

Purification and Characterization of New Special Ginsenosidase Hydrolyzing Multi-Glycosides of Protopanaxadiol Ginsenosides, Ginsenosidase Type I

Hongshan YU, Chunzhi ZHANG, Mingchun LU, Fang SUN, Yaoyao FU, and Fengxie JIN*

College of Bio & Food Technology, Dalian Institute of Light Industry Qinggong-yuan No 1; Ganjingzi-qu, Dalian 116034, People's Republic of China. Received May 8, 2006; accepted November 15, 2006

In this paper, the new type ginsenosidase which hydrolyzing multi-glycosides of ginsenoside, named ginsenosidase type I from *Aspergillus* sp.g48p strain was isolated, characterized and generally described. The enzyme molecular weight was about 80 kDa. Ginsenosidase type I can hydrolyze different glycoside of protopanaxadiol type ginsenosides (PPD); *i.e.*, can hydrolyze the 3(carbon)-*O*- β -glucoside of Rb₁, Rb₂, Rb₃, Rc, Rd; can hydrolyze 20(carbon)-*O*- β -glucoside of Rb₁, 20-*O*- β -xyloside of Rb₃, 20-*O*- α -arabinoside(p) of Rb₂ and 20-*O*- α -arabinoside(f) of Rc to produce mainly F₂, compound-K (C-K) and small Rh₂, but can not hydrolyze the glycosides of protopanaxatriol type ginsenoside (PPT) such as Re, Rf, Rg₁. So, when the ginsenosidase type I hydrolyzed ginsenosides, the enzyme selected ginsenoside-aglycone type, can hydrolyze different glycosides of PPD type ginsenoside; however no selected glycoside type, can hydrolyze multi-glycosides of PPD type ginsenosides. These properties were novel properties, and differentiated with the other previously described glycosidases.

Key words ginsenosidase type I; PPD ginsenoside; multi-glycoside hydrolysis of ginsenoside

Ginseng, the famous plant drug, has been used as an expensive traditional medicine in oriental countries. The main ginseng plants of this drug are *Panax ginseng* C. A. MEYER, *P. quinquefolium* L. (American ginseng), *P. natoginseng* (Sanchi ginseng, or Tienchi ginseng), *P. japonicus*, and other species of *Panax* genus.

One of physiological activity materials of ginseng plants is saponin (ginsenoside), known ginsenosides are over 40 kinds. Ginsenosides are divided three types, *i.e.* protopanaxadiol type (PPD), protopanaxatriol type (PPT), and oleanonic acid type saponins such as ginsenoside Ro. The ginsenoside Ra₁, Ra₂, Ra₃, Rb₁, Rb₂, Rb₃, Rc, Rd, F₂, Rg₃, Rg₅, Rh₂, and Rh₃ are protopanaxadiol type ginsenoside; Re, Rg₁, Rg₂, Rg₄, Rh₁, Rh₄ are protopanaxatriol type ginsenoside; these ginsenosides are dammarane saponins. Ginsenoside Ra₁, Ra₂, Ra₃, Rb₁, Rb₂, Rb₃, Rc, Rd, F₂, Re, Rg₁ are dammarane 20(*S*)-saponins, but the ginsenoside Rg₃, Rh₂, Rg₂, Rh₁ have 20(*S*) and 20(*R*)-forms.¹⁾ Structures of several compounds belonging to the respective common structure of protopanaxadiol and protopanaxatriol were shown in Figs. 1 and 2.

