Kinetics of a Cloned Special Ginsenosidase Hydrolyzing 3-O-Glucoside of Multi-Protopanaxadiol-Type Ginsenosides, Named Ginsenosidase Type III

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In this paper, the kinetics of a cloned special glucosidase, named ginsenosidase type III hydrolyzing 3-O-glucoside of multi-protopanaxadiol (PPD)-type ginsenosides, were investigated. The gene (ggpA) encoding this enzyme was cloned from a Terrabacter ginsenosidimutans strain and then expressed in E. coli cells. Ginsenosidase type III was able to hydrolyze 3-O-glucoside of multi-PPD-type ginsenosides. For instance, it could hydrolyze the 3- O-β-D-glucopyranosyl of Rb1 to gypenoside XVII, and then to further hydrolyze the 3-O-β-D-glucopyranosyl of gypenoside XVII to gypenoside LXXV. Similarly, the enzyme could hydrolyze the glucopyranosyls linked to the 3-O- position of Rb2, Rc, Rd, Rb3, and Rg3. With a larger enzyme reaction Km value, there was a slower enzyme reaction speed; and the larger the enzyme reaction Vmax value, the faster the enzyme reaction speed was. The Km values from small to large were 3.85 mM for Rc, 4.08 mM for Rb1, 8.85 mM for Rb3, 9.09 mM for Rb2, 9.70 mM for Rg3, 11.4 mM for Rd and 12.9 mM for F2; and Vmax value from large to small was 23.2 mM/h for Rc, 16.6 mM/h for Rb1, 14.6 mM/h for Rb3, 14.3 mM/h for Rb2, 1.81 mM/h for Rg3, 1.40 mM/h for Rd, and 0.41 mM/h for F2. According to the Vmax and Km values of the ginsenosidase type III, the hydrolysis speed of these substrates by the enzyme was Rĉ>Rb1>Rb3>Rb2>Rg3>Rd>F2 in order.

Keywords: Ginsenosidase type III, biotransformation, enzyme kinetic, PPD-type ginsenosides

Ginseng, the root of members of the Panax genus plant, plays a very important role in medical treatment, and it has been used as a traditional herbal medicine in Asian countries for over 2,000 years. The main molecular component responsible for the actions of ginseng is ginsenoside (ginseng saponin).

Ginsenosides are composed of aglycone and sugar moieties. Based on the structure of the aglycone, ginsenosides can be categorized into three broad types: protopanaxadiol-type ginsenosides (PPD), such as Rb1, Rb2, Rc, and Rd; protopanaxatriol type ginsenosides (PPT), such as Re, Rf, Rg1, and F1; and oleanane-acid-type saponins, such as Ro [6]. Although ginsenosides only have three kinds of aglycones, there are more than 60 kinds of ginsenosides identified by now because different sugar moieties link different carbon positions including 3-O- (C-3), 6-O- (C-6), and 20-O- (C-20). Ginsenosides have a wide spectrum of medicinal effects, such as antiinflammatory [21], anticancer [2], antihypertensive [13], and antioxidation [7] actions.

The high-content ginsenosides in wild ginseng have multi sugar moieties, so they present as a big size. These major ginsenosides in ginseng plant are Rb1, Rb2, Rc, Rd, Re, Rg1, etc. [9]. The small size ginsenosides presenting low content in wild ginseng are called minor ginsenosides. Ginsenoside sugar chains are closely related to their biological activity, and modification of their sugar chains may markedly change their biological activity. The activity of ginsenoside increases with the decrease of the number of the sugar moieties [10, 12]. Thus, minor ginsenosides are more effective for in vivo physiological action.

After oral intake of the ginseng, the big-size, low-solubility, and poor-permeability major ginsenosides cannot be absorbed by the human body directly [15]. These ginsenosides are usually deglycosylated into small size minor ginsenosides in the gastrointestinal tract by intestinal bacteria and/or
digestive enzymes, and then absorbed [5]. However, this transformation in the human body varies from person to person, because the intestinal bacteria are variable, and also depending on the conditions of the host, including diet, health, and stress. If the transformation occurs in vitro, these variations will be overcome.

According to the structural relationship of ginsenosides, it is a good way to prepare minor ginsenosides from major ginsenosides by hydrolyzing the sugar moieties using the method of biological enzymes. To prepare minor ginsenosides by the enzymatic method, researchers did plenty of work, and they found that many cultures of microorganisms can transform major ginsenosides, such as *Fusarium proliferatum* ECU2042 [14], *Absidia coerulea* [3], and *Mucor spinosus* [20]. Moreover, some enzymes have been purified from the cultures of microorganisms and other living beings [4, 11]. However, these studied enzymes exhibited the property of hydrolyzing only one type glycoside, or their hydrolysis on multi-glycosides was infrequent.

