Dynamic changes of multi-notoginseng stem-leaf ginsenosides in reaction with ginsenosidase type-I

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Abstract

Background: Notoginseng stem-leaf (NGL) ginsenosides have not been well used. To improve their utilization, the biotransformation of NGL ginsenosides was studied using ginsenosidase type-I from Aspergillus niger g.848.

Methods: NGL ginsenosides were reacted with a crude enzyme in the RAT-5D bioreactor, and the dynamic changes of multi-ginsenosides of NGL were recognized by HPLC. The reaction products were separated using a silica gel column and identified by HPLC and NMR.

Results: All the NGL ginsenosides are protopanaxadiol-type ginsenosides; the main ginsenoside contents are 27.1% Rb3, 15.7% C-Mx1, 13.8% Rc, 11.1% Fc, 7.10% Fa, 6.44% C-Mc, 5.08% Rb2, and 4.31% Rb1. In the reaction of NGL ginsenosides with crude enzyme, the main reaction of Rb3 and C-Mx1 occurred through Rb3/C-Mx1/C-Mx; when reacted for 1 h, Rb3 decreased from 27.1% to 9.82 %, C-Mx1 increased from 15.5% to 32.3%, finally into C-Mx and a small amount of C-K. When reacted for 1.5 h, all the Rb1, Rd, and Gyp17 were completely reacted, and the reaction intermediate F2 was produced to 8.25%, finally into C-K. The main reaction of Rc (13.8%) occurred through Rc/C-Mc1/C-Mc/C-K. The enzyme barely hydrolyzed the terminal xyloside on 3-Oe or 20-O-sugar-moiety of the substrate; therefore, 9.43 g C-Mx, 6.85 g C-K, 4.50 g R7, and 4.71 g Fc (hardly separating from the substrate) were obtained from 50 g NGL ginsenosides by the crude enzyme reaction.

Conclusion: Four monomer ginsenosides were successfully produced and separated from NGL ginsenosides by the enzyme reaction.

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1. Introduction

Ginseng is a popular, traditional medicinal herb. The Panax genus has approximately 14 species, but the most widely used ginseng are Korean ginseng (Panax ginseng), American ginseng (Panax quinquefolius), and Notoginseng (Panax notoginseng) [1]. Ginsenosides are important pharmacologically active compounds in ginseng and can be classified into two groups, dammarane ginsenosides and oleanane ginsenosides. Over 150 types of natural ginsenosides have been isolated from the ginseng plant parts, such as the roots, leaves, stems, fruits, and flower heads [2]. However, over 80–90% of the ginsenosides in Korean ginseng root and American ginseng root are Rb1, Rb2, Rc, Rd, Re, and Rg1 [3,4].

Over 70 types of ginsenosides have been isolated from the notoginseng plant parts, including the root, stem, leaf, flower, and fruit [5]; however, more than 90% ginsenosides of the notoginseng root are Rb1, Rg1, R1, Re, and Rd [4], and the main ginsenosides in...
The pharmacological activities of ginsenosides are closely related to the glycoside type, number, and position of the ginsenoside sugar moieties [9,10,14]. The high bioactive minor ginsenosides, such as Rg3, Rg2, Rg2, and Rh1, in the red ginseng and minor ginsenosides C-K, C-Mc, C-Mx, and C-Y of low number glycside can be produced from the major ginsenosides, namely Rb1, Rb2, Rc, Rd, Re, and Rg1, by enzyme hydrolysis [11–13]. The minor ginsenosides possess good pharmacological activities [14], such as anticancer [15–17], immunomodulatory, anti-inflammatory [18], anti-thrombus, anti-aging, anti-diabetes, and anti-stress activities [19–21].

To obtain the minor ginsenosides which have higher bioactivities and easy absorption properties, the transformation methods of microorganisms or enzyme conversion, and cloned ginsenosidase conversion have been reported [13,22]. Our laboratory previously reported four types of ginsenosidases: ginsenosidase-I (GE-I) can hydrolyze 3-O-glycosides of protopanaxadiol (PPD) ginsenosides [23–26], ginsenosidase-II (GE-II) can hydrolyze 20-O-glycosides of PPD ginsenosides [23], ginsenosidase-III (GE-III) can hydrolyze 3-O-glycosides of PPD ginsenosides [27], and ginsenosidase-IV (GE-IV) can hydrolyze 6-O- and 20-O-glycosides of protopanaxatriol ginsenosides [28].

