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

Jin, Xue-Feng1, Hong-Shan Yu1*, Dong-Ming Wang12, Ting-Qiang Liu12, Chun-Ying Liu1, Dong-Shan An3, Wan-Taek Im4, Song-Gun Kim3, and Feng-Xie Jin1*

1College of Biotechnology, Dalian Polytechnic University, Dalian 116034, People’s Republic of China
2College of Science, Yanbian University, Yanji City 133002, People’s Republic of China
3Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology, Daedeon 305-806, Korea
4Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daedeon 305-701, Korea

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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 (bgpA) 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 was able to hydrolyze the 3-O-β-D-(1→2)-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, Re, Rd, Rb3, and Rg3. With a larger enzyme reaction $K_m$ value, there was a slower enzyme reaction speed; and the larger the enzyme reaction $V_{max}$ value, the faster the enzyme reaction speed was. The $K_m$ values from small to large were 3.85 mM for Rc, 8.08 mM for Rb1, 11.4 mM for Rd, and 12.9 mM for F2; and $V_{max}$ value from large to small was 23.2 mM/h for Rc, 16.6 mM/h for Rb1, 8.85 mM for Rb3, 9.70 mM for Rd, and 0.41 mM/h for F2. According to the $V_{max}$ and $K_m$ values of the ginsenosidase type III, the hydrolysis speed of these substrates by the enzyme was Rc>Rb1>Rb3>Rb2>Rg3→Rd>F2 in order.

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

*Corresponding author
F-X. J.
Phone: +86-411-86307737; Fax: +86-411-86307737;
E-mail: fxjin@dlpu.edu.cn
H.-S. Y.
Phone: +86-411-86307737; Fax: +86-411-86307737;
E-mail: hongshan@dlpu.edu.cn

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-multi-glycosides of PPD-type ginsenosides; and the ginsenosidase type II can hydrolyze 20-O-multi-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-gluco-side 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 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 (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 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 isomeric n-butanol saturated by water for three times, and then the n-butanol layer was washed four times.
with isometric water. After that, the enzymatic reaction products in the n-butanol were dried by vacuum distillation and dissolved in 1 ml of methanol before the HPLC analysis.

One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 µmol of the substrate per hour.

**Determination of Enzyme Kinetics**

The values of the Michaelis constant (K\textsubscript{m}) and the maximal reaction velocity (V\textsubscript{max}) for ginsenosidase type III were determined by incubating the enzyme in 50 mM sodium phosphate (pH 7.0) at 40°C with ginsenoside Rb1 at concentrations of 10, 12.5, 17, 25, and 50 mM for 0.5, 1, 1.5, 2, 2.5, and 3 h; with Rb2, Rb3, or Rc at 10, 13.3, 20, and 40 mM for 0.5, 1, 1.5, 2, and 2.5 h; or with Rd, Rg3(S), or F2 at 1.0, 1.25, 1.67, 2.5, and 5 mM for 3, 6, 9, 12, 24, 36, and 48 h. The conversion was obtained by using Bandscan software to analyze the area and shade of the plot on the silica gel. Values for K\textsubscript{m} and V\textsubscript{max} were calculated from Lineweaver–Burk plots [8]. The transformation velocity of the hydrolysis on the PPD-type ginsenosides was calculated from the Michaelis–Menten equation [16]:

\[
V = \frac{V_{\text{max}} [S]}{K_{\text{m}} + [S]},
\]

V, velocity (mM/h); [S], concentration (mM); K\textsubscript{m}, Michaelis constant (mM); V\textsubscript{max}, maximum velocity (mM/h); V\textsubscript{0}, the transformation velocity at concentration of 10 mM (mM/h); V\textsubscript{i}, the transformation velocity at concentration of 1 mM (mM/h).

Reactions for each substrate were carried out in triplicate.