The main ginsenosides in the drug ginseng are the ginsenoside Rb₁, Rb₂, Rc, Rd, Re, Rf and Rg₁, and other ginsenoside content was low, the ginsenosides such as Rg₃, Rg₂, Rg₅, Rh₂, Rh₁, Rh₃ and Rh₄ are minor ginsenosides in the red ginseng or wild ginseng. These minor ginsenoside Rg₃, Rg₅, Rg₂, Rg₄, Rh₂, Rh₃, Rh₁ and Rh₄ have special physiological activities: for example, the ginsenoside Rh₂, Rh₃, Rg₃ and Rh₁ have a good anticancer property, but haven't sideeffects; the ginsenoside Rg₃ and Rg₂ have anti-thrombus and mediating endothelium. So, the minor ginsenosides are very useful for the drug and health food. However, it is very difficult to obtain the minor ginsenoside from the red and wild ginseng, because the contents in the red ginseng and wild ginseng are very low.¹⁾

The metabolism of ginsenosides by human intestinal bacteria was reported to express the ginsenoside hydrolyzing behaviors in human intestinal, to prove the ginseng saponin hy-

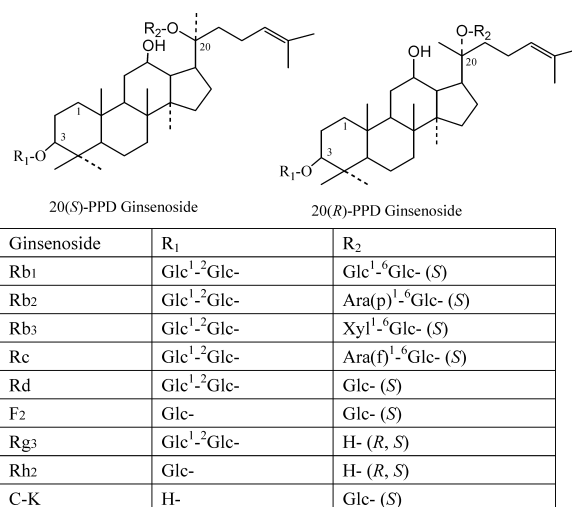


Fig. 1. Protopanaxadiol Type Ginsenosides (PPD)

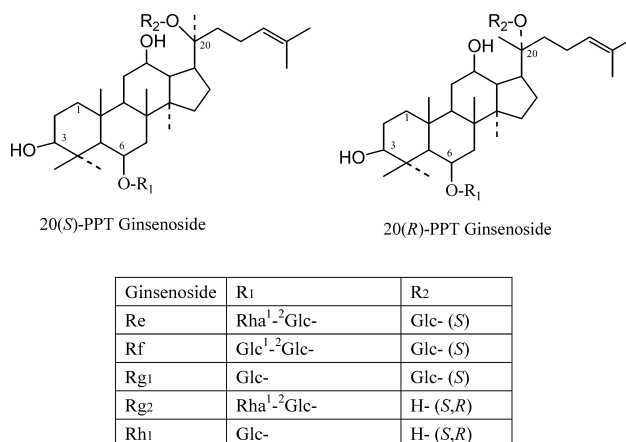


Fig. 2. Protopanaxatriol Type Ginsenosides (PPT)

* To whom correspondence should be addressed. e-mail: fxjin@dlili.edu.cn

hydrolyzing to minor ginsenoside absorbing by body after take orally for ginseng.²⁻⁵) To obtain minor ginsenoside, our laboratory previously reported the interesting ginseng or other saponin-glycosidases hydrolyzing ginseng or other saponin-sugar-moiety from microorganism, plant and liver.⁶⁻¹¹

In this paper, the new special ginsenoside-glycosidases (ginsenosidases hydrolyzing multi-glycoside of proto-panaxadiol type ginsenosides) from microorganism were isolated, characterized and generally described.

Experimental

Materials DEAE-Cellulose DE-52 was obtained from Whatman. The standard ginsenoside Rb₁, Rb₂, Rb₃, Rc, Rd, F₂, Rg₃, Rh₂, C-K, Re, Rf and Rg₁ were obtained from Dalian Green Bio co ltd. Thin-layer chromatography (TLC) plate was the silica gel G-60 F₂₅₄ (Merck). Standard proteins such as trypsinogen (24 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), glutamic dehydrogenase (53 kDa), albumin (66 kDa) and phosphorylase (97 kDa) were sigma products.

