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Purification and Characterization of Ginsenoside-β-Glucosidase

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Abstract : In this paper, the saponin enzymatic hydrolysis of ginsenoside Rg3 was studied. The ginsenoside β-glucosidase from FFCDL-48 strain mainly hydrolyzed the ginsenoside Rg3 to Rh2, the enzyme from FFCDL-00 strain hydrolyzed Rg3 to the mixture of Rh2 and protopanaxadiol (aglycon). The ginsenoside β-glucosidase from FFCDL-48 strain was purified with a column of DEAE-Cellulose to one spot in the SDS polyacrylamide gel electrophoresis. During the purification, the enzyme specific acitvity was increased about 10 times. The purified ginsenoside β-glucosidase can hydrolyze the Rg3 to Rh2, but do not hydrolyze the p-nitrophenyl-β-glucoside which is a substrate of original exocellulase such as β-glucosidase of cellulose. The molecular weight of ginsenoside β-glucosidase was 34,000, the optimal temperature of enzyme reaction was 50℃, and the optimal pH was 5.0.

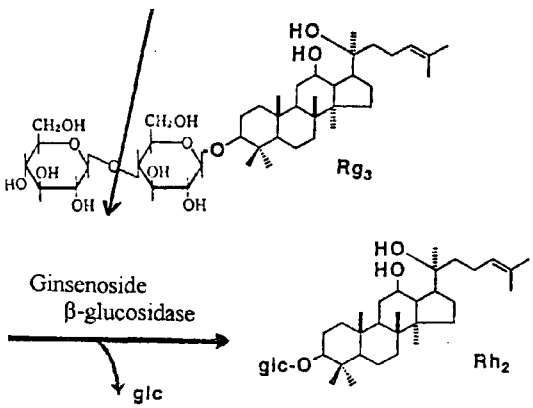
Key words : Ginsenoside Rg3, Rh2, ginsenoside β-glucosidase, ginsenoside hydrolysis

Introduction

Ginsenosides are one of the major physiological avtivite materials in the ginseng, and the known ginsenosides are over 30 kinds.[1] Belong to the saponin aglycon, ginsenosides are classified to three types, *i.e.* protopanaxadiol type saponin such as Ral, Ra2, Ra3, Rbl, Rb2, Rb3, Rc, Rd, Rg3 and Rh2; protopanaxatriol type saponin such as Re, Rf, Rgl, Rg2 and Rhl; and oleanolic acid type saponin such as Ro.[2]

Recently, the ginsenoside Rh2 and Rhl which contained in the red ginseng has attracted attention, because the ginsenoside Rh2 and Rhl highly restrain the caner cell.[3,4] However, it is difficult to obtain the ginsenoside Rh2 and Rhl from the natural ginseng, because its content in the natural ginseng is very low for the utilization in the medicine.

The ginsenoside Rh2 is a protopanaxadiol type saponin, the aglycon of ginsenoside Rh2 is same as the Rbl, Rc, Rd and Rg3. If the sugar-moiety of these ginsenosides were hydrolyzed by enzyme, the ginsenoside such as Rh2 can be easily produced. For example, the β-glucoside of ginsenside Rg3 was hydrolyzed by enzyme to obtain Rh2 as follows:



However, the original exo-cellulase such as β-

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glucosidase (EC 3.2.1.21) of cellobiose or cellulose can not hydrolyze the soponin β-glucoside, because the ginsenoside has the alycon-moiety which is different from that of the cellobiose or cellulose. Only special saponin enzyme can hydrolyze the β-glucoside of saponin.[5]

In this paper, the saponin enzyme which can hydrolyze the β-glucoside of ginsenoside Rg3 to the ginsenoside Rh2 was isolated and characterized.

Materials and Methods

1. Materials

The ginsenoside Rg3, Rh2 and protopanaxadiol aglycon were obtained from the Shenyang University of Phamaceutical Science, China; and from the Academy of Science of D.P.R. Korea.

The ginsenoside enzyme microorgainsm FFCDL-48 and FFCDL-00 strains were isolated and bred in our laboratory in 1995.[6]

(FFCDL, Food Fermentation Culture Collection of The Dalian Institute of Light Industry).

2. Enzyme Production

Microorganism culture: The microorgainsm of saponine enzyme was cultured at 28 to 30℃ with shaking in the medium containing 4% malt extraction and 2% ginseng extraction.

