



Research Article

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

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ARTICLE INFO

Article history:

Received 22 February 2017

Received in Revised form

5 June 2017

Accepted 11 October 2017

Available online 21 October 2017

Keywords:

dynamic changes

enzyme reaction

ginsenosidase type-I

notoginseng

notoginseng stem-leaf ginsenosides

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%, C-Mx was produced to 6.46%, 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-O- 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

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notoginseng stem-leaf (NGL ginsenosides) are Rb3, C-Mx1, Rc, Fa, Fc, and Rb2 [4,6]. The sugar moieties of major ginsenosides of high content in ginseng have 3–5 number glycosides; thus, the major ginsenosides have low bioavailability and low absorption by the human bodies. After the oral intake of ginseng, the sugar moieties of major ginsenosides are hydrolyzed by digestive enzymes and/or intestinal bacteria into minor ginsenosides with low number glycoside to be absorbed by human bodies [7]; however, these conversions are low, and the absorption of major ginsenosides is quite rare [8].

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, Rh2, and Rh1, in the red ginseng and minor ginsenosides C-K, C-Mc, C-Mx, and C-Y with low number glycoside 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- and 20-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-multi-glycosides of PPD ginsenosides, the enzyme hydrolysis sequence and hydrolysis 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.g48p strains can hydrolyze PPD ginsenosides Rb1, Rb2, Rc, and Rd into F2, C-K, and small amount of Rh2 [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, Gyp75, 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 *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.g48p 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 reports 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 vana-ginsenoside R7 and notoginsenoside 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-F₂₅₄ 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 react 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; ^{13}C : 150 MHz) NMR Spectrometer (Switzerland)] [25,26].

3. Results and discussion

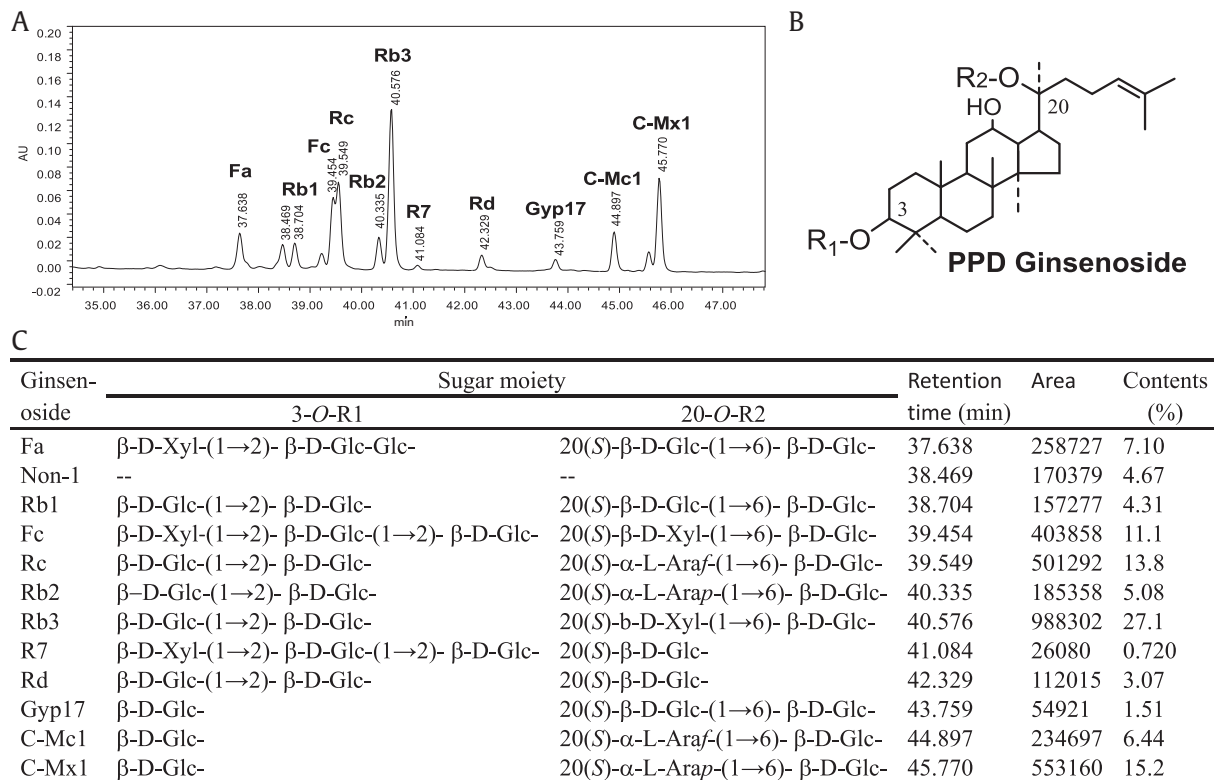
3.1. Analysis on the purchased NGL ginsenosides

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.