The microorganism was the *Aspergillus* sp.g48p strain isolating from traditional Chinese Koji (Daqu in Chinese).¹⁰

Enzyme Production The sp.g48p strain was cultured in the medium (200 ml in 1000 ml Erlenmeyer flask) containing 1% ginseng extraction and 4% extraction of wheat bran at 30 °C for 72 to 120 h. After removed the cells by centrifuging, the (NH₄)₂SO₄ powder was added to the cell free supernatant with stirring to 40% saturation and stored at 4 °C for 4 h, and to remove the protein precipitate by centrifuging; then the (NH₄)₂SO₄ powder was also added to 70% saturation and stored at 4 °C over night to collect the protein precipitate by centrifuging, and dialyzed on 0.01 M and pH 5 acetate buffer, diluted to 1/10 volumn of culture with 0.01 M and pH 5 acetate buffer, and removed non-dissolved material to obtain crude enzyme solution.

Enzyme Analysis The ginsenosidase was assayed using ginsenoside as the substrate. The assay mixture containing 0.1 ml of the substrate (0.1 to 0.5% ginsenoside solution) and 0.1 ml of the enzyme were incubated at 40 °C for 12 to 20 h. The reaction was stopped by the addition of 0.2 ml of *n*-butanol saturated water. The hydrolyzed product was removed to the butanol layer, and an aliquot of the butanol layer was carried out by TLC; the solvent, chloroform-methanol-water (70:30:5, v/v/v); and the produced ginsenosides on the silica gel G-60 F₂₅₄ plate was determined by scanning the TLC spots using a Shimadzu CS-930. One unit of the enzyme activity equals 1 μM of ginsenoside Rg₃ hydrolyzed per hour.^{6,12}

The product-ginsenosides from enzyme reaction were also examined by the HPLC method: The equipment was Waters 2695; the detector, Waters 2996 Photodiode Array Detector; and the column, C18 Hypersil 5μODS2 250×4.6. The mobile phase was A (acetonitrile) and B (water): 0—20 min, A 20%; 20—31 min, A from 20 to 32%; 31—40 min, A from 32 to 43%; 40—70 min, A from 45 to 100%; and 70—80 min, the column eluted by 100% A.

The HPLC sample was obtained that 1 ml of ginsenoside Rb₁, Rb₂, Rc or Rb₃ enzyme reaction mixture were respectively eluted on 10 ml volumn of AB-8 macroporous resin (from Tianjin Chemical Plant of Nankai University, P. R. China) column, and the resin column was washed with 80 ml 0.01 M, pH 5.0 acetate buffer, washed with 50 ml 40% alcohol; then eluted with 60 ml 83% alcohole to collect enzyme reaction products. These products were dried by vacuum distilled method, and dissolved with 1 ml methanol to apply HPLC.

Enzyme Purification The 10 ml of above crude enzyme solution was eluted on a DEAE-Cellulose DE-52 (Whatman) column (φ1.8×7.5 cm) to absorb the enzyme protein. Then the column was fractionated stepwise with 0.06, 0.12, 0.18, 0.24, 0.3, 0.4, 0.5 and 0.6 mol KCl in 0.01 mol and pH 5 acetic acid buffer (fraction, 3.0 ml/tube), and the fractions were examined enzyme activities hydrolyzing the ginsenoside Rg₃. Then the fraction enzyme hydrolyzing the ginsenoside Rg₃ were respectively dialyzed against 0.005 mol, pH 5.0 acetate buffer, freeze-dried, and dissolved in 0.3 ml of distilled water to remove non-dissolved by centrifuging, then farther purification was carried out with the method of vertical slab polyacrylamide electrophoresis¹³: after electrophoresis, the enzyme band of the polyacrylamide electrophoresis gel was excised, and dissolved in acetate buffer to remove non-dissolved material by centrifuging to get purified enzyme solution, and the solution was used to mensurating enzyme molecular weight and activities.

Enzyme Molecular Weight Purified enzyme was used to determine the molecular weight by SDS (sodium dodecyl sulfate) polyacrylamide gel elec-

trophoresis. SDS polyacrylamide gel electrophoresis¹⁴) was also carried out for standard proteins under the same conditions. The molecular weight of enzyme was determined by plotting the log of the molecular weights of the standard proteins.¹⁴) The standard proteins were trypsinogen (24 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), glutamic dehydrogenase (53 kDa), albumin (66 kDa) and phosphorylase (97 kDa).