Ginsenoside β-glucosidase purification: The culture was centrifuged, and the $(NH_4)_2SO_4$ pellets were slowly added to the cell-free supernatant with shaking 60% saturation and stored at 4℃ overnight. The mixture was centrifuged to collect the protein precipitate. This crude protein was dissolved in distilled water and dialyzed in 20 mM acetate buffer, pH 5.0. After removal of the non-dissolved fraction by centrifuge, the enzyme solution was fractionated on a DEAE-cellulose DE 52 (Whatman) column (ϕ 1.4×6.7 cm). The column was eluted stepwise with 0.04, 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl in 0.02 M acetate buffer, pH 5.0, and the ginsenoside enzyme peaks was collected and dried by freeze-drying for the study of enzyme properties. The enzyme purification and molecular weight were estimated by the SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis.[7] The molecular weight of marker proteins (Sigma) were trysinogen, 24,000; egg albumin, 45,000; bovine serum albumin, 66,000; and Tag enzyme, 99,000.

3. Enzyme assays

The saponin enzyme (ginsenoside β-glucosidase) activity was measured using the 0.15 mg/m*l* of ginsenoside Rg3 in 0.02 mM acetate buffer, pH 5.0, as a substrate. The 0.1 m*l* of enzyme solution was added to same volume of ginsenoside Rg3 solution to react at 40℃ for 6 to 12 h. After reaction, 0.3 m*l* of butanol was added to the reaction mixture, and then mixed and centrifuged. The produced new ginsenoside Rh2 in the butanol layer was detected by the method of thin layer chromatography (TLC).[8] The ginsenoside Rh2 on the silica plate was determined by the method.[8] of scanning the TLC spots by the Shimadzu TLC Scanner CS-930. One unit of enzyme activity was defined as the amount of enzyme producing 1 nmole of ginsenoside Rh2 per hour.

The β-glucosidase (PNPG enzyme *i.e.* original cellulose β-glucosidase) activity was determined by a colorimetric method[9] using *p*-nitrophenyl-β-glucoside (PNPG) as a substrate. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μM of *p*-nitrophenol per hour.

4. Analysis method

The maltose concentration in the microorganism medium was measured by the method of the dinitrosalicylic acid reagent.[10]

The protein was determined by the method of Folin phenol reagent.[11]

Results and Discussion

1. Enzyme fermentation

To examine the behaviors of saponin enzyme production in the FFCDL-48 strain fermentation, the cell growth, enzyme production, and the maltose reducing were measured in all the fermentation process as shown in Fig. 1.

It is shown from Fig. 1 that the highest ginsenoside β-glucosidase production of FFCDL-48 strain occurred after fermentation for 35 to 45 h,

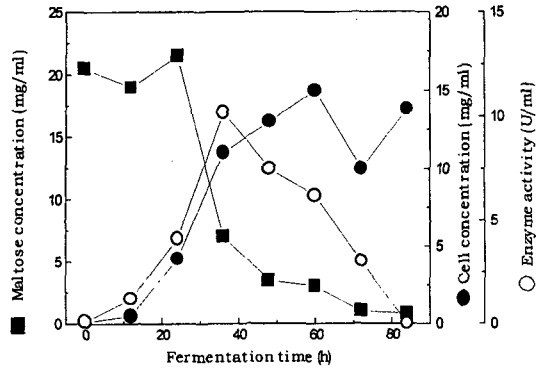
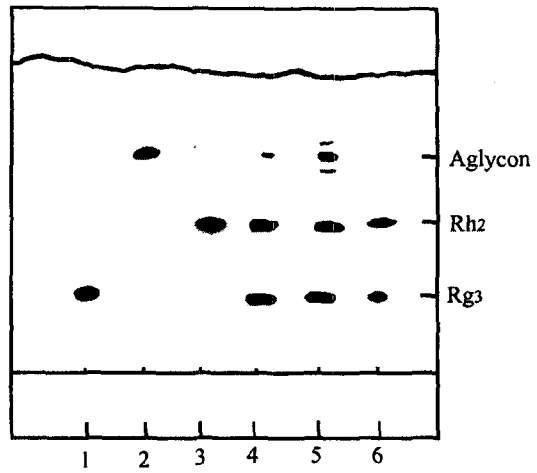


Fig. 1. Saponin enzyme fermentation behavior. fermentation was carried out at 28 to 30℃ with shaking.