Non-1, no-recognized. Ginsenoside contents, HPLC peak area ratio. The experiment, repeat 3 times.

Fig. 1. Composition of notoginseng stem-leaf ginsenosides in HPLC and their structures. (A) Notoginseng stem-leaf ginsenosides in HPLC. (B) Structure of protopanaxadiol ginsenoside. (C) Table of the sugar moiety of notoginseng stem-leaf ginsenosides and ginsenoside contents by HPLC (contents, peak area ratio %). The experiment was repeated three times.

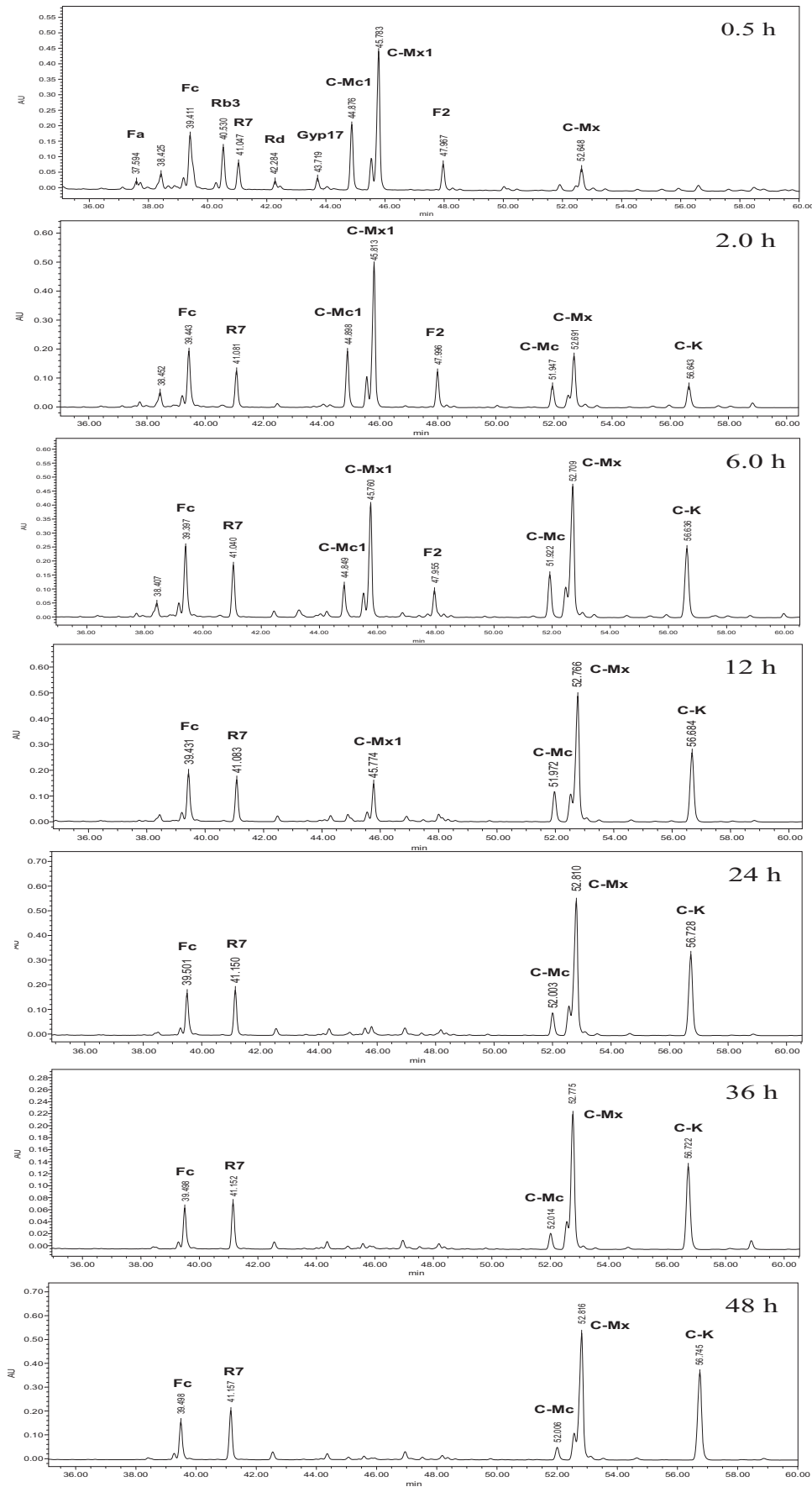


Fig. 2. In different reaction times, the dynamic conversion of notoginseng stem-leaf ginsenosides by crude ginsenosidase type-I from *A. niger* in HPLC. The experiment was repeated three times.

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-glycoside 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% differentiated 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 the all 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-Mx 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 3-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 ginsenosides Rb3 and C-Mx1 were mainly hydrolyzed to C-Mx; the ginsenosides 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* g848 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-Mx, 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

Table 1

Dynamic changes of notoginseng stem-leaf ginsenosides in different enzyme reaction times (area ratio %). The experiment was repeated three times

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	6.31	NP	
1	NP	4.01	NP	16.8	NP	NP	9.82	5.75	NP	2.36	14.6	32.3	6.06	NP	6.46	1.81	
1.5	NP	4.25	NP	13.3	NP	NP	NP	7.47	NP	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	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	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	NP	9.57	27.0	6.43	6.51	17.4	8.14	
6	NP	2.98	NP	12.2	NP	NP	NP	8.41	NP	NP	5.48	18.5	3.76	7.89	26.5	14.43	
12	NP	NP	NP	12.7	NP	NP	NP	10.38	NP	NP	NP	9.62	NP	8.22	38.4	20.81	
24	NP	NP	NP	11.2	NP	NP	NP	11.4	NP	NP	NP	NP	NP	4.43	43.8	29.1	
30	NP	NP	NP	11.2	NP	NP	NP	11.9	NP	NP	NP	NP	NP	5.48	42.3	29.2	
36	NP	NP	NP	11.1	NP	NP	NP	12.3	NP	NP	NP	NP	NP	4.79	42.0	29.8	
48	NP	NP	NP	10.3	NP	NP	NP	13.1	NP	NP	NP	NP	NP	3.69	42.4	30.6	