Results and Discussion

Enzyme Purification When (NH₄)₂SO₄ concentration reached 70% saturation for enzyme culture, most ginsenosidase type I was precipitated. Therefore, after removed the precipitate by (NH₄)₂SO₄ 40% saturation; the (NH₄)₂SO₄ powder was slowly added to 70% saturation, and the mixture stored at 4 °C overnight. The protein precipitate was the collected by centrifuging, and dissovved and dialyzed at he 0.01 M acetic acid buffer, pH 5.0. After removing the non-dissolved fraction by centrifuging, the crude enzyme solution was eluted on a DEAE-Cellulose DE-52 (Whatman) column (φ1.8×7.5 cm) to absorb the enzyme protein, and fractionated stepwise with respectively 45 ml of 0.06, 0.12, 0.18, 0.24, 0.3, 0.4, 0.5 and 0.6 mol KCl in 0.01 mol and pH 5 acetate buffer (fraction, 3.0 ml/tube). When the enzyme activities of the fractions were examined; the 41 to 47 fractions eluted by 0.12 mol KCl solution hydrolyzed the 0.1% ginsenoside Rg₃ to ginsenoside Rh₂ (Fig. 3), the fraction 43 enzyme activity was the highest than other fractions as shown in Fig. 4.

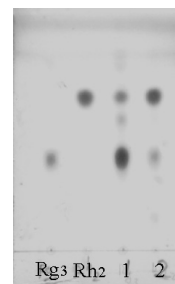


Fig. 3. Fraction 43 Enzyme Hydrolysis on Ginsenoside Rg₃

Rg₃ and Rh₂ standard ginsenosides; 1, reacted for 9 h; 2, reacted for 14 h. Substrate, 0.2%; reacted at 40 °C. Solvent, chloroform : methanol : water = 7 : 3 : 0.5.

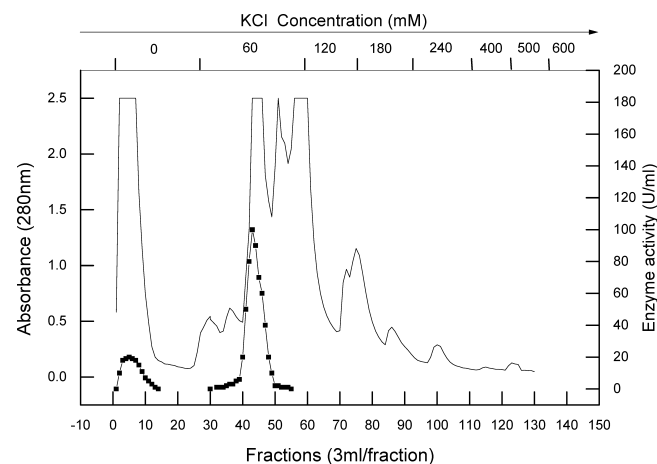


Fig. 4. Purification of Ginsenosidase Type I on DEAE-Cellulose DE52

Column, φ1.8×8.6; fraction, 5 ml/tube; solvent, 60, 12, 180, 240, 400, 500, 500 mM KCl in 0.02 M, pH 5.6 acetate buffer; ■, enzyme activity hydrolyzing Rg₃ to Rh₂; —, protein absorbance.

Table 1. The Enzyme Purification

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Fermentation	400	15320	1504	10.2	100
(NH ₄) ₂ SO ₄ precipitation	40	10440	840	11.9	68
DEAE-Cellelose (4 times)	12.0	1820	12.4	146	8.4
Electrophoresis	2.0	303	2.07	146	1.6

The 43 fraction was almost single band in the polyacrylamide electrophoresis¹³ to prove pure enzyme. But to caution, the farther purification was carried out: the 12 ml enzyme of the fraction 43 was dialysed against 0.005 mol, pH 5.0 acetate buffer, freeze-dried, and dissolved in 2 ml of distilled water to remove non-dissolved by centrifuging, then farther purified with the method of vertical slab polyacrylamide gel electrophoresis. The enzyme band of the polyacrylamide electrophoresis gel were excised, dissolved in above acetate buffer to remove non-dissolved material by centrifuging to obtain purified enzyme solution, these experiment carried out 6 times. The above solution was used to enzyme characterization.

