Purification and Characterization of Ginsenoside-α-L-Rhamnosidase

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In this paper the ginsenoside-α-(1→2)-L-rhamnosidase from microorganisms was purified and characterized. The enzyme hydrolyzed the 6-C, α-(1→2)-L-rhamnoside of 20(*S*) and 20(*R*)-ginsenoside Rg2 to produce the 20(*S*) and 20(*R*)-ginsenoside Rh1, but hardly hydrolyzed the α-rhamnoside of *p*NPR. The enzyme molecular weight was about 53 kDa. The optimum temperature of enzyme reaction was 40 °C, and the optimum pH was 5.

Key words ginsenoside-α-(1→2)-L-rhamnosidase; ginsenoside Rg2; ginsenoside Rh1

Ginseng, an ancient and famous herbal drug in oriental traditional medicines, has been used for more than 4000 years. Ginsenosides, dammarane saponins of ginseng, are one of the major physiologically active materials of ginseng, and over 40 kinds[1,2] are known. Those belonging to the ginsenoside aglycone, are classified in three types: protopanaxadiol type ginsenosides Ra1, Ra2, Ra3, Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rg5, Rh2 and Rh3; protopanaxatriol type ginsenosides Re, Rg1, Rg2, Rg4, Rh1 and Rh4; and the oleanolic acid type saponin Ro.

The main ginsenosides in cultivated ginseng are Rb1, Rb2, Rc, Rd, Re and Rg1; Rg3, Rg5, Rh2, Rh3, Rg2, Rg4, Rh1 and Rh4 are rare ginsenosides contained in red ginseng or wild ginseng. Recently, the rare ginsenosides have attracted attention, because they have higher physiological activity that is impotent in health food and medicine.[3] For example, the rare ginsenosides Rh2, Rh3 and Rh1 have an anticancer property; Rg3 has properties of anticancer and anti-thrombus, as is also ginsenoside Rg2[3] has properties of anticancer and anti-thrombus. It is difficult however, to obtain the rare ginsenosides from red ginseng or wild ginseng as their content is low.

To obtain rare ginsenoside, the sugar-moiety modification by enzymes of those present in higher amounts at cultivated ginseng is one possible method.[4] Our laboratory previously reported the ginsenoside-β-glucosidase hydrolyzing 3-C β-(1→2)-glucoside of ginsenoside Rg3 to ginsenoside Rh2 from the microorganisms of the ginseng plant.[5,6] In this paper, the ginsenoside-α-L-rhamnosidase hydrolyzing ginsenoside Rg2 to ginsenoside Rh1 (as shown in Fig. 1) was purified and characterized.

Experimental

Materials The 20(*S*) and 20(*R*)-ginsenoside Rg2, 20(*S*) and 20(*R*)-ginsenoside Rh1 were obtained from Prof. Longyun Li, The Jilin Institute of Traditional Medicine, Changchun, P. R. China. The ginsenoside enzyme microorganism, *Absidia* sp.39 (FFCDL-39) strain was isolated from the traditional Chinese Koji, Gaowen Daqu (**高温大麹**), and bred in our laboratory in 1995 (FFCDL, Food Fermentation Culture Collection of The Dalian Institute of Light Industry).

A thin-layer chromatography (TLC) plate was the silica gel plate (Kieselgel 60 F-254, Merck).

Enzyme Preparation The FFCDL-39 strain was cultured at 28 to 30 °C shaking in medium containing 4% malt extraction and 1.5% ginseng water extraction for 3 to 4 d. The culture was centrifuged to remove cells, then the pellets of $(NH_4)_2SO_4$ were slowly added to the supernatant 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 non-dissolved fraction by centrifugation, the enzyme solution was fractionated by the protein chromatography: instrument, BioRad BioLogic Medium Pressure Chromatography: Column, Bio-scale Q_2; solvent A, 25 mM Tris–HCl buffer, pH 8.2; solvent B, 25 mM Tris–HCl + 1 M NaCl buffer, pH 8.2; gradient, 0 to 80% B solution in 30 min. The purified enzyme was freeze-dried and used for the next experiment.