**RESULTS AND DISCUSSION**

**Production and Purification of Ginsenosidase Type III**

The gene of ginsenosidase type III, bgpA, from a Terrabacter ginsenosidimutans strain was amplified by PCR and then subcloned into pGEX4T-1 to generate a GST–bgpA gene fusion that could be expressed in E. coli under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter Ptac. Induction with 0.5 mM IPTG at 20°C for 12 h produced the maximum level of soluble active fusion enzyme. The GST–BgpA fusion protein purified by glutathione-Sepharose 4B chromatography was digested by thrombin to remove the GST moiety, and the resulting recombinant BgpA was purified by successive chromatography on DEAE-cellulose and Mono Q anion-exchange columns. This procedure resulted in a 20.8-fold purification and a recovery of 13% from the crude extract [1].

The purity of the purified ginsenosidase type III was examined by the method of HPLC (Fig. 1). Only one peak appeared on the HPLC spectrum at 5.423 min, indicating that the ginsenosidase type III was a pure enzyme.

In SDS-PAGE examination (Fig. 2), the enzyme also represented a single band on the gel. By calculating the mobility, the molecular mass of the ginsenosidase type III was approximately 72 kDa, which was similar to the size estimate based on the translated polypeptide sequence (71,140 Da).

The purified enzyme was used for the following experiments.

**Hydrolysis on PPD-Type Ginsenosides**

The above purified enzyme, named ginsenosidase type III, was examined for its hydrolysis on protopanaxadiol-type ginsenosides such as ginsenosides Rb1, Rb2, Rc, Rd, Rb3, and Rg3(S). The enzyme reacted with ginsenosides Rb1, Rb2, and Rc for 24 h, respectively; with ginsenoside Rd for 72 h; and with ginsenoside Rg3(S) for 48 h. The reaction products were examined by TLC, with the results shown in Fig. 3. The enzyme could not hydrolyze the PPT-type ginsenosides (data not shown).

The hydrolysates of Rb1 were gypenoside XVII (17) and gypenoside LXXV (75); the hydrolysates of Rb2 were ginsenosides C-O and C-Y; the hydrolysates of Rc were...
 were ginsenosides C-Mx1 and C-Mx; and the hydrolysates of Rg3(S) were ginsenosides Rh2 and PPD aglycone. Thus, ginsenoside type III first hydrolyzed the 3-O-β-D-(1 → 2)-glucopyranosyl of Rb1 to gypenoside XVII (17), and then hydrolyzed the 3-O-β-D-glucopyranosyl of gypenoside XVII (17) to gypenoside LXXV (75); first hydrolyzed the 3-O-β-D-(1 → 2)-glucopyranosyl of Rb2 to C-O, and then hydrolyzed the 3-O-β-D-glucopyranosyl of C-O to C-Y; and first hydrolyzed the 3-O-β-D-(1 → 2)-glucopyranosyl of Rc to C-Mc1, and then hydrolyzed the 3-O-β-D-glucopyranosyl of C-Mc1 to C-Mc. Ginsenosidase type III hydrolyzed Rd relatively slowly, where it first hydrolyzed 3-O-β-D-(1 → 2)-glucopyranosyl of Rd to F2, and then hydrolyzed the 3-O-β-D-glucopyranosyl of F2 to C-K; first hydrolyzed the 3-O-β-D-(1 → 2)-glucopyranosyl of Rh2 to C-Mx1, and then hydrolyzed the 3-O-β-D-glucopyranosyl of C-Mx1 to C-Mx; and first hydrolyzed the 3-O-β-D-(1 → 2)-glucopyranosyl of Rg3(S) to Rh2(S), and then hydrolyzed the 3-O-β-D-glucopyranosyl of Rh2(S) to its aglycone. The hydrolysis of the enzyme on Rg3(S) was similar to that on Rg3(S) (data not shown). The scientific names of these ginsenosides are shown in Table 1.

Table 1. Abbreviations.

