Purification and Characterization of Ginsenoside-β-Glucosidase from Ginseng

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The ginsenoside-β-glucosidase that hydrolyzes the β-(1→2)-glucoside of the ginsenoside Rg3 sugar moiety to ginsenoside Rh2 was isolated from the ginseng root, and the enzyme was purified and characterized. The enzyme was purified to one spot in SDS polyacrylamide gel electrophoresis, and its molecular weight was about 59 kDa. The optimum temperature of the ginsenoside-β-glucosidase was 60 °C, and the optimum pH was 5.0. Ca^{2+} ion had positive effect on ginsenoside-β-glucosidase, while Cu^{2+} had negative effect on it. The ginsenoside-β-glucosidase may be a special β-glucosidase that is different from the original exocellulase such as β-glucosidase (EC 3.2.1.21).

Key words 20(*S*)-ginsenoside Rh2; ginsenoside-β-glucosidase; ginseng enzyme; saponin enzyme

Ginseng, the root of *Panax ginseng* C. A. MEYER, is a medicinal plant used worldwide and has been reported to have various biological effects.[1—3] Ginsenosides isolated from ginseng are the main pharmacoactive molecules of ginseng.[4] They exhibit efficacy as anticancer, antistress, antihypertention, antidiabetes, and antinociception agents, and facilitate learning and improve weakness as tonics.[5] More than 34 ginsenosides, triterpene-derivatives containing sugars, have been isolated from the ginseng saponin fraction. The chemical structures of the individual ginsenosides have been identified.[6—12] Based on the ginsenoside aglycone, the ginsenosides can be classified into three groups: the protopanaxadiol-type; protopanaxatriol-type; and oleanolic acid-type saponins.[13]

Recently, ginsenoside Rh2, a rare saponin contained in wild ginseng and red ginseng, has attracted attention because of its high inhibition of cancer cell growth.[14] The ginsenoside Rh2 inhibited the growth of B16 melanoma cells,[15,16] and inhibited the tumor growth of human ovarian cancer cells inoculated into nude mice to the same extent as *cis*-diamminedichloroplatinum.[17,18] However, it is very difficult to obtain the ginsenoside Rh2 from wild and red ginseng, because its content is low. We reported that ginsenoside Rh2 was obtained from ginsenoside-β-glucosidase from the newly isolated microorganism FFCDL-48 strain by hydrolyzing the other protopanaxadiol saponins such as ginsenoside Rg3 into ginsenoside Rh2,[19] as shown in Fig. 1.

In this study, the ginsenoside-β-glucosidase in the ginseng plant which hydrolyzes the β-(1→2)-glucoside bond of ginsenosides was isolated, purified, and characterized.

Experimental

Materials The ginseng root used was 4-year-old ginseng from the No. 1 Ginseng Plant of Fusong, Jilin Province, China. The standard 20(*S*)-ginsenoside Rg3, 20(*S*)-ginsenoside Rh2, and other ginsenosides were obtained from the Academy of Science of D.P.R. Korea. The thin-layer chromatography (TLC) plate was a silica gel plate (Kieselgel 60 F-254, Merck).

Enzyme Extraction from Ginseng The fresh ginseng was broken into pieces and added to a 3-fold volume of 20 mM acetate buffer, pH 5.0. It was extracted at 40 °C for 2 h and the supernatant was obtained by centrifugation for use in saponin enzyme purification.

Purification of Ginsenoside-β-Glucosidase and Estimation of Molecular Weight Pellets of $(NH_4)_2SO_4$ were slowly added to the supernatant extracted from fresh ginseng with shaking to 70% saturation and stored at 4 °C overnight. The mixture was centrifuged to collect the protein precipitate. This crude protein was dissolved in distilled water and dialyzed against 20 mM acetate buffer, pH 5.0. After removing the nondissolved fraction by centrifugation, the enzyme solution was fractionated on a column (φ1.5×10 cm) of DEAE-cellulose DE-52 (Whatman). The column was eluted stepwise with 60, 120, 180, and 240 mM KCl in 20 mM Tris–HCl buffer, pH 7.4. The purified enzyme was dried by freeze-drying and used for the study of enzyme properties. The enzyme purity and molecular weight were estimated by the SDS polyacrylamide gel electrophoresis method using the carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), and Taq polymerase (99 kDa) as standard proteins.[20]

Analysis of Enzyme Activity Ginsenoside-β-glucosidase activity was measured using ginsenoside Rg3 0.15 mg/ml in 20 mM acetate buffer, pH 5.0 as the substrate. Enzyme solution 0.1 ml was added to the same volume of ginsenoside Rg3 solution and allowed to react at 60 °C for 24 h. Then butanol 0.2 ml was added to the reaction mixture. The product ginsenoside Rh2 in the butanol layer was detected by TLC.[21] The ginsenoside Rh2 on the silica plate was determined by scanning the TLC spots[14] using a Shimadzu CS-930. One unit of enzyme activity was defined as the amount of enzyme producing 1 nmole of ginsenoside Rh2 per h.