Enzyme Molecular Weight The enzyme molecular weight was estimated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis using Carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), and phosphorylase b (97 kDa) (Sigma products) as standard

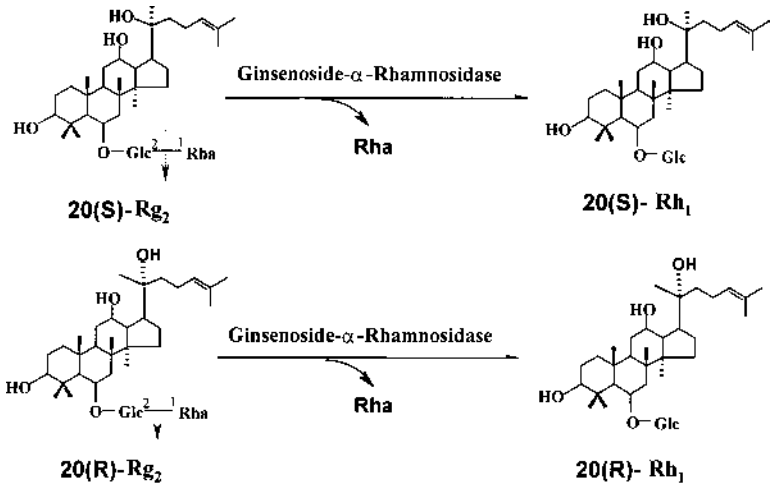

Fig. 1. Hydrolysis of 20(*S*) and 20(*R*)-Ginsenoside Rg2 by Ginsenoside-α-Rhamnosidase

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proteins.[7]

Protein Concentration The concentration of protein was measured by the Folin phenol reagent method of Lowry *et al.* using bovine serum albumin as a standard omit.[8]

Enzyme Analysis Ginsenoside-α-L-rhamnosidase: ginsenoside-α-L-rhamnosidase activity was measured using 20(*S*) or 20(*R*) ginsenoside Rg2 2.0 mg/ml in 20 mM acetate buffer, pH 5.0 as the substrate. One tenth milliliter of enzyme solution was added to the same volume of ginsenoside Rg2 solution and allowed to react at 40 °C for 10 to 20 h. Then butanol 0.2 ml was added to stop the reaction. The produced ginsenoside Rh1 was removed to the butanol layer, and the production was carried out by TLC; the solvent, $CHCl_3 : MeOH : H_2O = 70 : 30 : 5$ v/v; and the produced ginsenoside Rh1 on the silica plate was determined by scanning the TLC spots using a Shimadzu CS-930.[9] One unit of enzyme activity was defined as the amount producing 1nmole of ginsenoside Rh1 per hour.

α-Rhamnosidase activity. The α-rhamnosidase activity was determined by a colorimetric method using 2 mM *p*-nitrophenyl-α-rhamnoside (*p*NPR) as a substrate.[10] One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per hour.

Ginsenoside Structure The ginsenoside Rh1 produced from the enzyme reaction was purified by a 300 mesh silica gel (product from Qingdao Chemical Plant, China) column. The structure of ginsenoside produced from the enzyme reaction was detected by the nuclear magnetic resonance (NMR) spectrum method using a 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 fast-atom bombardment mass spectrometry (FAB-MS).

Results and Discussion

Enzyme Fermentation The behavior of enzyme fermentation by the FFCDL-39 strain is shown in Table 1.

As shown, cell growth reached maximum after fermenting for 60 h, but higher enzyme production was obtained after fermenting for 84 h. Therefore, the fermentation was carried out for 84 h, the culture was centrifuged to remove the cells, and the supernatant was used for enzyme purification.

Enzyme Purification When the concentration of $(NH_4)_2SO_4$ reached 70% saturation, most ginsenoside-α-L-rhamnosidase was precipitated. Therefore, pellets of $(NH_4)_2SO_4$ were slowly added to the enzyme culture supernatant to 70% saturation, stored at 4 °C overnight, and protein precipitate was collected by centrifuging. The collected crude enzyme protein was dissolved and dialyzed. After removing the non-dissolved fraction by centrifugation, the saponin enzyme was purified with the instrument for BioRad protein chromatography as shown in Fig. 2. Fractions 14 and 15 had ginsenoside-α-L-rhamnosidase activity, and the purified enzyme was one spot in the SDS electrophoresis.