In the purification, the enzyme almost purified at the step of DEAE-cellulose column, the yield of pure ginsenosidase type I was 8.4%, the specific activity of enzyme was increased about 14 times. This enzyme was single band in the polyacrylamide electrophoresis to prove pure enzyme. But to caution, the enzyme was carried out with the polyacrylamide electrophoresis, and passthrough the polyacrylamide electrophoresis step, the enzyme specific activity was not changed, but the yield was only 1.6% as shown in Table 1.

Enzyme Molecular Weight The pure enzyme from fraction 43 (ginsenosidase type I) is one spot in the SDS polyacrylamide electrophoresis¹⁴ to prove purified enzyme as shown in Fig. 5. When the molecular weight of enzyme was determined by plotting the log of the molecular weights of the standard proteins, molecular weight was about 8.0 kDa.

Enzyme Hydrolysis for Ginsenoside-Glycosides The pure enzyme from fraction 43 provisionally named "ginsenosidase type I" was examined the hydrolysis of protopanaxadiol type ginsenosides (PPD) such as Rb₁, Rb₂, Rb₃, Rc, Rd as shown in Fig. 6. It is shown that from Fig. 6, the ginsenosidase Type I not only can hydrolyze the 3-*O*- β -glucoside of Rg₃; but also can hydrolyzed 3-*O*- β -glucoside of Rb₁, Rb₂, Rb₃, Rc, Rd; can hydrolyze 20-*O*- β -glucoside of Rb₁, 20-*O*- β -xyloside of Rb₃, 20-*O*- α -arabinoside(p) of Rb₂ and 20-*O*- α -arabinoside(f) of Rc. The enzyme reaction products were examined by TLC method to produce F₂, compound-K or Rh₂ (Fig. 6).

When these products from ginsenosidase type I was examined with HPLC method, the products from ginsenoside Rb₁, Rb₂, Rb₃ and Rc were similar that the main products were ginsenoside F₂ (45.215 min peak), C-K (54.619 min peak), and small Rh₂ (56.104 min peaks); the Rb₁ product was as shown in Fig. 7; the HPLC peaks of the products from the ginsenoside Rb₂, Rb₃ and Rc by enzyme were reaction similar to those of from Rb₁; so, the HPLC peaks of enzyme products from Rb₂, Rb₃ and Rc was elided.

But, the ginsenosidase type I cannot hydrolyze the glycosides of protopanaxatriol type ginsenoside such as Re, Rf,

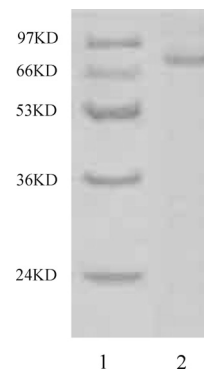


Fig. 5. SDS-Polyacrylamide Gel Electrophoresis of Ginsenosidase Type I

1, marker protein: trypsinogen (24 kDa), glyceraldehyde-3-phosphate-dehydrogenase (36 kDa), glutamic dehydrogenase (53 kDa), albumin (66 kDa) and phosphorylase (97 kDa); 2, ginsenosidase type I.