1; Rg3, 2; protopanaxadiol, 3; Rh2, 4; Rg3 hydrolysis by enzyme of 48 strain, 5; Rg3 hydrolysis by enzyme of 00 strain, 6; Rg3 hydrolysis by purified enzyme

Fig 2. This layer chromatogram of Rg3 hydroly sate by saponin enzyme.

and the saponin enzyme production was reached to about 12 U/ml, but the enzyme activity was rapidly decreased after 55 h. The enzyme production was closely related with the cell growth, the saponin enzyme production was associated with the cell growth. So, the enzyme fermentation time was defined 35 to 45 h in our experiment.

The fermentation behavior of FFCDL-00 strain was similar with that of the FFCDL-48 strain.

2. Enzyme hydrolysis on Rg3 β-glucoside

The ginsenoside Rg3 has two β-glucoside bonds. The two possible pathways of hydrolysis of Rg3 glucoside by saponin enzyme can be considered. The one is that β-glucoside of Rg3 was hydrolyzed to Rh2 by the ginsenoside β-glucosidase. The other pathway is that the cellobioside of Rg3 was hydrolyzed to protopanaxadiol by ginsenoside cellobiosidase, or the β-glucoside of Rh2 was hydrolyzed to the protopanaxadiol.

The hydrolysis of ginsenoside Rg3 β-glucoside by the saponin enzyme produced from FFCDL-48 and FFCDL-00 strains is shown in Fig. 2. Fig. 2 shows that the saponin enzyme from the FFCDL-48 strain hydrolyzed Rg3 glucoside to Rh2, and produce very small amount of protopanaxadiol. The purified enzyme from FFCDL-48 produced only Rh2. But the enzyme from FFCDL-00 strain hy- drolyzed the Rg3 to the mixture of Rh2 and protopanxadiol. It can be considered that the FFCDL-00 strain produced the saponin enzyme which hydrolyze the Rg3 or Rh2 to the protopanxdiol. In this paper, therefore, only the saponin enzyme from FFCDL-48 strain was carried out in the other experiment.

3. Enzyme purification

The culture of FFCDL-48 strain was centrifuged, and the $(NH_4)_2SO_4$ pellets were slowly added to the cell-free supernatant. When the $(NH_4)_2SO_4$ pellets reached to 60% saturation, the most of saponin enzyme was precipitated. The precipitates were centrifuged to collect the protein, and the crude protein was dissolved, dialyzed, fractionated on the DEAE-cellulose column. In the fractionation of DEAE-cellulose column, the ginsenoside β-glucosidase was eluted by the step of 0.1 M NaCl (Fig. 3). The peaks of ginsenoside β-glucosidase was freeze-dried for the other experiment. During the purification, the specific activity of saponin enzyme was increased about 10 times and the enzyme yield was 0.29% (Table 1).

4. Saponin enzyme properties

The purified saponin enzyme from FFCDL-48 strain was tested to hydrolyze the ginsenoside Rg3. After enzyme reaction for 6 h, the 50 to 60% of Rg3 was hydrolyzed to Rh2 (Fig. 2). However, the purified enzyme did not hydrolyze the *p*-

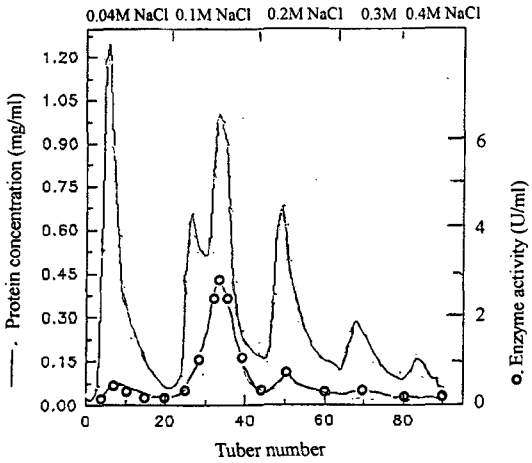


Fig. 3. Purification of saponin enzyme on a DEAE-cellulose column, cloumn size, ϕ 1.4×6.7 cm, Fraction, 2 ml/tube. Elution buffer, 0.04, 0.1, 0.2, 0.3 and 0.4 M NaCl in 0.02 M acetate buffer, pH 5.0.