In the purification, the enzyme yield was 2.8% as shown in Table 2. The purified enzyme was dried by freeze-drying and used to study enzyme properties.

Enzyme Molecular Weight The enzyme purity and molecular weight were estimated by SDS polyacrylamide gel electrophoresis[7] as shown in Figs. 3 and 4. The purified enzyme was one spot in the electrophoresis showing that the enzyme was a pure protein, and the molecular weight was about 53 kDa.

Enzyme Properties The enzyme reactions are shown in

Table 1. Ginsenoside-α-Rhamnosidase Production

Fermentation time (h)	0	12	24	36	48	60	72	84	96
Cell (mg/ml)	0.1	1.3	4.0	10.2	12.5	15.0	13.3	14.0	13.8
Substrate (maltose mg/ml)	20	18	22	7.6	4.2	3.9	2.1	1.8	1.7
Enzyme (U/ml)	0	0.3	3.7	4.9	7.9	25	40	45	39

Fermented at 28 to 30 °C by shaking.

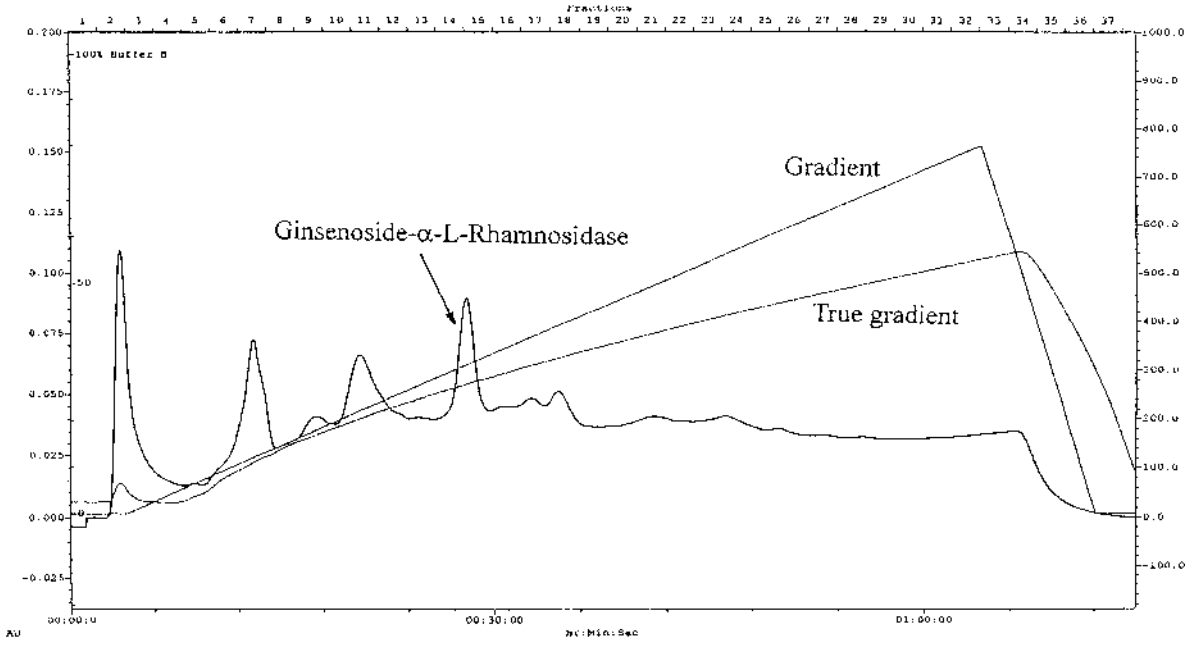

Fig. 2. Purification of Ginsenoside-α-Rhamnosidase in BioRad Protein Chromatography

Column, Bio-scale Q_2; solvent A, 25 mM Tris–HCl buffer, pH 8.2; solvent B, 25 mM Tris–HCl + 1 M NaCl buffer, pH 8.2; gradient, 0 to 80% solution B in 30 min.