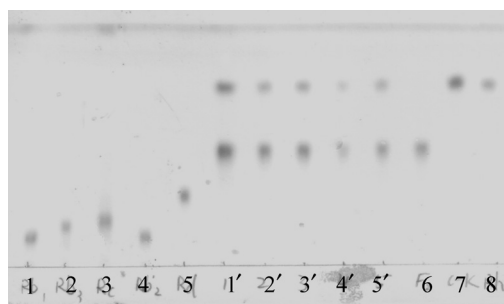


Fig. 6. Ginsenosidase Type I Hydrolysis on PPD Type Ginsenosides in TLC

Substrate, 0.2%; reacted at 40 °C for 18 h. 1 to 8: standard ginsenosides: 1, Rb₁; 2, Rb₂; 3, Rc; 4, Rb₂; 5, Rd, 6, F₂; 7, compound-K; 8, Rh₂. 1', 2', 3', 4', 5': products from ginsenoside Rb₁, Rb₂, Rc, Rb₂ and Rd by enzyme reaction. Solvent, chloroform: methanol: water=7:3:0.5.

Rg₁ in Fig. 8.

Therefore, the ginsenosidase type I can hydrolyze the 3-C and 20-C multi-glycoside of ginsenoside molecular such as Rb₁, Rb₂, Rb₃, Rc (PPD ginsenosides) to produce ginsenoside F₂ as a main product and C-K, and to produce small Rh₂; can not hydrolyze glycoside of protopanaxatriol type ginsenoside Re, Rf and Rg₁ (PPT type ginsenosides). It was shown that the ginsenosidase type I selected ginsenoside-aglycone type, however no selected glycoside type; hydrolyzed multi-glycosides of PPD type ginsenoside. The ginsenosidase type I reaction on PPD type ginsenoside such as Rb₁, Rb₂, Rb₃ and Rc was shown in Fig. 9.

Differences of Ginsenosidase Type I with the Original Glycosidase To understand the differences between the developed ginsenosidase and original glycosidase, the ginsenoside hydrolysis by enzymes was examined with the substrates of 0.2% of ginsenoside Rb₁, Rb₂, Rc, Rd at 40 °C for 20 h. The used ginsenosidase type I concentration was lower (pro-

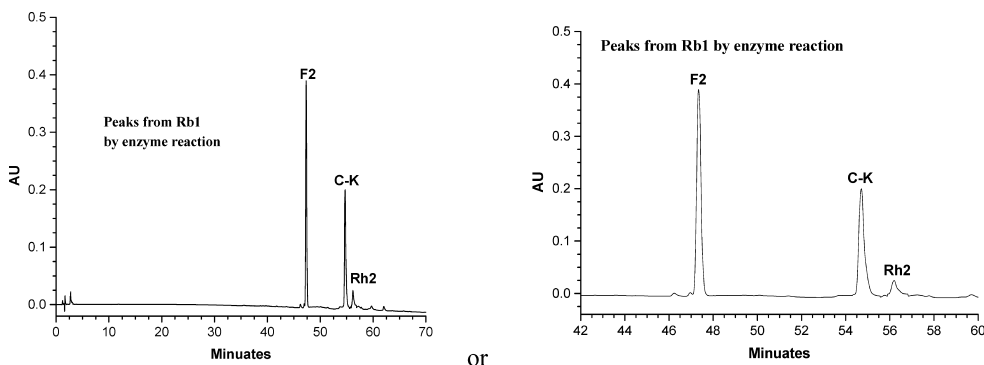


Fig. 7. The Products from Ginsenoside Rb₁ by Ginsenosidase Type I in HPLC

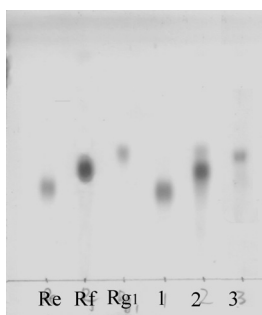


Fig. 8. Ginsenosidase Type I Hydrolysis on PPT Type Ginsenosides

Substrate, 0.2%; reacted at 40 °C for 18 h. Re, Rf and Rg₁, standard. 1, Re after enzyme reaction; 2, Rf after enzyme reaction; 3, Rg₁ after enzyme reaction. Solvent, chloroform : methanol : water = 7 : 3 : 0.5.