<table>
<thead>
<tr>
<th>Name</th>
<th>Scientific name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginsenoside Rb1</td>
<td>3-O-[β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl]-20-O-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside Rb2</td>
<td>3-O-[β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl]-20-O-[α-L-arabinopyranosyl-(1 → 6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside Rb3</td>
<td>3-O-[β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl]-20-O-[β-D-xylopyranosyl-(1 → 6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside Rc</td>
<td>3-O-[β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl]-20-O-[α-L-arabinofuranosyl-(1 → 6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside Rd</td>
<td>3-O-[β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl]-20-O-[β-D-glucopyranosyl]-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside Rg3(S)</td>
<td>3-O-[β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl]-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside Rg3(R)</td>
<td>3-O-[β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl]-20(R)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside F2</td>
<td>3-O-β-D-Glucopyranosyl-20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside Rh2(S)</td>
<td>3-O-β-D-Glucopyranosyl-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside Rh2(R)</td>
<td>3-O-β-D-Glucopyranosyl-20(R)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside C-K</td>
<td>20-O-β-D-Glucopyranosyl-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside C-O</td>
<td>3-O-β-D-Glucopyranosyl-20-O-[α-L-arabinopyranosyl-(1 → 6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside C-Y</td>
<td>20-O-[α-L-arabinopyranosyl-(1 → 6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside C-Mc1</td>
<td>3-O-β-D-Glucopyranosyl-20-O-[α-L-arabinofuranosyl-(1 → 6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside C-Mc</td>
<td>20-O-[α-L-arabinofuranosyl-(1 → 6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside C-Mx1</td>
<td>3-O-β-D-Glucopyranosyl-20-O-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Ginsenoside C-Mx</td>
<td>20-O-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Gypenoside XVII</td>
<td>3-O-β-D-Glucopyranosyl-20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol</td>
</tr>
<tr>
<td>Gypenoside LXXV</td>
<td>20-O-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol</td>
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</table>
In order to further verify the products from the reaction, HPLC was used to examine the reaction products. Rb1 was hydrolyzed quickly by the enzyme. When reacted for 24 h, the substrate Rb1 was completely transformed, whereas when reacted for 16 h, Rb1 was not transformed completely. The results of HPLC examining the products of Rb1 reacted for 16 h, Rb2 and Rc reacted for 24 h, and Rd reacted for 72 h are shown in Fig. 4. As revealed from the TLC of the enzyme reaction. The substrate concentrations ([S]) of Rb1 were 10, 12.5, 17, 25, and 50 mM; the 1/[S] values were 0.1, 0.08, 0.06, 0.04, and 0.02 (mM)^{-1}, respectively. Only a small quantity of Rb1 was transformed after 0.5 h, thus the velocity (V) was determined from the conversion amount of the substrate in the initial 0.5 h. The V values in accordance with the sequence of above concentrations were 11.76, 12.5, 13.51, 14.49, and 15.15 mM/h, and the 1/V values were 0.085, 0.06, 0.057, and 0.046 (mM h)^{-1}, respectively. Then a Lineweaver–Burk plot of 1/V against 1/[S] was obtained (Fig. 7). The kinetic parameters of ginsenosidase type III hydrolyzing ginsenoside Rb1 were calculated using the Lineweaver–Burk plot. The K_m value was 4.08 ± 0.36 mM, and V_max was 16.6 ± 1.5 mM/h.

The kinetic parameters of ginsenosidase type III for ginsenoside Rb2 were estimated by incubating the purified enzyme with Rb2 at concentrations of 10, 13.3, 20, and 40 mM under standard assay conditions for 0.5, 1, 1.5, 2, and 2.5 h at 40°C. The Lineweaver–Burk plot (data not shown) was obtained for the conversion of Rb2 in the initial 0.5 h, because only a small quantity of Rb2 was transformed after 0.5 h. Therefore, the kinetic parameters were 9.09 ± 0.90 mM for K_m and 14.3 ± 1.4 mM/h for V_max.

The kinetic parameters of ginsenosidase type III for ginsenosides Rb3 and Rc were estimated by incubating the purified enzyme with Rb3 or Rc at concentrations of 10, 13.3, 20, and 40 mM under standard assay conditions for 0.5, 1, 1.5, 2, and 2.5 h at 40°C. The Lineweaver–Burk plot (data not shown) was obtained for the conversion of Rb3 and Rc in the initial 0.5 h. The K_m value was 8.85 ± 0.87 mM with a V_max of 14.6 ± 1.4 mM/h; the K_m for Rc was 3.85 ± 0.32 mM with a V_max of 23.2 ± 2.2 mM/h.