Although some GE-I from different Aspergillus strains have the same properties which can hydrolyze 3-O- and 20-O-multiply-glycosides of PPD ginsenosides, the enzyme hydrolysis sequence and hydrolisis ability to different types of glycosides on the 3-O- and 20-O-sugar moiety of PPD ginsenosides are different [23–26]. For example, GE-I (molecular weight, 80 kDa) from Aspergillus sp. g.848 strains can hydrolyze PPD ginsenosides Rb1, Rb2, Rc, and Rd into F2, C-K, and small amount of Rb2 [23]. GE-I (molecular weight, 74 kDa) from the A. niger g.48 strain can hydrolyze both 3-O- and 20-O-glucosides of Rb1 and Rb3 via two pathways: thus, the enzyme produces many intermediates such as Gyp17, Gyp,75, Rd and F2 from Rb1, and C-Mx1, C-Mx, Rd, and F2 from Rb3 in the enzyme reaction, finally into minor ginsenoside C-K; furthermore, the enzyme first hydrolyzes 3-O-glucoside of Rb2 and Rc into C-Y and C-Mc and then hydrolyzes 20-O-Ara of C-Y and C-Mc into minor ginsenoside C-K [24]. The special GE-I (molecular weight, 75 kDa) from the A. niger g.848 strain [25,26] first hydrolyzes 20-O-Glc of ginsenoside Rb1 to Rd and then hydrolyzes 3-O-Glc of Rd into F2 and C-K; however, the enzyme first hydrolyzes 3-O-Glc of Rb2 and Rc and then hydrolyzes 20-O-Ara into C-K; the enzyme first hydrolyzes 3-O-Glc of Rb3 and then slowly hydrolyzes 20-O-Xly into C-K.

Because GE-I from Aspergillus sp. g.48p strains produces many intermediates from Rb1 and Rb3 in the enzyme reaction, the crude GE-I from A. niger g.848 strain is suitable for the production of minor ginsenosides C-Mc, C-Y, F2, and C-K from the PPD ginsenoside of American ginseng [25] and for the production of minor ginsenosides C-Mx and C-K from NGL ginsenosides [26]; however, the results did not study the dynamic changes for different types of NGL ginsenosides and did not study the unknown ginsenosides (high content) from the NGL ginsenoside enzyme reaction [26].

Therefore, in this paper, the dynamic changes for different types of NGL ginsenosides (low-cost) were studied using the crude GE-I from A. niger g.848 strain of low cost; and the products from the enzyme reaction were separated using a silica-gel column to obtain the vina-ginsioside R7 and notoginsioside Fc in addition to the minor ginsenosides C-Mx and C-K; the structures of R7, Fc, C-Mx, and C-K were also identified using NMR.

2. Materials and methods

2.1. Materials

The ginsenosides Rb1, Rb2, Rb3, Rc, Rd, and standard ginsenosides F2, Gyp17, C-K, C-Mc, C-Mc1, C-Mx1, C-Mx, Fa, Fc, R7, C-Y, and C-O were obtained from GreenBio Co., Ltd. (Dalian, Liaoning province, People’s Republic of China) and Tianle Co., Ltd. (Shenyang, Liaoning province, People’s Republic of China). The NGL ginsenosides were purchased from Huizhou Shennong-Bencao Health Products Co., Ltd. (Huizhou, Guangdong province, People’s Republic of China). The A. niger g.848 strain was isolated from traditional Chinese koji [25,26]. The 60-F25 silica gel plates from Merck (Darmstadt, Germany) were used for the TLC analysis. The AB-8 macroporous resin and D-280 anion exchange resin were purchased from Nankai University (Tianjin, People’s Republic of China).

2.2. Crude enzyme production and enzyme analysis

The crude GE-I enzyme was prepared with the culture of A. niger g.848 strain according to a previously reported method [25,26] using a medium containing 0.05% NGL ginsenosides and 5% wheat bran extraction.

The optimal temperature and pH of the crude enzyme were examined with the ginsenoside Rb3 because it is the typical ginsenoside in the NGL ginsenosides. First, 0.2 mL crude enzyme was mixed with 0.2 mL of 2% of Rb3 in 0.02M acetate buffer (pH 5.0) and allowed to react at 30°C, 35°C, 40°C, 45°C, 50°C, and 60°C and at pH 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, and 8.0. Next, 0.4 mL of water-saturated n-butanol was added to the reaction mixture to stop the enzymatic reaction. The reaction product (n-butanol layer) was analyzed by HPLC to examine the optimal temperature and pH of the GE-I enzyme.

In the determination for good enzyme reaction concentration of NGL ginsenosides, the substrate (NGL ginsenosides) concentration was fixed to 2%, 4%, 5%, 6%, 7%, and 8% in 0.02M acetate buffer (pH 5.0); next, 0.2 mL of crude enzyme was mixed with the same volume of substrate to react at 45°C for 24–48 h. Then, 0.4 mL of water-saturated n-butanol was added to the reaction mixture to stop the enzymatic reaction. The n-butanol layer was dried, dissolved in 1 mL of methanol, and analyzed by HPLC to examine the good reaction substrate concentration.

2.3. Dynamic changes for different types of NGL ginsenosides in enzyme reaction

Under the aforementioned optimal reaction conditions, 10 g of NGL ginsenosides was dissolved in 200 mL of 0.02M acetate buffer (pH 5.0), mixed with the same volume of crude enzyme (final substrate concentration of NGL ginsenosides was 2.5%), and allowed to react at 45°C in a bioreactor (RAT-5D, Shanghai Shenshun Ltd, Shanghai, People’s Republic of China). According to the different enzyme reaction times, such as 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 6 h, 12 h, 24 h, 30 h, 36 h, and 48 h, 1 mL of reaction samples were taken, respectively; then, 3 mL of methanol was added to the 1 mL reaction samples to stop the enzymatic reaction; after centrifugation, the supernatant was dried, adsorbed on the 15 mL of AB-8 resin column, and eluted with water (5 times volume of column) to remove soluble impurity such as sugar; the column was
eluted with 84% alcohol (5 times volume of column) to elute ginsenosides from the reaction. The eluted ginsenosides were dried and dissolved in 3 mL of methanol for the HPLC analysis.

