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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 Mg^{2+} ion, and inhibited by Cu^{2+} and Fe^{2+} 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 Tienchi 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

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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, R1, 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*), Rh1(*S*), F1, notoginsenoside R1, 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 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 $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$ powder was added to a 70% saturation and the mixture was stored at 4°C overnight. Thereafter, the mixture was centrifuged to harvest the crude enzyme, which was dialyzed in a 0.01 M phosphate buffer (pH 6.0) and subsequently diluted to 1/20 volume of the culture with 0.02 M and pH 6.0 phosphate buffers, and then non-dissolved material was removed by centrifugation to obtain a concentrated crude enzyme solution.

Purification of Ginsenosidase Type IV

The WH-500 USB Protein Chromatographic Working Station, with an HDL ultraviolet detector, and a BSZ-160 fraction collector (Shanghai Kingdom Biochemical Instrument Co. Ltd, Shanghai, China) was used for the enzyme purification process. Ten ml of the concentrated crude enzyme solution was applied to a column (Φ2.0 cm×10 cm) of DEAE-cellulose DE-52 (Whatman Ltd., Maidstone, UK) and the proteins were fractionated stepwise with 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, 3.0 ml/fraction). The enzymatic activity of each fraction was then assayed based on the hydrolysis of ginsenoside Re, and the fractions that exhibited hydrolyzing activity were respectively dialyzed against a 0.01 M phosphate buffer (pH 6.0), freeze-dried, and dissolved in 1/10 (w/v) distilled water. Then vertical slab polyacrylamide gel electrophoresis was carried out for further purification of the ginsenosidase. After electrophoresis, the enzyme band on the vertical slab polyacrylamide gel was cut and mashed in a phosphate buffer, and the non-dissolved materials were removed by centrifugation to obtain a purified enzyme solution. This enzyme solution was then evaluated for its molecular weight and enzymatic properties. The purity of the enzyme protein was also examined by the method of HPLC using a TOSOH TSK-Gel-2000 SW chromatographic column (Tosoh Bioscience, Tokyo, Japan).

Enzyme Assay

The enzymatic activity of the ginsenosidase was assayed using 1.0 mg/ml ginsenoside in a 0.02 M phosphate buffer (pH 6.0) as the substrate. An assay mixture containing 0.1 ml of the substrate (0.1% ginsenoside solution) and 0.1 ml of the enzyme were incubated at 40°C for 12 to 48 h. Next, 0.2 ml of *n*-butanol, saturated in water, 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.

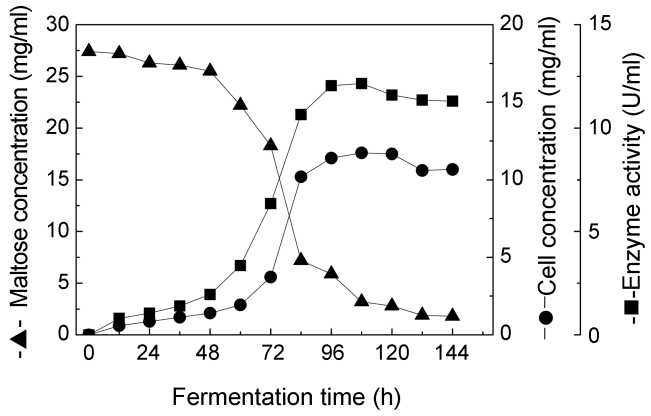

Fig. 1. Enzyme fermentation behavior.
Fermentation was carried out at 28−30°C with shaking.

Enzyme Purification

The cell-free culture of the *Aspergillus* sp. 39g strain was treated with $(NH_4)_2SO_4$ at 40% saturation to remove any precipitate, and further treated with $(NH_4)_2SO_4$ 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) to obtain the pure enzyme solution. When the HPLC method was used to check the purity of the enzyme, only one peak appeared on the HPLC spectrum at 5.513 min (Fig. 3A); the enzyme could hydrolyze the ginsenosides Re and R1 (Fig. 4).

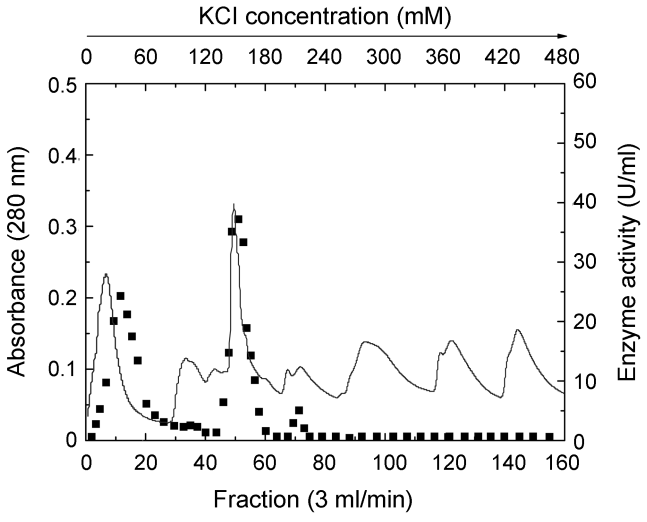

Fig. 2. Purification of ginsenosidase type IV on a DEAE-Cellulose DE52 column.
Column, Φ2.0 cm×10 cm; fraction, 3 ml/fraction; solvent, 0.06, 0.12, 0.18, 0.24, 0.3, 0.36, and 0.42 M KCl in a 0.02 M phosphate buffer (pH 6.0); ■, enzyme activity hydrolyzing Re to Rg1; —, protein absorbance at 280 nm.