The larger the K_m value, the slower the hydrolysis speed. The K_m value of these four substrates from small to large was 3.85 mM for Rc, 4.08 mM for Rb1, 8.85 mM for Rb3, and 9.09 mM for Rb2. Moreover, the larger the V_max value, the faster the hydrolysis speed. The V_max value from large to small was 23.2 mM/h to Rc, 16.6 mM/h to Rb1, 14.6 mM/h to Rb3, and 14.3 mM/h to Rb2. Therefore, the rule from K_m was the same as that from V_max. It was revealed from the kinetic results that the hydrolysis speed of ginsenosidase type III to Rc was fastest, next to Rb1, Rb3, and Rb2 in order.

According to the Michaelis–Menten equation [Eq. (1)], the transformation velocities (V) of Rc, Rb1, Rb3, and terminal α-(1→6)-l-arabinofuranosyl residue is linked to the β-α-glucopyranosyl at the 20-O- position.
Fig. 5. Ginsenosidase type III hydrolysis on Rb1, Rb2, Rc, and Rd.
Kinetics of Ginsenosidase Type III on Rg3(S)

Ginsenoside Rg3(S) has two glucose molecules at the 3-O-position, but has no sugar moiety at the 20-O-position. Ginsenosidase type III hydrolyzed Rg3(S) slowly, so the substrate concentration used for assay was relatively lower. The substrate concentrations used to assay were 1.0, 1.25, 1.7, 2.5, and 5 mM. The Lineweaver–Burk plot (data not shown) was obtained for the conversion in the initial 3 h. The $K_m$ value was 9.70 ± 0.92 mM, and $V_{max}$ was 1.81 ± 0.16 mM/h. The transformation velocity ($V_i$) of Rg3(S) at concentration of 1 mM was 0.169 mM/h.

Kinetics of Ginsenosidase Type III on Rd and F2

The structure of Rd and F2 are different to that of the above PPD ginsenosides. Rd and F2 both contain one glucose molecule at the 20-O-position, Rd has two glucose molecules at the 3-O-position, and F2 has one glucose molecule at each of the 3-O-positions. The enzyme hydrolyzed Rd and F2 slowly too. The substrate concentrations were 1.0, 1.25, 1.7, 2.5, and 5 mM. The Lineweaver–Burk plot (data not shown) was obtained for the conversion in the initial 3 h. The $K_m$ values for Rd and F2 were 11.4 ± 1.1 and 12.9 ± 1.2 mM, and $V_{max}$ values 1.40 ± 0.13 and 0.41 ± 0.04 mM/h, respectively. Thus, according to the regularity of $K_m$ and $V_{max}$, the hydrolysis speed of Rd by ginsenosidase type III was slower than that of F2.

Moreover, the transformation velocities ($V_i$) of Rd and F2 at concentration of 1 mM were 0.113 and 0.0295 mM/h, respectively.

Comparison of the Enzyme Kinetics on PPD-Type Ginsenosides

The kinetics parameters results of ginsenosidase type III are summarized in Table 2. The $K_m$ values for Rc, Rb1, Rb3, Rb2, Rg3(S), Rd, and F2 were 3.85, 4.08, 8.85, 9.09, 9.70, 11.4, and 12.9 mM, and the $V_{max}$ values for these substrates were 23.2, 16.6, 14.6, 14.3, 1.81, 1.40, and 0.41 mM/h, respectively. The hydrolysis velocities of the enzyme on Rc, Rb1, Rb3, and Rb2 at concentration of 10 mM were 16.8, 11.8, 7.75, and 7.50 mM/h; the hydrolysis velocities of the enzyme on Rg3(S), Rd, and F2 at concentration of 1 mM were 0.169, 0.113, and 0.0295 mM/h,
respectively. It is shown from the above results that the hydrolysis speed of ginsenosidase type III exhibited obvious difference to different PPD ginsenosides.