Exocellulase β-glucosidase (EC 3.2.1.21) activity was determined by a colorimetric method using *p*-nitrophenyl-β-glucoside (PNPG) as the substrate.[22] One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per h.

Concentration of Protein The concentration of protein was measured by the method of Lowry *et al.*[23]

Structure of Ginsenoside from Enzyme Reaction The structure of ginsenoside produced from Rg3 enzyme reaction was detected by the nuclear magnetic resonance (NMR) spectrum method using Bruker DR×400 with pyridine-d_5 as the solvent. The mass spectrum was determined using a JEOL DX×303 instrument and bombarding the sample with the fast-atom bombardment mass spectrometry (FAB-MS).

Results and Discussion

Enzyme Purification When the concentration of $(NH_4)_2SO_4$ reached 70% saturation, most of the saponin enzyme was precipitated. The precipitates were centrifuged to collect the protein, and the crude protein was dissolved, dia-

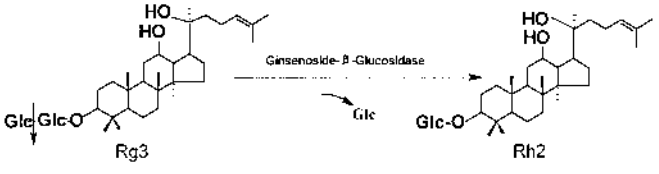

Fig. 1. Hydrolysis of Ginsenoside Rg3 by Ginsenoside-β-Glucosidase

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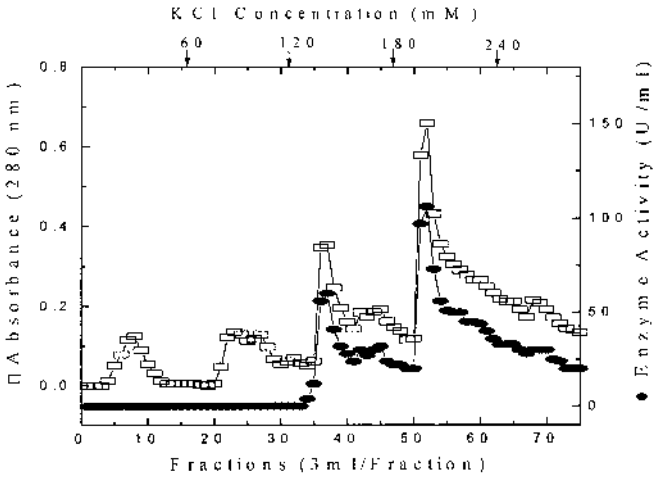

Fig. 2. Purification of Ginsenoside-β-Glucosidase on DEAE-Cellulose DE-52

Column, ϕ1.5×10 cm; fraction, 3 ml/tube; elution buffer, 60, 120, 180, and 240 mM KCl in 20 mM Tris–HCl buffer (pH 7.4).

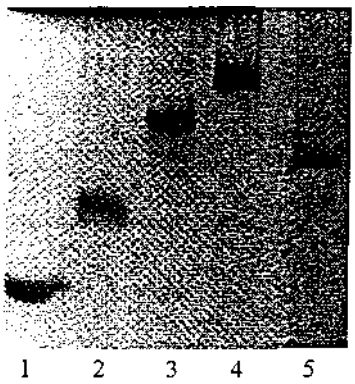

Fig. 3. SDS-Polyacrylamide Gel Electrophoresis of Ginsenoside-β-Glucosidase

1, carbonic anhydrase (29 kDa); 2, ovalbumin (43 kDa); 3, bovine serum albumin (66 kDa); 4, taq polymerase (99 kDa); 5, ginsenoside-β-glucosidase.