Table 2. Ginsenoside-α-L-Rhamnosidase Purification

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification
Fermentation	100	4570	307	14.9	100	1.0
$(NH_4)_2SO_4$ precipitation	10.0	392	19.1	20.6	8.6	1.4
Chromatography	4.0	130	2.2	60.0	2.8	4.0

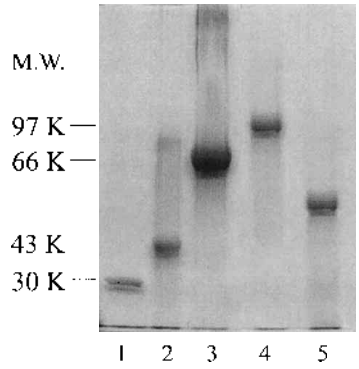

Fig. 3. SDS–Polyacrylamide Gel Electrophoresis of Ginsenoside-α-Rhamnosidase

1, carbonic anhydrase (29 or 30 kDa); 2, ovalbumin (43 kDa); 3, bovine serum albumin (66 kDa); 4, phosphorylase b (97 kDa); 5, ginsenoside-α-rhamnosidase.

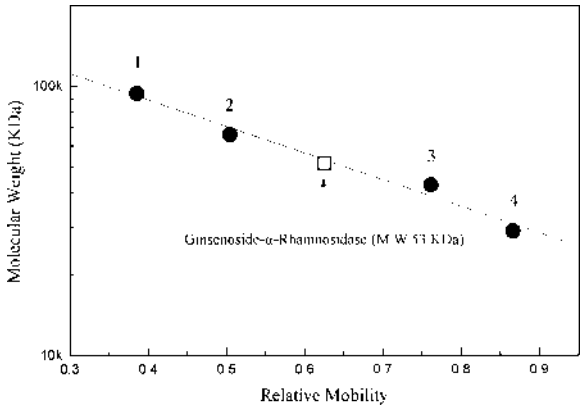

Fig. 4. Molecular Weight of Ginsenoside-α-Rhamnosidase on SDS–Polyacrylamide Gel Electrophoresis

1, phosphorylase b (97 kDa); 2, bovine serum albumin (66 kDa); 3, ovalbumin (43 kDa); 4, carbonic anhydrase (29 or 30 kDa).

Table 3. Enzyme Reaction of Different Substrates

Substrate	20(*S*)-Ginsenoside Rg2	20(*R*)-Ginsenoside Rg2	*p*NPR
Pure enzyme (U/ml)	33.6	32.6	1.8
Fermented enzyme (U/ml)	45.7	44.0	23.8

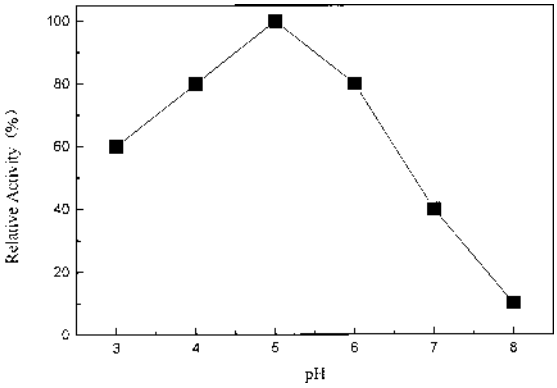

Fig. 5. Optimum pH of Ginsenoside-α-Rhamnosidase

100% enzyme activity, 30 U/ml; reaction temperature, 40 °C.