If the $K_m$ value is smaller and the $V_{max}$ value is higher, the enzyme hydrolysis velocity will be faster. On the basis of this regularity, when the kinetic parameters of the enzyme for these substrates were compared, the hydrolysis speed of Rc by ginsenosidase type III was fastest, followed by Rb1, Rb3, Rb2, Rg3(S), Rd, and F2 in order.

In conclusion, the ginsenosidase type III, a novel special glucosidase, can hydrolyze 3-O-glucoside of multi-PPD ginsenosides, such as Rb1, Rb2, Rc, Rd, Rb3, and Rg3. This enzyme shows differences with the other previously reported ginsenosidases, such as ginsenosidase type I [18] and ginsenosidase type II [19]. Ginsenosidase type I can hydrolyze not only 3-O-glucose 3-O-β-glucoside of PPD-type ginsenosides, but also 20-O-mono-glycosides of PPD-type ginsenosides such as 20-O-β-glucoside of Rb1, 20-O-β-xylloside of Rb3, 20-O-α-arabinoside(p) of Rb2, and 20-O-α-arabinoside(f) of Rc. Ginsenosidase type II cannot hydrolyze 3-O-glucoside of PPD ginsenosides, but can hydrolyze 20-O-multi-glycosides of PPD-type ginsenosides such as 20-O-β-glucoside of Rb1, 20-O-β-xylloside of Rb3, 20-O-α-arabinoside(p) of Rb2, and 20-O-α-arabinoside(f) of Rc. All these three ginsenosidases cannot hydrolyze the PPT-type ginsenosides.

The ginsenosidase type III exhibited different hydrolysis speeds to different substrates.

The structures of ginsenosides Rc, Rb1, Rb3, and Rb2 exhibit high similarity. They all have two glucosyls at the 3-O- position, and two different sugar moieties at the 20-O- position. The $K_m$ values for Rc, Rb1, Rb3, and Rb2 were 3.85, 4.08, 8.85, and 9.09 mM; the $V_{max}$ values for these substrate were 23.2, 16.6, 14.6, and 14.3 mM/h; and the hydrolysis velocities at concentration of 10 mM calculating from $K_m$ and $V_{max}$ by the Michaelis–Menten equation were 16.8, 11.8, 7.75, and 7.50 mM/h, respectively. If $K_m$ is smaller and $V_{max}$ is larger, the hydrolysis speed is faster, and the above $K_m$ and $V_{max}$ values present good regularity. The hydrolysis velocity also exhibits obvious regularity. It is revealed from the above kinetic parameters that the fastest hydrolysis velocity happened to Rc, followed by Rb1, Rb2, and Rb3.

Rg3(S) has two glucose molecules at the 3-O- position, but no sugar moiety links to the 20-O- position. Rd and F2 both have one glucose molecule at the 20-O- position. Rd has two glucose molecules at the 3-O- position, whereas F2 has one glucose molecule at each of the 3-O- positions. The $K_m$ values for Rg3(S), Rd, and F2 were 9.70, 11.4, and 12.9 mM, and the $V_{max}$ values for these substrate were 1.81, 1.40, and 0.41 mM/h, respectively. The hydrolysis velocities at concentration of 1 mM were 0.169, 0.113, and 0.0295 mM/h, respectively. Thus, in these three substrates, the hydrolysis speed of Rg3(S) was fastest; that of Rd was slower; that of F2 was slowest.

The larger the enzyme reaction $K_m$ value, the slower will be the enzyme reaction speed; the larger the enzyme reaction $V_{max}$ value, the faster will be the enzyme reaction speed. It is shown above that the $K_m$ value of ginsenosidase type III was Rc<Rb1<Rb3<Rb2<Rg3(S)<Rd<F2; and the $V_{max}$ value of the enzyme was Rc<Rb1<Rb3<Rb2<Rg3(S)>Rd>F2; so, the hydrolysis speed of these substrates by ginsenosidase type III was Rc>Rb1>Rb3>Rb2>Rg3(S)>Rd>F2 in order.

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**References**