Non-1, not recognized. NP, no product

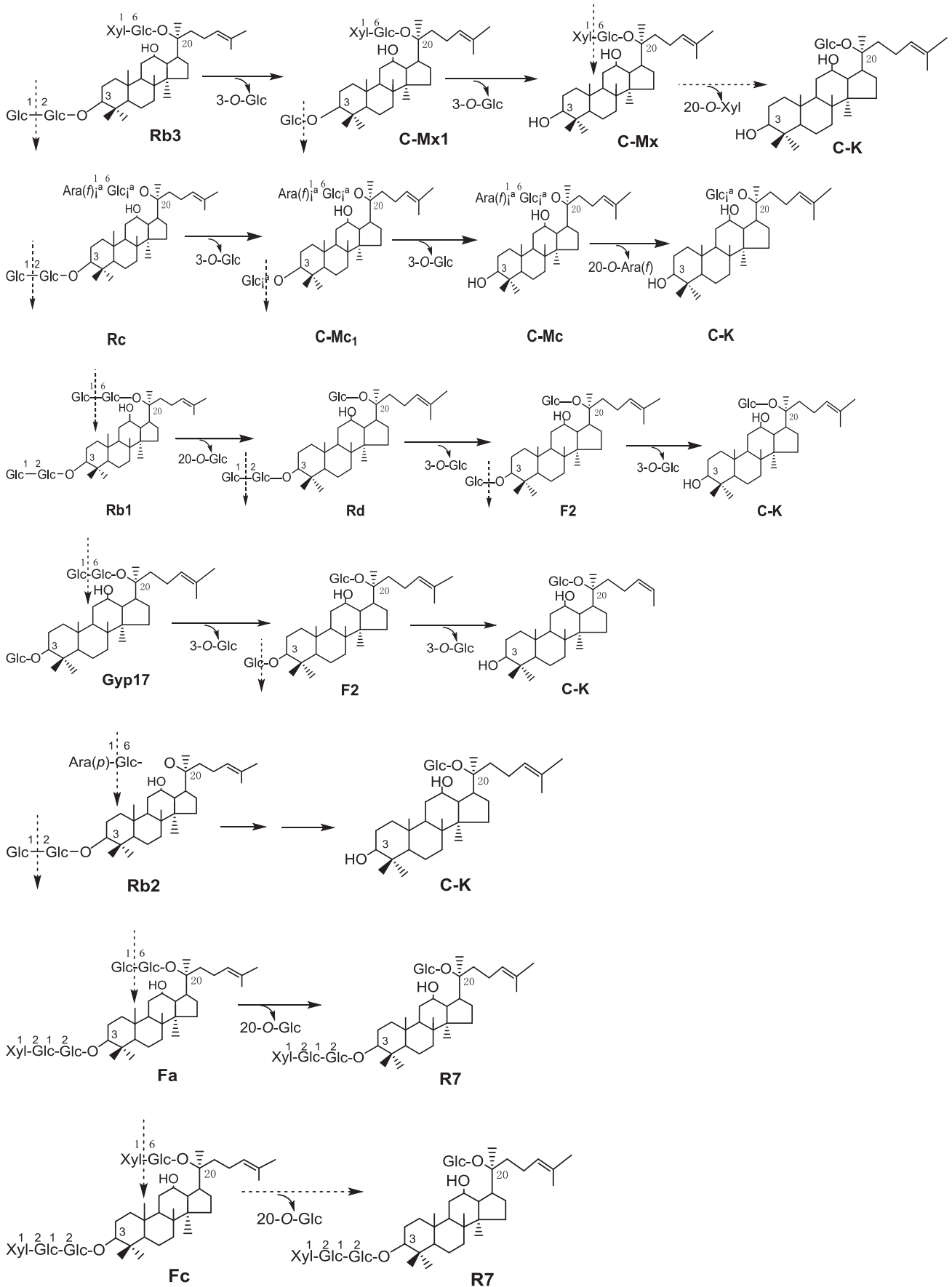
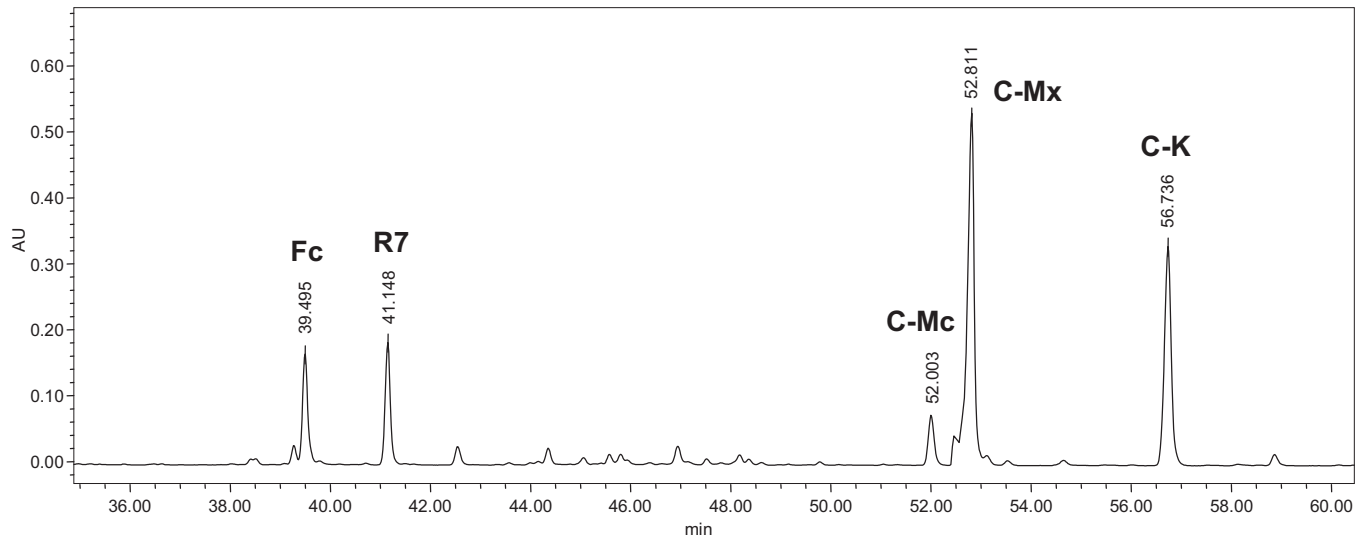


Fig. 3. Main biotransformation pathway of notoginseng stem-leaf ginsenosides by the crude ginsenosidase type-I from the *A. niger* g.848 strain.