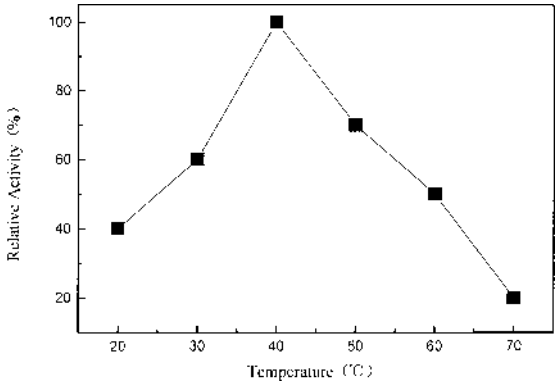

Fig. 6. Optimum Temperature of Ginsenoside-α-Rhamnosidase

100% enzyme activity, 30 U/ml; reaction pH, 5.0.

Table 3. The fermented enzyme has ginsenoside-α-L-rhamnosidase activity and *p*NPR α-L-rhamnosidase activity, but the purified enzyme has only the former activity, and the latter activity is very low, proving this is a special enzyme. The ability of ginsenoside-α-rhamonosidase to hydrolyze the α-L-rhamnoside of 20(*S*) and 20(*R*) ginsenoside Rg2 was nearly.

The pH effect on enzyme reaction was examined at 3, 4, 5, 6, 7, and 8 pH, the optimum pH of ginsenoside-α-rhamnosidase was 5 (Fig. 5), and the enzyme was stable at pH 4 to 7.

The effect of temperature on enzyme reaction is shown in Fig. 6. The optimum temperature of the ginsenoside-α-rhamnosidase was 40 °C, and the enzyme was stable at 30 to 40 °C.

Structure of Ginsenoside from Enzyme Reaction The ginsenoside from the 20(*S*) and 20(*R*)-ginsenoside Rg2 by the enzyme reaction was separated using a silica gel column and crystallized in cold methanol after dissolution in hot

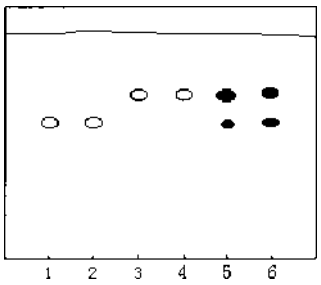

Fig. 7. Thin Layer Chromatogram of Hydrolyzing 20(*S*) and 20(*R*)-Ginsenoside Rg2 by Ginsenoside-α-Rhamnosidase

Developing solvent, $CHCl_3$: MeOH : H_2O = 70 : 30 : 5. 1, 20(*S*)-ginsenoside Rg2; 2, 20(*R*)-ginsenoside Rg2; 3, 20(*S*)-ginsenoside Rh1; 2, 20(*R*)-ginsenoside Rh1; 5, hydrolyzing 20(*S*)-ginsenoside Rg2 by enzyme; 6, hydrolyzing 20(*R*)-ginsenoside Rg2 by enzyme.

methanol.[11] The TLC *Rf* of ginsenosides from the enzyme reaction was the same as that of ginsenoside Rh1 (Fig. 7).

Purified ginsenosides from the enzyme reaction were used to determine its structure in the Laboratory of Phytochemistry, Kunming Institute of Botany, Academia Sinica, P. R. China. The ^{1}H- and ^{13}C-NMR spectral data are the same as those of reference 20(*S*) and 20(*R*)-ginsenoside Rh1.[12] Therefore, the ginsenoside from 20(*S*)-ginsenoside Rg2 by the enzyme reaction was 20(*S*)-ginsenoside Rh1, and the ginsenoside from 20(*R*)-ginsenoside Rg2 by the enzyme reaction was 20(*R*)-ginsenoside Rh1.

The above experiment showed that the ginsenoside-α-L-rhamnosidase from FFCDL-39 strain hydrolyzed the α-(1→2)-L-rhamnoside of 6-C ginsenoside Rg2, but hardly hydrolyzed the α-rhamnoside of *p*NPR; the product from 20(*S*)-ginsenoside Rg2 by enzyme reaction was 20(*S*)-ginsenoside Rh1, and the product from the 20(*R*)-ginsenoside Rg2 was 20(*R*)-ginsenoside Rh1. The molecular weight of ginsenoside-α-L-rhamnosidase was about 53 kDa.

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