2.4. Preparation of minor ginsenosides C-Mx, C-K, R7, and Fc from NGL ginsenosides using crude enzyme

First, 50 g of substrate (NGL ginsenosides) was dissolved in 20 times the volume of substrate weight (V/W) of 0.02M acetate buffer (pH 5.0), mixed with the same volume of crude enzyme from A. niger g.848 strain (final substrate concentration of NGL ginsenosides was 2.5%), and allowed to reacted at 45°C for 24 h in the bioreactor. Then, 95% alcohol was added to stop the enzymatic reaction; after centrifugation, the reaction mixture was treated with AB-8 macroporous resin and D-280 anion-exchange resin column according to the previously reported method [25,26] to obtain a mixture product containing ginsenosides C-Mx, C-K, R7, and Fc.

The enzyme reaction products were separated by a silica gel column [25,26]. The column was first eluted with the mixture of chloroform and methanol [9.0:1.0 (V/V)]; each fraction was approximately 200–250 mL. After the complete elution of minor ginsenosides C-K and C-Mx, the column was also eluted with the mixture of chloroform, methanol, and water [7.0: 3.0: 0.3 (V/V)]; each fraction was approximately 200–250 mL. According to the TLC detection, fractions with the same component were collected and dried by vacuum distillation.

2.5. TLC, HPLC, and NMR analysis

TLC and HPLC analyses were performed using the previously reported methods [25,26]. The structures of the product ginsenosides C-Mx, C-K, R7, and Fc from the enzyme reaction were analyzed using the previously reported method of NMR [Bruke AVANCE 600 (1H: 600 MHz; 13C: 150 MHz) NMR Spectrometer (Switzerland)] [25,26].

3. Results and discussion

3.1. Analysis on the purchased NGL ginsenosides

Along with the product producing batch number, or the products from different manufacturers, the composition of NGL ginsenosides was different; therefore, the NGL ginsenosides were recognized by HPLC. Furthermore, 4 mg of NGL ginsenosides was dissolved in 1 mL methanol, and the different types of NGL ginsenosides were examined by HPLC as shown in Fig. 1.

Fig. 1 shows that all types of NGL ginsenosides are PPD ginsenosides with same aglycone and different sugar moieties. If the HPLC peak area ratio of ginsenosides is assumed to be the ginsenoside content ratio, the content ratio of different types of NGL ginsenosides (according to the content of high and low order) would be as follows: Rb3, 27.1%; C-Mx1, 15.7%; Rc, 13.8%; Fc, 11.1%; Fa, 7.10%; C-Mc, 6.44%; Rb2, 5.08%; Rb1, 4.31%; Rd; 3.07%; Gyp17, 1.51%; and R7, 0.72%. Thus, the main ginsenosides of NGL ginsenosides are Rb3 (27.1%), C-Mx1 (15.2%), Rc (13.8%), and Fc (11.1%). The NGL ginsenosides are used to following studies.

3.2. Enzyme production and enzyme reaction condition

When the A. niger g.848 strain was cultured by shaking in the medium containing 0.05% NGL ginsenosides (as an enzyme inducer) and 5% wheat bran, the good enzyme production was obtained at 30°C culturing for 5–6 d.
Fig. 2. In different reaction times, the dynamic conversion of notoginseng stem-leaf ginsenosides by crude ginsenosidase type-I from A. niger in HPLC. The experiment was repeated three times.
When 0.1 mL crude enzyme from the *A. niger* g.848 strain was mixed with the same volume of substrates (2% of Rb1, Rb2, Rb3, and Rc) in 0.02M acetate buffer (pH 5.0) and allowed to react at 45°C for 3 h, the enzyme could hydrolyze 3-O- and 20-O-multi-glycosides of ginsenoside Rb1, Rb2, Rb3, and Rc, according to the TLC analysis; therefore, the crude enzyme from the *A. niger* g.848 strain is GE-I [23–26].

The good enzyme reaction in 2% Rb3 was obtained at 45°C and pH 5.0. The good enzyme reaction was obtained at 45°C and pH 5.0 with 2.5% NGL ginsenosides concentration. All the above results were similar to those in a previously reported study [26]; only the substrate concentration of NGL-ginsenosides was 2.5% differented it with the 3% of previously reported study [26].