A



B

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

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.

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

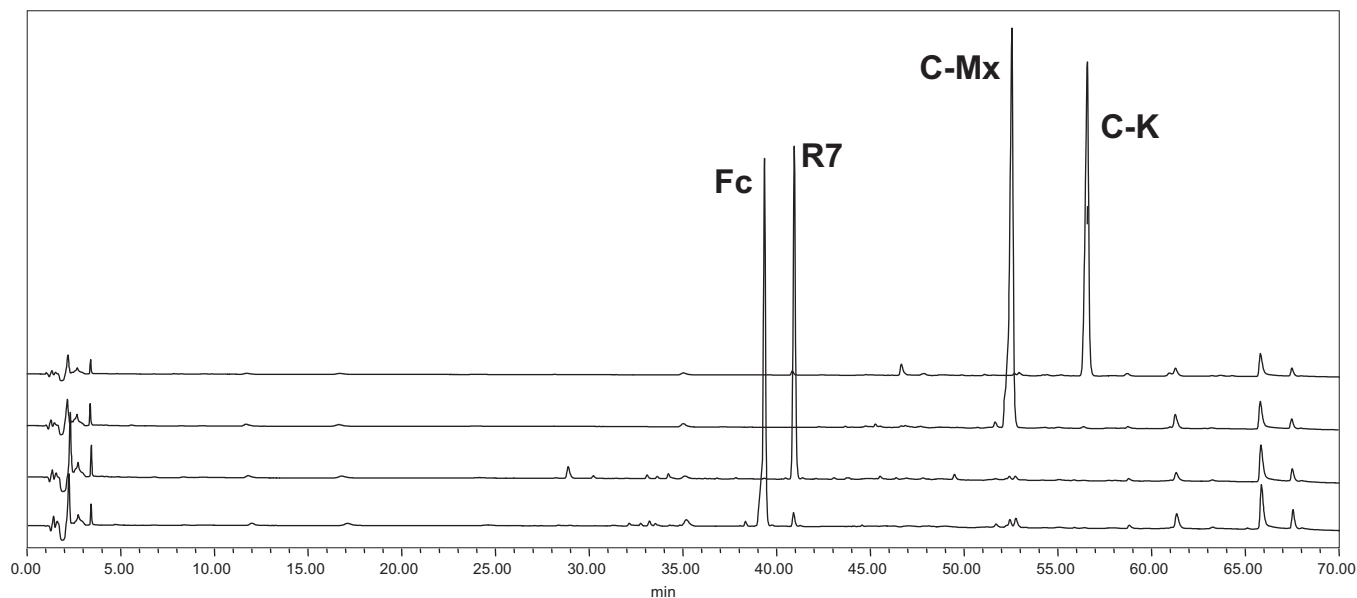


Fig. 5. Separated monomer ginsenosides from the reaction mixture of notoginseng stem-leaf ginsenosides in HPLC.

Table 2

The ^{13}C NMR spectroscopic data of enzyme reaction products C-Mx, C-K, R7, and Fc. In this work, assignments were based on ^1H , ^{13}C , DEPT, HSQC NMR experiments and compared with the reference of [29–31].