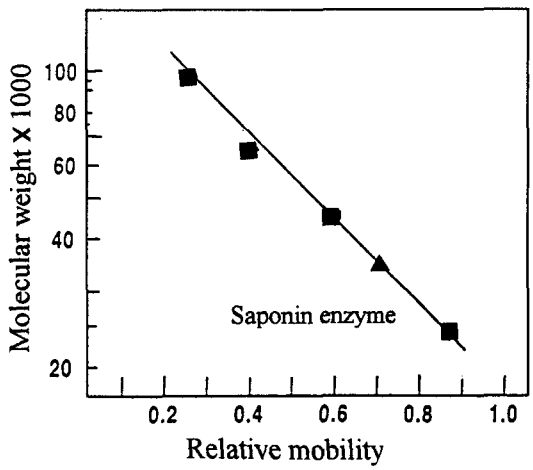


Fig. 4. Molecular weight of saponin enzyme in electrophoresis. egg abumin, 45,000 bovine serum albumin, 66,000, tag enzyme 99,000.

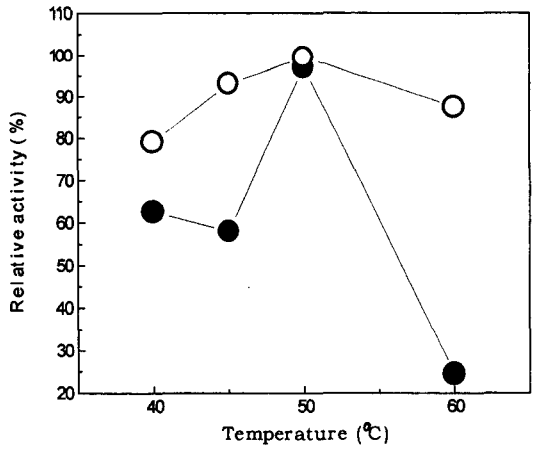


Fig. 5. Optimal temperature of saponin enzyme. ●; enzyme from FFCDL-48 strain, ◉; crude enzyme from FFCDL-00 strain, 100% enzyme activity, 2 U/ml, enzme reaction for 6 h.

nitrophenyl-β-glucoside, *i.e.* the saponin enzyme did not recognized the PNPG activity. Therefore, the saponin enzyme from FFCDL-48 strain is a special β-glucosidase, and the saponin enzyme is different from the original exo-cellulase such as β-glucosidase of cellulose.

The molecular weight of purified enzyme was also determined. In the SDS polyacrylamide gel electrophoresis, the enzyme was one spot, the molecular weight was about 34,000 (Fig. 4).

The optimal temperature of saponin enzyme from FFCDL-48 strain and crude enzyme from FFCDL-00 strain were 50℃ (Fig. 5); but the crude enzyme from FFCDL-00 strain was somewhat thermostable than that from FFCDL-48 strain. The optimal pH of saponin enzyme was 5.0 (Fig. 6).

In the reaction of ginsenoside β-glucosidase, only the partial ginsenoside Rg3 were hydrolyzed to the Rh2. When the enzyme reaction time was extended to 24 h, the yield of ginsenoside Rh2 did not increased than the product of 6 h reaction.

It is shown from above experiment that the ginsenoside β-glucosidase is a special β-glucosidase, the enzyme can hydrolyze one β-glucoside of ginseno-

Table 1. Purification of saponin enzyme

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Cuture supernatant	200	1200	357	3.4	100
$(NH_4)_2SO_4$ precipitation	10.4	101	18.7	5.4	52
DEAE-cellulose	3	36	1.05	35	0.29

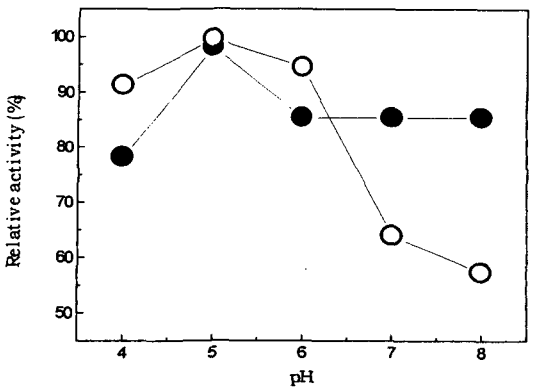


Fig. 6. Optimal pH of saponin enzyme ●; enzyme from FFCDL-48 strain, ○; crude enzyme from FFCDL-00 strain, 100% enzyme activity, 2 U/ml; enzyme reaction for 6 h.

side Rg3 to the ginsenoside Rh2, but do not hydrolyze the p-nitrophenyl-β-glucoside which is a substrate of original exo-cellulase such as β-glucosidase of cellulose.

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