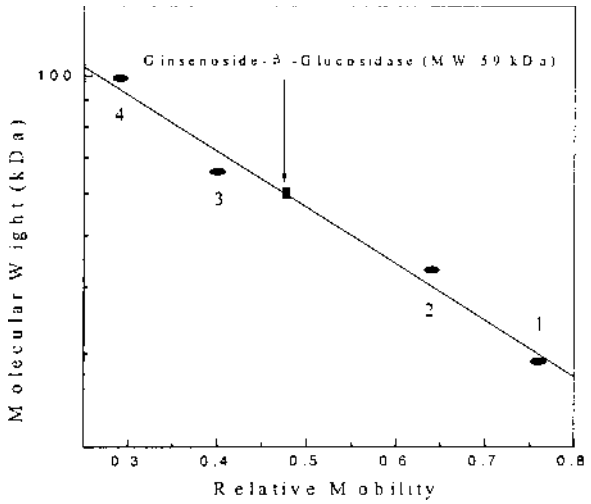

Fig. 4. Molecular Weight of Ginsenoside-β-Glucosidase on SDS-Polyacrylamide Gel Electrophoresis

1, carbonic anhydrase (29 kDa); 2, ovalbumin (43 kDa); 3, bovine serum albumin (66 kDa); 4, taq polymerase (99 kDa).

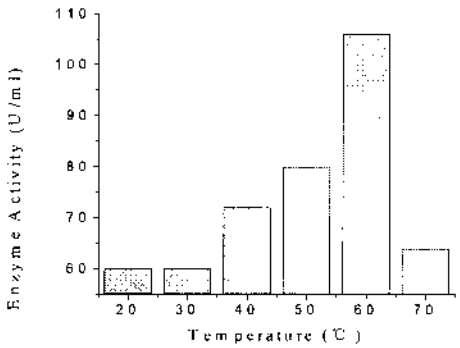

Fig. 5. Optimum Temperature of Ginsenoside-β-Glucosidase

Buffer, 20 mM acetate buffer; temperature, 60 °C.

Table 1. Purification of Ginsenoside-β-Glucosidase

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification
Extraction of ginseng	1800	13287	1408	9.4	100	1
$(NH_4)_2SO_4$ precipitation	100	10629	959	11.1	80	1.2
DEAE-cellulose	18	1913	12.9	148	14.4	16

lyzed, and fractionated on a DEAE-cellulose column. The result is shown in Fig. 2. The enzyme peak eluted by the step of 180 mM KCl solution hydrolyzed ginsenoside Rg3, and formed one spot in polyacrylamide gel electrophoresis, confirming that this enzyme was a pure protein. The enzyme peak eluted by the step of 120 mM KCl solution had ginsenoside hydrolysis activity, but was not a pure protein. The pure enzyme activity (yield) was 14.4% of total enzyme used in the purification, and the specific activity of pure saponin enzyme was 148 U/mg, an increase of approximately 16 fold (Table 1).

Enzyme Molecular Weight and Other Properties The purified saponin enzyme from the DEAE-cellulose column formed one spot in SDS polyacrylamide gel electrophoresis (Fig. 3), and its molecular weight is about 59 kDa (Fig. 4), which is different from the 34 kDa of the ginsenoside-β-glucosidase from the FFCDL-48 strain.[19)]

The saponin enzyme of the ginseng plant is stable at less than 60 °C, and its optimum temperature is 60 °C (Fig. 5), but the optimum temperature of the enzyme from the FFCDL-48 strain is 50 °C.[19)] The optimum pH of the ginseng enzyme is 5.0 (Fig. 6), the same as that of the FFCDL-48 strain enzyme.[19)]

The effects of metallic ions on the saponin enzyme are summarized in Table 2. Mg^{2+}, Fe^{3+}, and Zn^{2+} ions have no significant effect on the activity of the enzyme, Ca^{2+} ion has

a positive effect, while Cu^{2+} ion has a negative effect.

Hydrolysis of Ginsenosides by Ginsenoside-β-Glucosidase Ginsenoside Rg3 was dissolved in 20 mM acetate buffer, pH 5.0 (0.15 mg/ml), and purified enzyme solution 0.1 ml was added to the same volume of ginsenoside solution and allowed to react at 60 °C for 24 h. Then butanol 0.2 ml was added to the reaction mixture with shaking. The products in the butanol layer were detected by TLC (Fig. 7). The results showed that ginsenoside Rg3 belonging to the protopanaxadiol-type saponin can be hydrolyzed by the enzyme. It can be concluded that there the β-glucosidase present in ginseng hydrolyzes the β-(1→2)-glucoside bond of ginsenosides. We therefore called it ginsenoside-β-glucosidase.