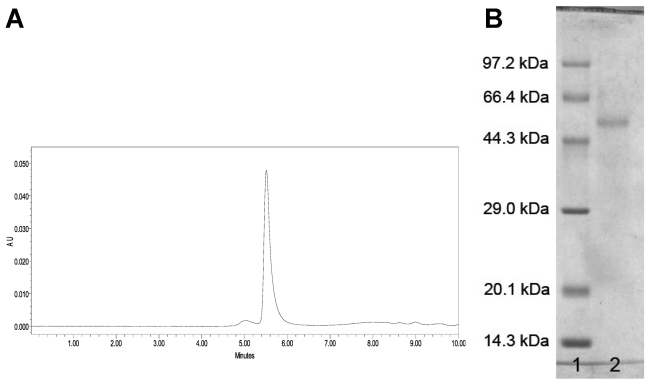

Fig. 3. SDS–PAGE and HPLC of ginsenosidase type IV.
A. HPLC for ginsenosidase type IV. **B**. SDS–polyacrylamide gel electrophoresis of ginsenosidase type IV. **1**, Marker proteins: lysozyme (14.3 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (29.0 kDa), ovalbumin (44.3 kDa), serum albumin (66.4 kDa), and phosphorylase b (97.2 kDa); **2**, ginsenosidase type IV. Protein quantity, 3 μg.

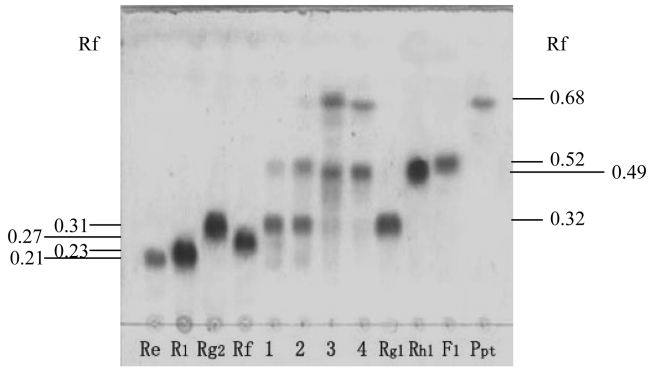

Fig. 4. Ginsenosidase type IV hydrolysis on PPT-type ginsenosides in TLC.
Re, R1, Rg2(*S*), Rf(*S*), Rg1, Rh1(*S*), F1, Ppt: standard ginsenosides; **1**, Enzyme reaction product from Re, reacted at 40°C for 12 h; **2**, Enzyme reaction product from R1, reacted at 40°C for 48 h; **3**, Enzyme reaction product from Rg2(*S*), reacted at 40°C for 16 h; **4**, Enzyme reaction product from Rf, reacted at 40°C for 16 h; Substrate, 1.0 mg/ml; Solvent, chloroform:methanol:water = 7:3:0.5.

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-*O*-α-L-(1→2)-rhamnoside of Re, and the 6-*O*-β-D-(1→2)-xyloside of R1, into Rg1, and subsequently hydrolyze the 6-*O*-β-D-glucoside of Rg1 into F1. In addition, the enzyme can hydrolyze the 6-*O*-α-L-(1→2)-rhamnoside of Rg2(*S*), and the 6-*O*-β-D-(1→2)-glucoside of Rf(*S*), into Rh1(*S*), and further hydrolyze the 6-*O*-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.

Therefore, ginsenosidase type IV can hydrolyze the 6-*O*-multi-glycosides of PPT-type ginsenosides such as the

Table 1. Results of enzyme extraction and purification.

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold (χ fold)	Yield (%)
Crude supernatant	400	17,860	1,355	13.2	1.0	100
$(NH_4)_2SO_4$ precipitation	40	11,236	669	16.8	1.3	63
DEAE-cellulose (4 times)	12	461	4.71	98.1	7.4	2.6
Electrophoresis	2.0	113	1.12	101	7.6	0.63