Our laboratory previously reported on some novel ginsenosidases such as ginsenosidase type I [18] and ginsenosidase type II [19]; the ginsenosidase type I can hydrolyze 3-O- and 20-O-mono-glycosides of PPD-type ginsenosides; and the ginsenosidase type II can hydrolyze 20-O-mono-glycosides of PPD-type ginsenosides. We also reported the cloning of an enzyme by “expressing the recombined bgpA gene of a *Terrabacter ginsenosidimutans* strain in *E. coli* cells,” hydrolyzing 3-O-glucoside of ginsenoside Rb1 into gypenoside XVII and gypenoside LXXV [1]; but this report did not study the enzyme hydrolysis on other PPD-type ginsenosides such as Rb2, Rb3, Rc, Rd, Rg3, and F2.

In this paper, the enzyme hydrolysis on multi-PPD-type ginsenosides, and the enzyme kinetics for hydrolyzing seven kinds of PPD ginsenosides including Rb1, Rb2, Rb3, Rc, Rd, Rg3(5), and F2, were studied, and the enzyme was named ginsenosidase type III.

**Materials and Methods**

**Materials**

Ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Rg3(5), F2, and C-K were purchased from Dalian Green Bio, Ltd. (Dalian, China).

Standard proteins of phosphorylase b (97.2 kDa), serum albumin (66.4 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa) were purchased from Takara Bio Inc. (Otsu, Japan).

**Production and Purification of Ginsenosidase Type III**

The gene of the special glucosidase (ginsenosidase type III) from *Terrabacter ginsenosidimutans* sp. nov was inserted into pGEX-4T-1 (GE Healthcare, USA) to generate a glutathione S-transferase (GST)–*bgpA* gene fusion. Then the recombinant pGEX-*bgpA* was introduced into *E. coli*. The transformants harboring the plasmid were grown in LB-ampicillin medium at 40°C until the cultures reached an OD of 0.6 at 600 nm, and protein expression was induced by adding 0.5 mM isopropyl-[β-D]-thiogalactopyranoside. The culture was incubated for 12 h at 20°C to produce enzyme. Centrifugation was carried out to obtain the cell-free crude cell extract. The recombinant BgpA was purified by DEAE-cellulose DE-52 chromatography (Whatman Ltd., Maidstone, UK), followed by Mono Q anion-exchange chromatography (GE Healthcare, USA) [1]. The purified enzyme solution was used to evaluate its molecular mass and enzyme kinetic parameters.

Enzyme purity was examined by HPLC with a Waters 2695 Separations Module with the Waters 2996 Photodiode Array Detector (Waters Corp., Milford, USA). A TOSOH TSK-Gel-2000 SW chromatographic column (Φ7.8 mm×300 mm) was used in the HPLC examination (Tosoh Bioscience, Tokyo, Japan). The mobile phase was 0.02 mol/l (pH 6.7) phosphate buffer 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.

To prepare the sample of enzyme used for HPLC, 2 mg of enzyme protein was dissolved in 1 ml of 0.02 mol/l (pH 6.7) phosphate buffer containing 2% SDS and 0.03% mercaptoethanol.

**Measurement of Enzyme Molecular Mass**

The molecular mass was determined with the method of Weber et al. [17], using a 5% (w/v) stacking polyacrylamide gel and 12% (w/v) separating gel. The calibration curve was done using standard 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). Protein bands were visualized with Coomassie brilliant blue R-250.

**Enzyme Analysis**

The activity of purified ginsenosidase type III was determined using ginsenosides as substrates in 50 mM sodium phosphate buffer, pH 7.0, at 40°C. Assay mixtures containing 0.1 ml of the substrate (0.1% ginsenoside solution) and 0.1 ml of the enzyme were incubated for 0.5, 1, 1.5, 2, 2.5, 3, 16, 24, and 72 h. Next, 0.2 ml of the n-butanol saturated by water was added to the reaction mixture to stop the reaction. The reaction product in the n-butanol layer was then analyzed by TLC and HPLC.

Thin-layer chromatography (TLC) was carried out using a silica gel G 60 F254 plate (Merck) with developing solvent consisting of chloroform, methanol, and water [7:2.5:0.5 (v/v/v)] (under layer), or n-butanol, ethyl acetate, and water [4:1:2 (v/v/v)] (upper layer), and the produced ginsenosides on the silica gel plate were determined by scanning the TLC spots using a Shimadzu CS-930 [18].

The product-ginsenosides from the enzyme reaction were also examined by HPLC (Waters 2695 Separations Module with Waters 2996 Photodiode Array Detector). A Knauer C-18 chromatographic column (5 µm, Φ3 mm×300 mm) was used to analyze samples (Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer GmbH, Berlin, Germany): Measuring wavelength, 203 nm; injected volume was 10 µl, with a flow rate of 0.6 ml/min; and column temperature, 25°C. The mobile phase was A (acetoniitrile) 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 43% to 100%.

The sample of enzymatic reaction product for the HPLC was obtained as follows: the enzymatic reaction product in the mixture was extracted using the isometric n-butanol saturated by water for three times, and then the n-butanol layer was washed four times