3.3. Dynamic changes for different types of NGL ginsenosides in enzyme reaction

Under the aforementioned optimal reaction conditions, 5 g of NGL ginsenosides was dissolved in 100 mL of 0.02M acetate buffer (pH 5.0), mixed with the same volume of crude GE-I from *A. niger* g.848 (final substrate concentration of NGL ginsenosides in reaction solution was 2.5%), and allowed to react at 45°C in the bioreactor. According to the different enzyme reaction times 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 6 h, 12 h, 24 h, 30 h, 36 h, and 48 h, 1 mL of reaction samples were taken in different reaction times; then, 3 mL of methanol was added to stop the enzymatic reaction; after centrifugation, the supernatant was dried, adsorbed on the 15 mL of AB-8 resin column, and eluted with water to remove soluble impurity such as sugar, eluted with five times the volume of 84% alcohol, dried, dissolved in 3 mL of methanol for HPLC analysis. The dynamic changes for various types of NGL ginsenosides in the different enzyme reaction time are shown in Fig. 2 and Table 1.

Table 1 and Fig. 2 show that all the ginsenosides Rb1, Rc, and Rb2 were completely reacted after 0.5 h; Fa and Rd were completely reacted after 1 h; Rb3 and Gyp17 were completely reacted after 1.5 h; unknown Non-1 and C-Mc1 were completely reacted after 12 h; and C-Mx1 was completely reacted after 24 h. The substrate ginsenoside Fc gradually increased in the reaction for 0.5–6 h and finally came back to the original concentration after reaction for 24–48 h; the ginsenoside R7 gradually increased from 0.72% to 13.1% during the enzyme reaction.

In the enzyme reaction products, the ginsenosides C-Mc and C-K were produced about 42–43% and 29–30%, respectively; the reaction intermediate F2 was about 3.76–8.25% during the reaction time from 0.5 h to 6 h and disappeared after reaction for 12 h. C-Mc was produced to 4.18% after reaction for 1.5 h, increased to 8.22% in reaction for 12 h, and finally decreased to about 4%.

Analyzing conversion of the ginsenoside Rb3 and C-Mx1 by enzyme: when reacted for 1 h by enzyme, the ginsenoside Rb3 decreased from 27.1% to 9.82%, C-Mx1 increased from 15.2% to 32.3%, and C-Mx was produced to 6.48%. Thus, the enzyme hydrolyzed 3-O-Glc of Rb3 to C-Mx1 and 3-O-Glc of C-Mx1 into C-Mx. Furthermore, C-Mx decreased from 43.8% to 42%, thereby proving that the enzyme very weakly hydrolyzes 3-O-Glc of C-Mx1 into C-Mx and 20-O-Xyl of C-Mx1 to C-K. In the enzyme reaction of Rc and C-Mc1, when reacted for 1 h, Rc (13.8%) was completely hydrolyzed, and C-Mc1 increased from 6.44% to 14.1%; when reacted for 2 h, C-Mc1 decreased to 11.8%, and produced 5.24% C-Mc; C-Mc was increased to 8.22%, and finally decreased to about 4%. It can be considered that the enzyme hydrolyzed 3-O-Glc of C-Mc1 to C-Mc, and finally hydrolyzed 20-O-Araf of C-Mc to C-K. In the enzyme reaction of Rb1, Rd, and Gyp17, when reacted for 0.5–1.5 h, all Rb1, Rd, and Gyp17 were completely hydrolyzed, and the reaction intermediate F2 was produced to 8.25%; then, the ginsenoside F2 gradually decreased and disappeared after reaction for 12 h. It can be considered that the enzyme hydrolyzed 20-O-Glc of Rb1 to Rd, and 3-O-Glc of Rd to F2; the enzyme hydrolyzed 20-O-Glc of Gyp17 to F2 and 20-O-Glc of F2 finally to C-K. In the enzyme reaction of Rb2, when reacted for 0.5 h, Rb2 (5.08%) was completely reacted; although the reaction intermediates C-O and C-Y were not recognized, the ginsenoside Rb2 should be changed to C-K. In the enzyme reaction of ginsenosides Fa and Fc, when reacted for 0.5 h, Fa decreased from 7.1% to 1.9% and disappeared after reaction for 1 h. At the same time, the ginsenoside R7 increased to about 6%, thereby proving that the enzyme hydrolyzed 20-O-Glc of Fa into R7. When reacted for 0.5–1 h, the ginsenoside Fc increased from 11.1% to 16.8%, then gradually decreased to about 11% during the reaction; thus, the enzyme weakly hydrolyzed 20-O-Xyl of Fc into R7. Therefore, the ginsenoside Rb3 and C-Mx1 were mainly hydrolyzed to C-Mx; the ginsenoside Rb1, Rd, Gyp17, Rb2, and most of Rc were hydrolyzed to C-K; the ginsenoside Fa was hydrolyzed to R7; the ginsenoside Fc was weakly hydrolyzed to R7. In brief, GE-I from *A. niger* g.848 strain can hydrolyze the 3-O- or 20-O-glycoside, such as Glc and Ara of NGL ginsenosides to C-K, but hardly hydrolyzed the terminal xyloside on the 20-O-sugar moiety of Rb3 or C-Mx1 or C-Mx or Fc and 3-O- sugar moiety of the ginsenoside Fa or Fc or R7 (Fig. 2 and Table 1); thus, C-Mc, C-K, R7, and Fc were mainly found in the final reaction mixture. When producing the product C-Mx from NGL ginsenosides by the crude GE-I from the *A. niger* g.848 strain, the good enzyme