Comparison between Ginsenoside-β-Glucosidase and β-Glucosidase Ginsenoside-β-glucosidase, β-glucosidase (EC 3.2.1.21) from almonds (Sigma), or exocellulase β-glucosidase (EC 3.2.1.21) from the *Clostridium thermocopriae* JT3-3 strain[23] solution 0.1 ml were respectively added to the same volume of ginsenoside Rg3 solution (0.15 mg/ml) in 20 mM acetate buffer, pH 5.0, and then the mixtures were reacted at 60 °C for 24 h. After reaction, butanol 0.2 ml was added to the reaction mixture, shaken, and stored to dissolve the ginsenoside in the butanol layer. The saponins in the butanol layer were detected to examine ginsenoside Rh2 production by TLC (Fig. 7). It was shown that ginsenoside-β-glucosidase from ginseng hydrolyzes over 60% of Rg3 to Rh2, but the β-glucosidase from almonds and from the *C. thermocopriae* strain do not hydrolyze Rg3. This indicates that the saponin enzyme from the ginseng plant may be a special β-glucosidase, β-(1→2)-ginsenoside hydrolase.

Comparing the hydrolysis of different *p*-nitrophenyl-β-saccharosides such as glucoside, galactoside, arabinoside, xyloside, and rhamnoside, the β-glucosidase from the *C. thermocopriae* strain can only hydrolyze the PNPG, and the enzyme from almonds can hydrolyze all the substrates except for *p*-nitrophenyl-β-rhamnoside (Table 3). However, the enzyme from the ginseng plant only slightly hydrolyzes these substrates when using more than 100 U/ml of enzyme. This demonstrates that ginsenoside-β-glucosidase may be a special β-glucosidase that is different from the original exocellulase such as β-glucosidase (EC 3.2.1.21).

Structure of Ginsenoside from Rg3 Enzyme Reaction The ginsenoside from the Rg3 enzyme reaction was separated on a silica gel column and crystallized in 80% methanol-water. The TLC *Rf* of crystal ginsenoside was the same as that of ginsenoside Rh2. The crystal (mp, 218—220 °C; $[\alpha]^{25}$, +21.8° [c=0.93, MeOH]) was the same as

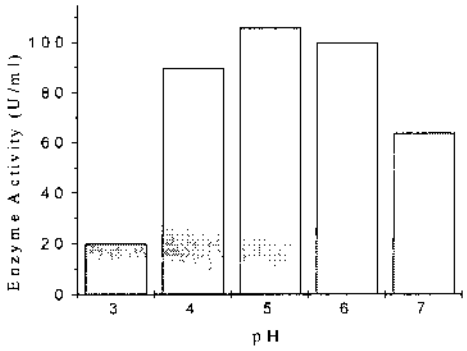

Fig. 6. Optimum pH of Ginsenoside-β-Glucosidase
Buffer, 20 mM acetate buffer; pH 5.0.

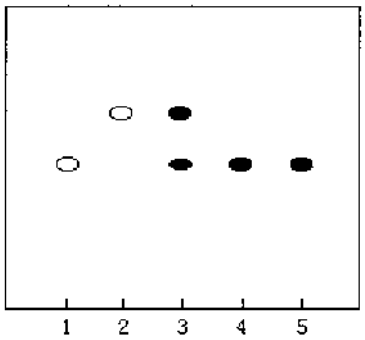

Fig. 7. Thin-Layer Chromatogram of Ginsenoside Rg2 and Rg3 Hydrolyzed by Ginsenoside-β-Glucosidase and β-Glucosidase
Developing solution, $CHCl_3$: MeOH : H_2O=70 : 30 : 5. 1, ginsenoside Rg3; 2, ginsenoside Rh2; 3, hydrolysis of ginsenoside Rg3 by ginsenoside-β-glucosidase; 4, hydrolysis of ginsenoside Rg3 by β-glucosidase from almonds; 5, hydrolysis of ginsenoside Rg3 by β-glucosidase from *C. thermocopriae* strain.

Table 2. Effect of Metallic Ion on Ginsenoside-β-Glucosidase (Relative Activity %)

Concentration (mM)	$CaCl_2$	$MgSO_4$	$FeCl_3$	$ZnSO_4$	$CuSO_4$
0	100	100	100	100	100
1	—	—	—	96	69
5	118	96	100	90	49
10	125	87	100	90	18
50	136	90	95	—	—
100	130	90	90	—	—

— Not determined.

Table 3. Enzyme Hydrolysis of Different Standard Substrates

Substrate	Activity of ginsenoside β-glucosidase (U/ml)	Activity of β-glucosidase from almonds (U/ml)	Activity of β-glucosidase from *C. thermocopriae* strain (U/ml)
PNPG	1.28	6.26	4.92
p-Nitrophenyl-β-D-galactoside	0.67	6.29	0.26
p-Nitrophenyl-β-L-arabinoside	0.41	6.28	0.12
p-Nitrophenyl-β-D-xyloside	0.09	6.26	0.24
p-Nitrophenyl-α-L-rhamnoside	0	0	0

that of 20(*S*)-ginsenoside Rh2.