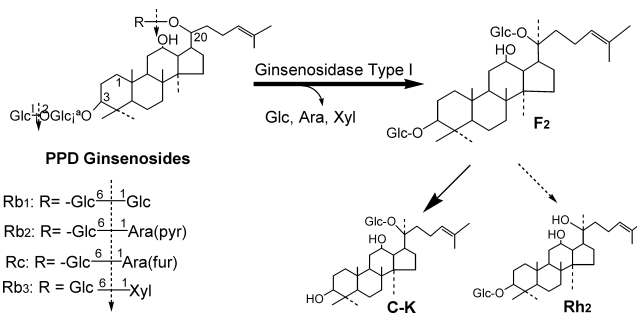


Fig. 9. Ginsenosidase Type I Hydrolysis on PPD Type Ginsenosides

.....> slowly reacted.

Table 2. Ginsenosidase Different with Original Glycosidase

Enzymes	Substrate	Substrate hydrolysis (%)	Hydrolyzed glycoside position
Ginsenosidase I	Rb ₁ , Rb ₂ , Rb ₃ , R _c , R _d Re, Rf, Rg ₁	100 —	3-C and 20-C —
<i>β</i> -Glucosidase from <i>Clostridium</i> and <i>Bacillus</i> strain (EC 3.2.1.21)	Rb ₁ , Rb ₂ , Rb ₃ , R _c , R _d	—	—
Crude enzyme of <i>Clostridium</i> and <i>Bacillus</i> strain	Rb ₁ , Rb ₂ , Rb ₃ , R _c , R _d	—	—
Almond <i>β</i> -glucosidase (EC 3.2.1.21)	Rb ₁ , Rb ₂ , Rb ₃ , R _c , R _d	—	—
Enzyme products of Novozymes, China (mixture enzyme)			
AMG (glucosidase from mold)	Rb ₁ , Rb ₂ , Rb ₃ , R _c , R _d	— +	—
Viscozymel (cellulase hemicellulase mixture)	Rb ₁ , Rb ₂ , Rb ₃ , R _c , R _d	— +	—

Substrate, 0.2%; reacted at 40 °C for 16 h in pH 5.0; —, not reacted; — +, cannot recognized the distinct hydrolysis by TLC.

tein concentration, 0.5 mg protein/ml) concentration enzyme; and other glycosidase such as pure *β*-glucosidase, *α*-arabinosidase, and glycosidase products from Novozymes, China (mixture enzyme) were higher concentration (protein concentration, 5 to 10 mg protein/ml), the enzyme reaction was examined as shown in Table 2.

The pure ginsenosidase type I can hydrolyze the 3(carbon)-*O*-*β*-glucoside of Rb₁, Rb₂, Rb₃, R_c, R_d; can hydrolyze 20(carbon)-*O*-*β*-glucoside of Rb₁, 20-*O*-*β*-xyloside of Rb₃, 20-*O*-*α*-arabinoside(p) of Rb₂ and 20-*O*-*α*-arabinoside(f) of R_c to produce mainly F₂, and compound-K, low Rh₂ as shown in Figs. 6 and 7; but cannot hydrolyze the glycoside of protopanaxatriol type ginsenoside (PPT) such as Re, Rf, Rg₁, discribed above (Figs. 6—9).

However, the cellulose *β*-glucosidase (EC 3.2.1.21) (protein concentration, 5 to 10 mg protein/ml) from *Clostridium thermocopriae*,¹²⁾ *Bacillus* sp.AX strain¹⁵⁾ and *Bacillus*

sp.JF¹⁶⁾ did not hydrolyze *β*-glucoside and any glycosides of ginsenoside Rb₁, Rb₂, Rb₃, R_c and R_d by the TLC method. The crude enzyme from *Clostridium thermocopriae*¹²⁾ and *Bacillus* sp.JF¹⁶⁾ did not hydrolyze any glycoide of the ginsenoside Rb₁, Rb₂, R_c and R_d by the TLC and HPLC methods. The *β*-glucosidase (EC 3.2.1.21) from almond was badly recognized to distinctly hydrolyze the *β*-glucoside of ginsenoside Rb₁ and R_d, did not hydrolyze any glycoside of Rb₂ and R_c by the TLC and HPLC method.