| Reaction time (h) | Substrate ginsenosides (%) | Produced ginsenosides (%) |
|------------------|-----------------------------|---------------------------|
|                  | Fa  | Non-1 | Rb1 | Fc | Rc | Rb2 | Rb3 | R7 | Rd | Gyp17 | C-Mc1 | C-Mx1 | F2 | C-Mc | C-Mx | C-K |
| 0                | 7.100 | 4.67 | 4.31 | 11.1 | 13.8 | 5.08 | 27.1 | 0.72 | 3.03 | 1.51 | 6.44 | 15.2 | NP | NP | NP | NP |
| 0.5              | 1.90 | 3.78 | NP | 16.4 | NP | NP | 9.46 | 6.08 | 1.89 | 2.71 | 14.1 | 31.3 | 5.89 | NP | NP | NP | NP |
| 1                | NP | 4.01 | NP | 16.8 | NP | NP | 9.82 | 5.75 | 2.36 | 14.6 | 32.3 | 6.06 | NP | NP | NP | NP |
| 1.5              | NP | 4.25 | NP | 13.3 | NP | NP | NP | 7.47 | NP | 13.6 | 34.5 | 8.25 | 4.18 | 10.2 | 4.30 |
| 2                | NP | 4.02 | NP | 13.1 | NP | NP | NP | 7.75 | NP | 11.8 | 31.4 | 7.58 | 5.24 | 13.2 | 5.96 |
| 2.5              | NP | 3.80 | NP | 12.5 | NP | NP | NP | 7.74 | NP | 10.5 | 28.8 | 7.26 | 6.12 | 15.8 | 7.52 |
| 3                | NP | 3.76 | NP | 13.1 | NP | NP | NP | 8.10 | NP | 9.57 | 27.0 | 6.43 | 6.51 | 17.4 | 8.14 |
| 6                | NP | 2.98 | NP | 12.2 | NP | NP | NP | 6.48 | NP | 18.5 | 3.76 | 7.89 | 26.5 | 14.43 |
| 12               | NP | NP | NP | 12.7 | NP | NP | NP | 10.38 | NP | 9.62 | 8.22 | 38.4 | 20.81 |
| 24               | NP | NP | NP | 11.2 | NP | NP | NP | 11.4 | NP | NP | NP | 4.43 | 43.8 | 29.1 |
| 30               | NP | NP | NP | 11.2 | NP | NP | NP | 11.9 | NP | NP | NP | 5.48 | 42.3 | 29.2 |
| 36               | NP | NP | NP | 11.1 | NP | NP | NP | 12.3 | NP | NP | NP | 4.79 | 42.0 | 29.8 |
| 48               | NP | NP | NP | 10.3 | NP | NP | NP | 13.1 | NP | NP | NP | 3.69 | 42.4 | 30.6 |

Non-1, not recognized. NP, no product.
Fig. 3. Main biotransformation pathway of notoginseng stem-leaf ginsenosides by the crude ginsenosidase type-I from the A. niger g.848 strain.
Fig. 4. Ginsenoside composition of reaction mixture from 50 g of notoginseng stem-leaf ginsenosides in HPLC (after reaction for 24 h). (A) Ginsenoside composition of reaction mixture in HPLC. (B) Ginsenoside contents of reaction mixture. The experiment was repeated three times.

### Ginsenosides Retention time (min) Content (%)

| Ginsenosides | Retention time (min) | Content (%) |
|--------------|----------------------|-------------|
| Fc           | 39.495               | 11.2        |
| R7           | 41.148               | 11.8        |
| C-Mc         | 52.003               | 4.20        |
| C-Mx         | 52.811               | 43.2        |
| C-K          | 56.736               | 29.6        |
reaction time was 24 h; when producing the product C-K, the good enzyme reaction time was 24–36 h; when obtaining the product R7, the good reaction time was 24–48 h; when obtaining C-Mc, the good enzyme reaction time was 12 h; when obtaining Fc, the good reaction time was 1 h. The main biotransformation pathways of NGL ginsenosides by the crude GE-I from the A. niger g.848 strain are shown in Fig. 3.

3.4. Preparation and separation of ginsenoside C-Mx, C-K, R7, and Fc from NGL ginsenosides by the crude enzyme reaction

Under the aforementioned optimal reaction conditions, 50 g of NGL ginsenosides were dissolved in 1,000 mL of 0.02M acetate buffer (pH 5.0), mixed with the same volume of crude enzyme (final substrate concentration was 2.5%), and allowed to react at 45°C for

![Graph](image)