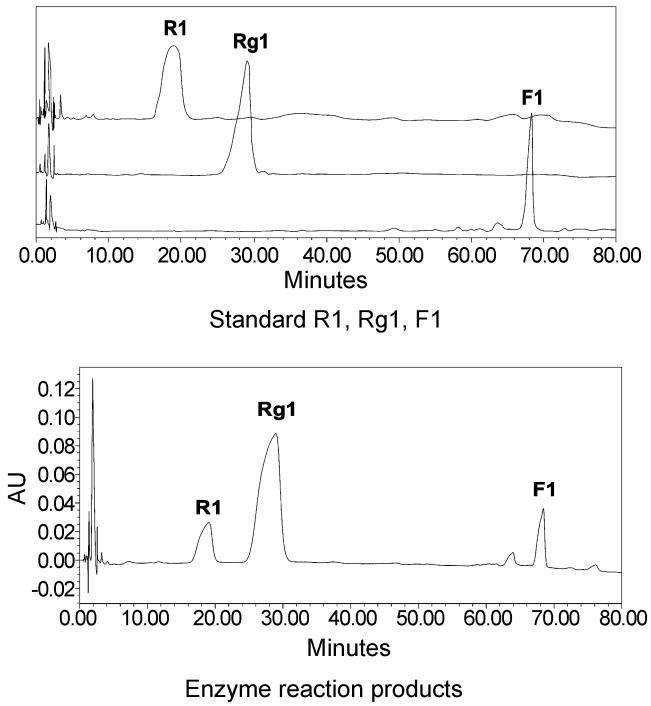

Fig. 5. Products from ginsenoside R1 by ginsenosidase type IV in HPLC.
Substrate, 1.0 mg/ml R1, at 40°C for 48 h.

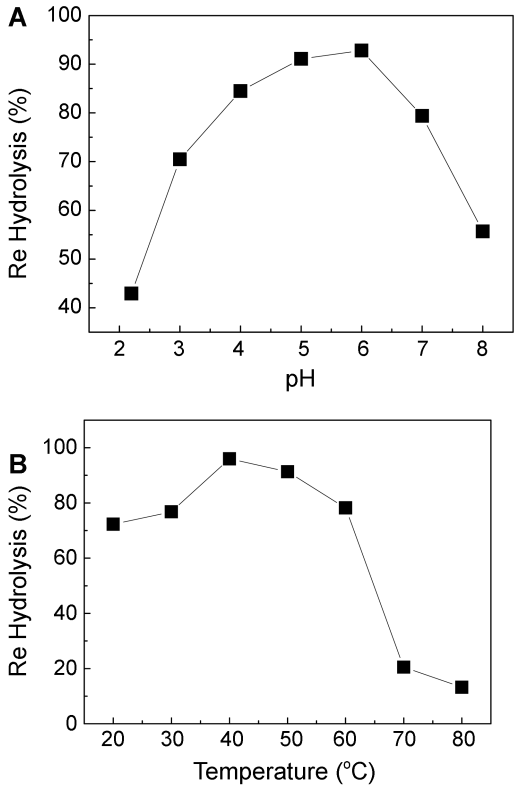

Fig. 7. Effects of pH and temperature on ginsenosidase type IV.
Enzyme, 10 U/ml; substrate, Re concentration 1.0 mg/ml. **A**. Reacted for 12 h at 40°C. **B**. Reacted for 12 h at pH 6.0. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%.

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

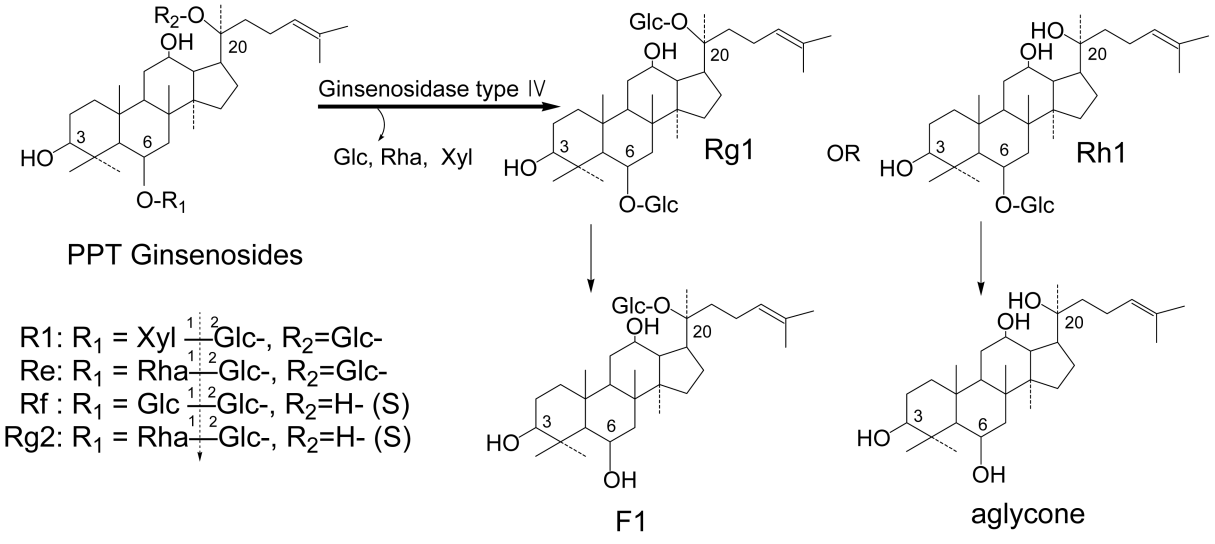

Fig. 6. Ginsenosidase type IV hydrolysis on PPT-type ginsenosides.

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)-rhamnoside 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, Rc, 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 rhamnoside, 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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