The higher concentration glycosidase of the enzyme products from Novozymes, China such as AMG (glucosidase from mold) enzyme and Viscozymel (cellulase hemicellulase mixture) was cannot recognized to hydrolyze distinctly the any glycoside of the ginsenoside Rb₁, Rb₂, R_c and R_d by the TLC and HPLC methods.

Above enzyme reaction was colligated to Table 2.

The ginsenosidase type I hydronglyze the multi-glycosides

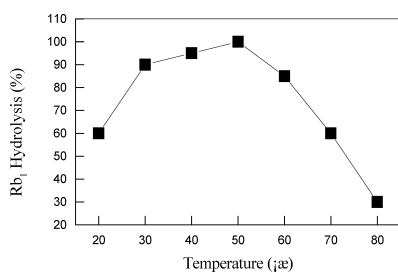


Fig. 10. Temperature Effect on Enzyme Reaction
Enzyme, about 100 U/ml; reacted in 0.5% Rb₁, pH 5.0 for 16 h.

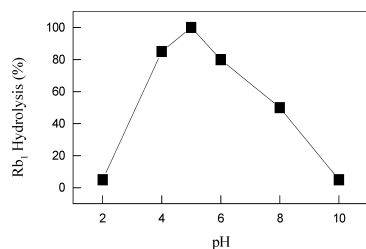


Fig. 11. The pH Effect on Enzyme Reaction
Enzyme, about 100 U/ml; reacted in 0.5% Rb₁, at 40 °C for 16 h.

of 20- and 3-position in PPD type ginsenoside, does not hydrolyze the glycoside of PPT type ginsenoside; *i.e.*, the enzyme properties in hydrolyzing ginsenoside-glycoside depends on the types of ginsenoside aglycone, without recognizing glycoside substructure; can hydrolyze multi-glycoside such as glucoside, arabinoside and xyloside of PPD type ginsenosides. These ginsenosidase type I properties differentiate it with that of glycosidases: one type enzyme hydrolyzed one type glycoside, described in Enzyme Nomenclature by NC-IUBMB (Nomenclature Committee of the International Union Biochemistry and Molecular Biology described in <http://www.qmul.ac.uk/iubmb/enzyme>).

Temperature Effect The ginsenosidase type I from fraction 43 was stable in 20 to 40 °C, the activity reducing was not recognized for 48 h at 20 to 40 °C. The optimum temperature was 50 °C (ginsenoside Rb₁ hydrolysis as shown in Fig. 10), but the enzyme no stable at 50 °C for long time; therefore, the temperature of enzyme reaction was used at 40 °C. The temperature effect on ginsenosidase type I hydrolysis of ginsenoside Rb₂, Rb₃, Rc and Rd was similar with the ginsenoside Rb₁.

Metal Ion and pH Effect The ginsenosidase type I was stable in pH 4.5 to 7.0. The optimum pH on ginsenoside Rb₁

hydrolysis by enzyme was pH 5.0 (Fig. 11); the pH effect on enzyme hydrolysis of ginsenoside Rb₂, Rb₃, Rc and Rd was similar with the ginsenoside Rb₁.

The 50 to 100 mM Ca⁺⁺ and Mg⁺⁺ ions slightly activated the ginsenosidase type I activity, but the Cu⁺⁺ and Pb⁺⁺ ions inhibited the enzyme activity.

It is shown from above experiments that the ginsenosidase type I hydrolysis on ginsenoside selected ginsenoside-aglycone: hydrolyzed multi-glycoside of PPD type ginsenoside, not hydrolyzed the glycoside of PPT type ginsenoside. But the enzyme hydrolysis not selection on glycoside kinds, can hydrolyzed multi-glycosides such as β-glucoside, β-xyloside and α-arabinoside of PPD type ginsenosides such as Rg₃, Rb₁, Rb₂, Rb₃, Rc and Rd. These results describe its novel properties to those of the other previously described glycosidases (On type enzyme hydrolyzed one kind glycoside).

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