**Table 2**
The 

| Carbon site | C-Mx | C-K | R7 | Fc | Carbon site | C-Mx | C-K | R7 | Fc |
|-------------|-----|-----|----|----|-------------|-----|-----|----|----|
| Aglycone moiety | | | | | | | | | |
| C-1 | 39.55 | 39.72 | 39.35 | 39.37 | 3-0-Glc (inside) | | | | |
| C-2 | 28.41 | 28.41 | 26.91 | 26.92 | C-1’ | | | | |
| C-3 | 79.50 | 79.43 | 89.07 | 89.07 | C-2’ | | | | |
| C-4 | 39.70 | 39.58 | 39.88 | 39.88 | C-3’ | | | | |
| C-5 | 56.51 | 56.53 | 56.54 | 56.54 | C-4’ | | | | |
| C-6 | 18.91 | 18.93 | 18.59 | 18.59 | C-5’ | | | | |
| C-7 | 35.32 | 35.34 | 35.28 | 35.28 | C-6’ | | | | |
| C-8 | 40.22 | 40.24 | 40.18 | 40.18 | Glc (1→2) | | | | |
| C-9 | 50.47 | 50.47 | 50.35 | 50.35 | C-1’ | | | | |
| C-10 | 37.90 | 37.52 | 30.91 | 30.98 | C-2’ | | | | |
| C-11 | 31.05 | 31.10 | 30.91 | 30.98 | C-3’ | | | | |
| C-12 | 70.27 | 70.35 | 70.32 | 70.27 | C-4’ | | | | |
| C-13 | 49.67 | 49.65 | 49.62 | 49.64 | C-5’ | | | | |
| C-14 | 51.55 | 51.59 | 51.57 | 51.54 | C-6’ | | | | |
| C-15 | 30.89 | 30.95 | 30.91 | 30.98 | 3-0-Xyl | | | | |
| C-16 | 26.79 | 26.81 | 26.79 | 26.79 | C-1’ | | | | |
| C-17 | 51.75 | 51.79 | 51.76 | 51.75 | C-2’ | | | | |
| C-18 | 49.67 | 49.65 | 49.62 | 49.64 | C-3’ | | | | |
| C-19 | 16.47 | 16.50 | 16.11 | 16.15 | C-4’ | | | | |
| C-20 | 83.98 | 83.46 | 83.46 | 83.46 | C-5’ | | | | |
| C-21 | 25.75 | 25.94 | 22.52 | 22.43 | 3-0-Glc | | | | |
| C-22 | 36.34 | 36.32 | 36.28 | 36.31 | C-1’ | | | | |
| C-23 | 23.28 | 23.37 | 23.26 | 23.29 | C-2’ | | | | |
| C-24 | 126.2 | 126.12 | 126.11 | 126.17 | C-3’ | | | | |
| C-25 | 131.1 | 131.07 | 131.06 | 131.14 | C-4’ | | | | |
| C-26 | 22.41 | 22.52 | 25.91 | 25.95 | C-5’ | | | | |
| C-27 | 18.08 | 17.94 | 17.91 | 18.08 | 20-O-Xyl | | | | |
| C-28 | 28.83 | 28.86 | 28.23 | 28.23 | C-1’ | | | | |
| C-29 | 16.21 | 16.19 | 16.83 | 16.83 | C-2’ | | | | |
| C-30 | 17.58 | 17.56 | 17.53 | 17.57 | C-3’ | | | | |

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24 h. Then, 95% alcohol was added in the reaction mixture to stop the enzyme reaction; the supernatant was concentrated, treated with the AB-8 resin column and de-coloration D-280 resin column, and dried to obtain 35.8 g reaction product; the weight yield was about 71.6% for substrate. The enzyme reaction mixture was detected by HPLC as shown in Fig. 4.

Fig. 4 shows that the enzyme reaction product mainly contained the ginsenosides C-Mx, C-K, R7, and Fc; the contents are as follows: C-Mx, 43.2%; C-K, 29.6%; Fc, 11.2%; R7, 11.8%; C-Mc, 4.2%. Furthermore, 35.7 g of ginsenoside reaction-products were separated using the silica gel column to obtain monomer ginsenosides: 9.43 g of C-Mx, 6.85 g of C-K, 4.50 g of R7, and 4.71 g of Fc; the purity of the separated monomer ginsenosides was over 90% as detected by HPLC (Fig. 5).

It was considered that the minor ginsenoside C-Mx was mainly produced from Rb3 (27.1%) and C-Mx1 (15.2%); therefore, the C-Mx yield was high (yield, 9.43 g; i.e., 18.9% of the weight yield for substrate). The ginsenoside C-K was mainly produced from Rb1, Rd, Gyp17 (8.9% in substrate), Rb2 (5.1% in substrate), and Rc (13.8% in substrate); therefore, 6.85 g of C-K was obtained, and the weight yield was 13.7% for substrate. The ginsenoside R7 was produced from Fa and Fc; so, the R7 yield was 4.5 g. The obtained 4.71 g of ginsenoside Fc (hardly separated from NGL ginsenosides) was that in the substrate; during the enzyme reaction, the Fc content was 11.1% in substrate, 16.8% in 1 h reaction, 13.1% in 3 h reaction, finally about 11% to prove that Fc was weakly changed to R7.

The monomer ginsenosides C-Mx, C-K, and vina-ginsenoside R7 were successfully obtained from low-cost NGL ginsenosides using the low-cost crude enzyme from A. niger g.848 strain, and the notoginsenoside Fc was successfully separated.