Carbon site	C-Mx	C-K	R7	Fc	Carbon site	C-Mx	C-K	R7	Fc
Aglycone moiety					3-O-Glc (inside)				
C-1	39.55	39.72	39.35	39.37	C-1'	—	—	104.91	104.91
C-2	28.41	28.41	26.91	26.92	C-2'	—	—	83.08	83.11
C-3	79.50	79.43	89.07	89.07	C-3'	—	—	78.07	78.08
C-4	39.70	39.58	39.88	39.88	C-4'	—	—	71.79	71.72
C-5	56.51	56.53	56.54	56.54	C-5'	—	—	77.89	77.89
C-6	18.91	18.93	18.59	18.59	C-6'	—	—	62.99	62.98
C-7	35.32	35.34	35.28	35.28	Glc (1→2)				
C-8	40.22	40.24	40.18	40.18	C-1''	—	—	103.30	103.31
C-9	50.47	50.47	50.32	50.35	C-2''	—	—	84.66	84.66
C-10	37.90	37.52	37.05	37.05	C-3''	—	—	78.40	78.11
C-11	31.05	31.10	30.91	30.98	C-4''	—	—	71.31	71.24
C-12	70.27	70.35	70.32	70.27	C-5''	—	—	77.93	77.93
C-13	49.67	49.65	49.62	49.64	C-6''	—	—	63.01	63.12
C-14	51.55	51.59	51.57	51.54	3-O-Xyl				
C-15	30.89	30.95	31.03	30.87	C-1'''	—	—	106.56	106.57
C-16	26.79	26.81	26.79	26.79	C-2'''	—	—	76.08	76.09
C-17	51.75	51.79	51.76	51.75	C-3'''	—	—	79.42	78.81
C-18	16.49	16.53	16.44	16.43	C-4'''	—	—	70.85	71.30
C-19	16.47	16.50	16.11	16.15	C-5'''	—	—	67.55	67.56
C-20	83.98	83.46	83.46	83.59	20-O-Glc				
C-21	25.75	25.94	22.52	22.43	C-1'	98.22	98.40	98.41	98.24
C-22	36.34	36.32	36.28	36.31	C-2'	75.00	75.27	75.28	75.02
C-23	23.28	23.37	23.26	23.29	C-3'	78.20	78.40	78.42	78.40
C-24	126.2	126.12	126.11	126.17	C-4'	71.23	71.80	71.92	71.90
C-25	131.1	131.07	131.06	131.14	C-5'	78.10	78.22	78.80	77.07
C-26	22.41	22.52	25.91	25.95	C-6'	70.20	63.04	63.11	70.18
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''	106.0	—	—	105.98
C-29	16.21	16.19	16.83	16.83	C-2''	74.97	—	—	74.99
C-30	17.58	17.56	17.53	17.57	C-3''	77.06	—	—	79.48
					C-4''	71.77	—	—	70.85
					C-5''	67.11	—	—	67.13

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 vana-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, vana-ginsenoside R7 and notoginsenoside Fc, as shown in Fig. 6.

The ginsenoside C-Mx was 20-O-[β -D-xylopyranosyl-(1 \rightarrow 6)- β -D-glucopyranosyl]-24-en, 3 β , 12 β , 20(S)-triol; the ginsenoside C-K was 20-O- β -D-glucopyranosyl-dammar-24-en, 3 β , 12 β , 20(S)-triol; the R7 was 3-O-[β -D-xylopyranosyl-(1 \rightarrow 6)- β -D-glucopyranosyl]-20-O- β -D-glucopyranosyl-dammar-24-en, 3 β , 12 β , 20(S)-triol; the Fc, 3-O-[β -D-xylopyranosyl-(1 \rightarrow 6)- β -D-glucopyranosyl]-0-O-[β -D-xylopyranosyl-(1 \rightarrow 6)- β -D-glucopyranosyl]-dammar-24-en, 3 β , 12 β , 20(S)-triol.

