolved in a 0.02 M phosphate buffer (pH 6.0) at 40°C. The activity assayed in the absence of metal ions was recorded as 100%.

**Fig. 1.** Enzyme fermentation behavior. Fermentation was carried out at 28–30°C with shaking.

**Fig. 2.** Purification of ginsenosidase type IV on a DEAE-Cellulose DE52 column. Column, Φ2.0 cm × 10 cm; fraction, 3 ml/fraction; solvent, 0.06, 0.12, 0.18, 0.24, 0.3, 0.36, and 0.42 M KCl in a 0.02 M phosphate buffer (pH 6.0); —, enzyme activity hydrolyzing Re to Rg1; —, protein absorbance at 280 nm.
During the purification, the yield of ginsenosidase after DEAE-cellulose column separation was about 2.6%, with the specific activity of the enzyme increasing 7.4 times. However, the specific activity of the enzyme was not changed significantly; the yield was only 0.63% after further separation by cutting the PAGE gel, as shown in Table 1.

**Molecular Mass of Ginsenosidase Type IV**

The enzyme purity was examined by HPLC and the results are displayed in Fig. 3A. SDS-polyacrylamide gel electrophoresis was used to estimate the molecular mass of ginsenosidase type IV. The purified enzyme from fractions 50 to 52 formed one band on the SDS-polyacrylamide gel (Fig. 3B). Standard proteins ran together with the enzyme, and the enzyme molecular mass was determined by plotting the log of the molecular masses of the standard proteins. Thus, the molecular mass of the ginsenoside type IV was found to be approximately 56 kDa (Fig. 3B).

**Enzyme Hydrolysis for PPT-Type Ginsenoside Glycosides**

The purified enzyme from fractions 50 to 52, provisionally named “ginsenosidase type IV,” was examined for its hydrolysis of protopanaxatriol-type ginsenosides (PPT), such as ginsenosides Re, Rf(S), Rg2(S), and notoginsenoside R1, with the results shown in Fig. 4.

The hydrolysates of Re and R1 were ginsenosides Rg1 and F1; the hydrolysates of Rg2(S) and Rf(S) were ginsenosides Rh1(S) and its aglycone. Thus, ginsenosidase type IV can hydrolyze the 6-<sup>O</sup>-α-L-(1→2)-rhamnoside of Re, and the 6-<sup>O</sup>-β-D-(1→2)-glucoside of Rf into Rh1(S). In addition, the enzyme can hydrolyze the 6-<sup>O</sup>-α-L-(1→2)-rhamnoside of Rg2(S), and the 6-<sup>O</sup>-β-D-(1→2)-glucoside of Rf(S), into Rh1(S), and further hydrolyze the 6-<sup>O</sup>-β-D-glucoside of Rh1(S) into its aglycone. However, the enzyme cannot hydrolyze the glycosides of protopanaxadiol-type ginsenosides (PPD) such as Rb1, Rb2, Rb3, Rc, or Rd.

The enzyme reaction products were further purified using an AB-8 Diaion resin and then subjected to HPLC analyses. The hydrolysis products from R1 are shown in Fig. 5. The retention time of standard ginsenosides was 19.091 min for R1, 28.942 min for Rg1, and 68.438 min for F1. The reaction products from R1 contained ginsenosides Rg1 and F1, indicating that the R1 was hydrolyzed by the enzyme into the ginsenosides Rg1 and F1. The result of HPLC for the enzyme reaction products was identical to that of the TLC in Fig. 4. The HPLC results of enzyme reaction products from Re, Rf(S), and Rg2(S) are elided.

**Table 1. Results of enzyme extraction and purification.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold (χ fold)</th>
<th>Yield (%)</th>
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</thead>
<tbody>
<tr>
<td>Crude supernatant</td>
<td>400</td>
<td>17,860</td>
<td>1,355</td>
<td>13.2</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation</td>
<td>40</td>
<td>11,236</td>
<td>669</td>
<td>16.8</td>
<td>1.3</td>
<td>63</td>
</tr>
<tr>
<td>DEAE-cellulose (4 times)</td>
<td>12</td>
<td>461</td>
<td>4.71</td>
<td>98.1</td>
<td>7.4</td>
<td>2.6</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>2.0</td>
<td>113</td>
<td>1.12</td>
<td>101</td>
<td>7.6</td>
<td>0.63</td>
</tr>
</tbody>
</table>
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...rhamnoside of Re and Rg2(S), the xyloside of R1, and the glucoside of Rf(S). The ginsenosidase type IV reactions on PPT-type ginsenosides are shown in Fig. 6.

Optimal pH, Temperature, and Effects of Metal Ions
The effects of pH and temperature on ginsenosidase type IV hydrolyzing the ginsenoside Re were evaluated, and the results are shown in Fig. 7.

It is observable from Fig. 7 that the optimal pH (Fig. 7A) and temperature (Fig. 7B) for ginsenosidase type IV are 6.0 and 40°C, respectively.

The effects of various metal ions on the ginsenosidase were also investigated. The activity of ginsenosidase was
slightly increased by 50 to 100 mM of the Mg\(^{2+}\) ion, but inhibited by the Cu\(^{2+}\) and the Fe\(^{2+}\) ions.

**DISCUSSION**

The results indicate that ginsenosidase type IV, isolated from the *Aspergillus* sp. 39g strain, hydrolyzes 6-O-multi-glycosides of PPT-type ginsenosides such as the 6-O-α-L-(1→2)-rhamnopyranoside of Re, the Rg2(S), 6-O-β-D-(1→2)-xyloside of R1, the 6-O-β-D-(1→2)-glucoside of Rf(S), and the 6-O-β-D-glucoside of Rg1 and Rh1(S); but cannot hydrolyze PPD-type ginsenosides. The molecular mass of the enzyme is approximately 56 kDa. In relation to optimal conditions, when using the ginsenoside Re as a substrate, the enzyme reached its highest activity at a pH of 6.0, and a temperature of 40°C.

Ginsenosidase type IV can be compared with previously reported ginsenosidases, including ginsenosidase type I [20], ginsenosidase type II [22], and ginsenoside-α-L-rhamnosidase [21]. Ginsenosidase type I can hydrolyze 3-O- and 20-O-multi-glycosides of PPD-type ginsenosides such as Rb1, Rb2, Rb3, Re, and Rd. Ginsenosidase type II can hydrolyze 20-O-multi-glycosides of PPD-type ginsenosides such as Rb1, Rb2, Rb3, and Rc. Ginsenoside-α-L-rhamnosidase can hydrolyze the 6-O-α-L-(1→2)-rhamnoside of 20(S) and 20(R)-ginsenoside Rg2 to produce the 20(S) and 20(R)-ginsenoside Rh1. Both the ginsenosidase type I and ginsenosidase type II cannot hydrolyze PPT-type ginsenosides. The ginsenoside-α-L-rhamnosidase has only been examined in relation to the hydrolysis of Rg2.

By contrast, the ginsenosidase type IV isolated from the *Aspergillus* sp. 39g strain can hydrolyze 6-O-multi-glycosides such as rhamnopyranoside, xyloside, and glucoside of PPT-type ginsenosides; but cannot hydrolyze 3-O- or 20-O-glycosides of PPD-type ginsenosides. Therefore, the properties of ginsenosidase type IV differ from the glycosidases currently described in Enzyme Nomenclature by the NC-IUBMB, where typically one enzyme hydrolyzes one type of glycoside. It therefore represents the advent of a novel enzyme.

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