3.5. Structure of the enzyme reaction product ginsenosides C-Mx, C-K, R7, and Fc by NMR

In the HPLC method, the product monomers obtained from the enzymatic hydrolysis of NGL ginsenosides were proved to be C-Mx, C-K, R7, and Fc by comparing with the standard C-Mx, C-K, R7, and Fc (Figs. 4 and 5). In order to ensure the accuracy of the results obtained, the NMR method was used to determine the structures of the enzyme reaction products. The $^{13}$C NMR (600 MHz, pyridine-$d_5$) spectral data of minor ginsenoside products are shown in Table 2. The data of Table 2 correspond with previous reports [29–31], and the enzyme reaction products should be minor ginsenosides C-Mx and C-K, vina-ginsenoside R7 and notoginsenoside Fc, as shown in Fig. 6.

The ginsenoside C-Mx was 20-O-[β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl]-24-en, 3β,12β,20(3S)-triol; the ginsenoside C-K was 20-O–D-glucopyranosyl-dammar-24-en, 3β,12β,20(3S)-triol; the R7 was 3-O-[β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl]-dammar-24-en, 3β,12β,20(3S)-triol; the Fc, 3-O-[β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl]-dammar-24-en, 3β,12β,20(3S)-triol.

In conclusion, all the different types of NGL ginsenosides are PPD ginsenosides with same aglycone. If the HPLC peak area ratio of

![Fig. 6. Structures of ginsenosides C-Mx, C-K, R7, and Fc.](image-url)
ginsenosides is assumed to be the ginsenoside content ratio, the ginsenoside contents are as follows: Rb3, 27.1%; C-Mx1, 15.7%; Rc, 13.8%; Fc, 11.1%; Fa, 7.10%; C-Mc, 6.44%; Rb2, 5.08%; Rb1, 4.31%; Rd; 3.07%; Gyp17, 1.51%; and R7, 0.72%. In the reaction of NGL ginsenosides with crude enzyme, the main reaction of Rb3 and C-Mx1 was Rb3 → C-Mx1 → C-Mx; when reacted for 1 h, Rb3 decreased from 27.1% to 9.82%, C-Mx1 increased from 15.5% to 32.3%, C-Mc was Rb3 3.07%; Gyp17, 1.51%; and R7, 0.72%. In the reaction of NGL ginsenoside contents are as follows: Rb3, 27.1%; C-Mx1, 15.7%; Rc, 9.43 g; C-Mx, 6.85 g; C-K, 4.50 g (producing by enzyme reaction), and 4.71 g Fc (hardly separating from NGL-ginsenosides) were separated using the silica gel column. The purity of monomers C-Mx, C-K, R7, and Fc is over 90%, and the structures of these monomers were recognized by NMR.

**Conflicts of interest**

All contributing authors declare no conflicts of interest.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jgr.2017.10.001.

**References**

[1] Jin FX. Biotransformation of natural products. Beijing: Chemical Industry Press; 2009. p. 74–113 [in Chinese].

[2] Christensen LP. Ginsenosides chemistry, biosynthesis, analysis, and potential health effects. Adv Food Nutr Res 2009;55:1–99.

[3] Liu CY, Song JG, Li PF, Yu HS, Jin FX. Ginsenoside contents in three different ginsengs. J Dalian Polytechn Univ 2011;30:79–82.

[4] Li PF, Liu CY, Guo JY, He D, Yu HS. Jin FX. Comparison of ginsenosides on different ginseng stem. J Anhui Agric Sci 2010;34:13077–9.

[5] Zhang JT. Ginseng is the King of Herbs—Ginseng chemistry, physiology activity and pharmacokinetics. Beijing: Chemical Industry Press, 2008. p. 34–44 [in Chinese].

[6] Huang F, Xiang JF, Wu JX. Research progress on the extraction and separation of ginsenosides from Notoginseng stem life. J Chinese Med Mater 2009;32:999–1005.

[7] Kobashi K. Glycosides are natural prodrugs—evidence using germ-free and gnotobiotic rats associated with a human intestinal bacterium. J Trad Med 1998;15:1–13.

[8] Tawab MA, Bahr U, Karas M, Wurglics M, Schubert-Zsilavecz M. Degradation of ginsenosides in humans after administration. Drug Metab Dispos 2003;31:1065–71.

[9] Hasegawa H. Proof of the mysterious efficacy of ginseng: basic and clinical trials: metabolic activation of ginsenoside: deglucosylation by intestinal bacteria and esterification with fatty acid. J Pharmacol Sci 2004;95:153–7.

[10] Popowich DC, Kitts DD. Structure-function relationship exists for ginsengsides in reducing cell proliferation and inducing apoptosis in the human leukemia (THP-1) cell line. Arch Biochem Biophys 2002;406:1–8.

[11] Cui CH, Kim SC, Im WT. Characterization of the ginsenoside transforming recombinant β-glucosidase from Actinomyces mirum and biocconversion of major ginsenosides into minor ginsenosides. Appl Microbiol Biotechnol 2013;97:649–59.

[12] Hasegawa H, Sung JH, Matsumiya S, Uchiyama M. Main ginseng saponin metabolites formed by intestinal bacteria. Planta Med 1996;62:453–7.