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

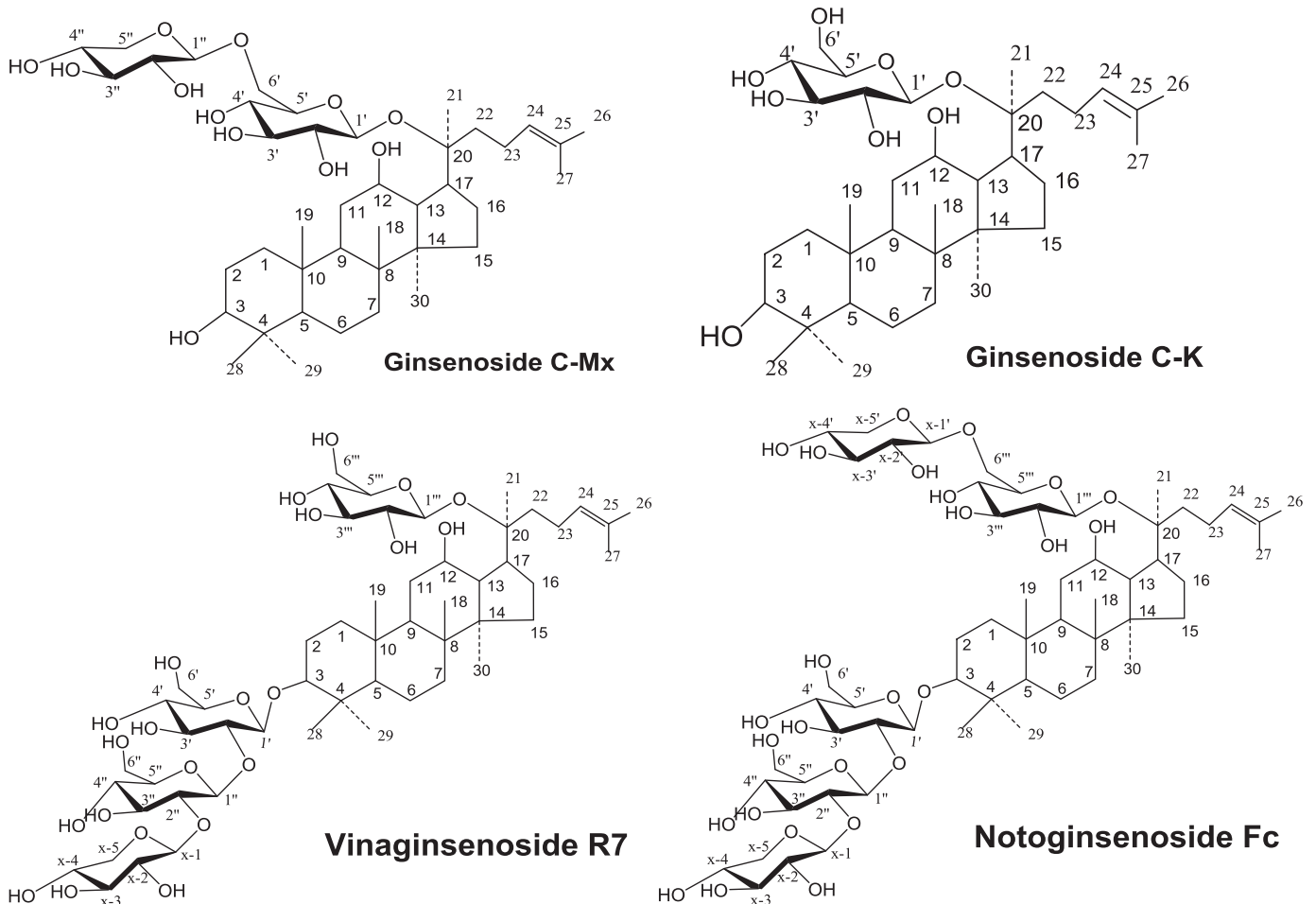


Fig. 6. Structures of ginsenosides C-Mx, C-K, R7, and Fc.

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-Mx was produced to 6.46%, finally into C-Mx and small amount of C-K. C-K was mainly produced from Rb1, Rd, Gyp17, Rb2, and Rc; when reacted for 1.5 h, 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%) was Rc→C-Mc1→C-Mc→C-K.

In brief, the GE-1 enzyme from *A. niger* g848 strain can hydrolyze 3-O- or 20-O-glycoside, such as Glc and Ara of NGL ginsenosides to C-K, but weakly hydrolyzed the terminal xyloside on the 20-O-sugar-moiety of Rb3 or C-Mx1 or C-Mx finally into C-K, weakly hydrolyzed the terminal xyloside on the 20-O-sugar-moiety of Fc into R7, and hardly hydrolyzed the terminal xyloside on the 3-O-sugar-moiety of the ginsenoside Fc or R7 to remain in the reaction solution; thus, C-Mx, C-K, R7, and Fc were mainly found in the final reaction mixture. To solve the problem of hydrolysis, the terminal xyloside on the 3-O-sugar-moiety of the ginsenosides Fa, Fc, and R7, new enzymes are needed.

After 50 g NGL ginsenosides reaction, the monomer products of 9.43 g C-Mx, 6.85 g C-K, 4.50 g R7 (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.

Acknowledgments

This work was supported by Major Projects of China National Science and Technology No. 2012ZX09503001-003, by the National High-end Foreign Expert Project of China, No. GDT20152100019.

Appendix A. Supplementary data

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

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