The ginsenoside crystal from the enzyme reaction was used to determine its structure in the Laboratory of Phytochemistry, Kunming Institute of Botany, Academia Sinica, P. R. China. The molecular formula of crystal gisenoside was analyzed as $C_{36}H_{62}O_8$ from the negative FAB-MS, in which there appeared a quasimolecular ion peak at *m*/*z* 621 $[M-H]^-$. The ^{1}H- and ^{13}C-NMR spectral data are the same as those of 20(*S*)-ginsenoside Rh2.[24] Therefore the ginsenoside from Rg3 is a 20(*S*)-ginsenoside Rh2, *i.e.*, a 3-*O*-(β-D-glucopyranosyl)-dammar-24-en-3β,12β,20(*S*)-triol.

As shown above, the ginseng root has a ginsenoside-β-glucosidase, the enzyme can hydrolyze the β-(1$\rightarrow$2)-glucoside bond of ginsenoside Rg3 to ginsenoside Rh2, and the enzyme molecular weight is 59 kDa.

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References

1) Nah S. Y., Park E. W., *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 8739—8743 (1995).
2) Baek N. I., Kim D. S., Lee Y. H., Park J. D., Lee C. B., Kim S. I., *Arch. Pharm. Res.*, **18**, 164—168 (1995).
3) Kim Y. H., Park K. H., Rho H. M., *Panax ginseng. J. Biol. Chem.*, **271**, 24539—24543 (1996).
4) Kaku T., Miyata T., Uruno T., Sako I., Kinoshita A., *Arzeim-Forsch* (*Drug Res.*), **25**, 343 (1975).
5) Nah S., *Korean J. Ginseng Sci.*, **21**, 1—12 (1997).
6) Shibata S., Tanaka O., Sado M., Iita Y., Tsushima S., *Tetrahedron Lett.*, **1963**, 795—800.
7) Shibata S., Tanaka O., Soma K., Iita Y., Ando T., Nakamura H., *Tetrahedron Lett.*, **1965**, 207—213.
8) Nagai Y., Tanaka O., Shibata S., *Tetrahedron Lett.*, **1971**, 881—892.
9) Kaku T., Kawashima Y., *Arzneimittelforschung*, **30**, 936—943 (1980).
10) Kitagawa I., Taniyama T., Hayashi T., Toshikawa M., *Chem. Pharm. Bull.*, **31**, 3353—3356 (1983).
11) Shao C. J., *Chung Yao Tung Pao*, **9**, 172—173 (1984).
12) Baek N. I., Kim D. S., Lee Y. H., Park J. D., Lee C. B., Kim S. I., *Planta Med.*, **62**, 86—87 (1996).
13) Sakamoto I., Morimoto K., Tanaka O., *Yakugaku Zasshi*, **95**, 1456—1461 (1975).
14) Park M. T., Cha H. J., Jeong J. W., Lee H. Y., Kim S. I., Baek N. I., Kim O. H., Kim K. W., *J. Ginseng Res.*, **22**, 216—221 (1998).
15) Ota T., Fujikawa-Yamamoto K., Zong Z. P., Yamazaki M., Odashima S., *Cancer Res.*, **47**, 3863—3867 (1987).
16) Ota T., Maeda M., Odashima S., *J. Pharm. Sci.*, **80**, 1141—1146 (1991).
17) Park J. A., Lee K. Y., Oh Y. J., Kim K. W., Lee S. K., *Cancer Lett.*, **121**, 73—81 (1997).
18) Nakada H., Kikuchi Y., Tode T., Hirata J., Kita T., Ishii K., Kudoh K., Nkata I., Shinomiya N., *Jpn. Cancer Res.*, **89**, 733—740 (1998).
19) Yu H. S., Ma X. Q., Guo Y., Jin F. X., *J. Ginseng Res.*, **23**, 50—54 (1999).
20) Weber K., Osborn M., *J. Biol. Chem.*, **244**, 4406—4412 (1969).
21) Zou C. J., Xu J. D., *Zhongcaoyao*, **13**, 355—359 (1982). (In Chinese)
22) Jin F. X., Tada K., *J. Ferment. Biotech.*, **67**, 81—83 (1989).
23) Lowry O. H., Rosobrough N. J., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
24) Chen Y. P., Meng Q., Song C. C., Ma X. Y., Wang G. S., Xu J. D., *Zhongguo Yaoxue Zazhi*, **32**, 273—275 (1997). (In Chinese)