[13] Park CS, Yoo MH, Noh KH, Oh DK. Biotransformation of ginsenosides by hydrolyzing sugar moieties of ginsenosides using microbial glycosidases. Appl Microbiol Biotechnol 2010;87:9–19.

[14] Leung KW, Wong AS. Pharmacology of ginsenosides: a literature review. Chin Med 2010;5:20–2.

[15] Choi S, Kim TW, Singh SV. Ginsenoside Rh2-mediated G1 phase cell cycle arrest in human breast cancer cells is caused by p15 Ink4B and p27 Kip1-dependent inhibition of cyclin-dependent kinases. Pharm Res 2009;26:2280–8.

[16] Choi SH, Shin TJ, Hwang SH, Lee BH, Kang J, Kim HJ. Differential effects of ginsenoside metabolites on HERG channel currents. J Ginseng Res 2011;35:159–68.

[17] Kim YJ, Yamabe N, Choi P, Lee JW, Ham J, Kang KS. Effective thermal deglycosylation of Rd and its contribution to the improved anticancer activity of ginseng. J Agric Food Chem 2013;61:9185–91.

[18] Kim SJ, Kang BY, Cho SY, Sung DS, Chang HK, Yeon HM, Kim DH, Sim YC, Lee YS. Compound K induces expression of hyaluronan synthase 2 gene in transformed human keratinocytes and increases hyaluron in hairless mouse skin. Biochem Biophys Res Commun 2004;316:348–55.

[19] Kim M, Ahn BY, Lee JS, Chung SS, Lim S, Park SG, Jung HS, Lee HK, Park KS. The ginsenoside Rg3 has a stimulatory effect on insulin signaling in L6 myotubes. Biochem Biophys Res Commun 2009;389:70–3.

[20] Sun S, Wang CZ, Tong XL, Xiao L, Fishbein AWQ, He TC, Du W, Yuan CS. Effects of steaming the root of Panax notoginseng on chemical composition and anticancer activities. Food Chem 2010;118:307–14.

[21] Sala F, Mulet J, Choi S, Jung SY, Nah SY, Rhim H, Valore M, Ciaid, Mala S. Effects of ginsenoside Rg2 on human neuronal nicotinic acetylcholine receptors. J Pharmacol Exp Ther 2002;301:1052–9.

[22] Jin FX, Yu HS, Fu Yaoao, An DS, Im WT, Lee ST, Jaime A, Teixeira da Silva: biotransformation of ginsenosides (ginseng saponins). Int J Biomed Pharm Sci 2012;6(Special Issue):33–44.

[23] Yu HS, Zhang CZ, Lu MC, Sun F, Fu YY, Jin FX. Purification and characterization of new special ginsenoside hydrolyzing multi-glycides of protopanaxadiol ginsenosides, ginsenoside type I. Chem Pharm Bull 2007;55:231–5.

[24] Liu CY, Jin YH, Yu HS, Sun CK, Gao P, Xiao YK, Zhang TY, Xu LQ, Im WT, Jin FX. Biotransformation pathway and kinetics of ginsenosides type I hydrolyzing 3-O- and 20-O-mono- and 20-O-di- glycosides of PPD type ginsenosides. Process Biochem 2014;49:811–20.

[25] Liu CY, Zhou RX, Sun CK, Jin YH, Yu HS, Zhang TY, Xu LQ, Jin FX. Preparation of minor ginsenoside C-Mc, C-Y, F2 and C-k from American ginseng PPD-ginsenoside using special ginsenoside type-I from Aspergillus niger g648. J Ginseng Res 2012;35:221–9.

[26] Liu CY, Zou KZ, Yu HS, Sun CK, Zhang TY, Xu LQ, Jin YH, Jin FX. Preparation of minor ginsenoside C-Mx and C-K from notoginseng leaf ginsenosides by a special ginsenoside type-I. Process Biochem 2015;50:2158–67.

[27] Jin XF, Yu HS, Wang DM, Liu TQ, Liu CY, An DS, Im WT, Kim SG, Jin FX. Kinetics of a cloned special ginsenoside hydrolyzing 3-O-glucoside of multiprotopanaxadiol-type ginsenosides, named ginsenoside type III. J Microbiol Biotechnol 2012;22:343–51.

[28] Wang DM, Yu HS, Song JG, Xu YF, Jin FX. Enzyme kinetics of ginsenosides type IV hydrolysing 6-O-multi-glycides of protopanaxatriol type ginsenosides. Process Biochem 2012;47:133–4.

[29] Tanaka I, Kasai R. Saponins of ginseng and related plants. Progress in the chemistry of organic natural products, vol. 46. Berlin: Springer; 1984. p. 1–76.

[30] Duc NM, Kasai R, Ohtani K, Ito A, Nham NT, Yasumitsu K, Tanaka O. Saponins from Vietnamese ginseng, Panax vietnamensis HA et Grushv. Collected in central Vietnam.II. Chem Pharm Bull 1994;42:115–22.

[31] Yang TK, Kasai R, Zhou J, Tanaka O. Dammarrane saponins of leaves and seeds of Panax Notoginseng. Phytochemistry 1983;22:1473–4.