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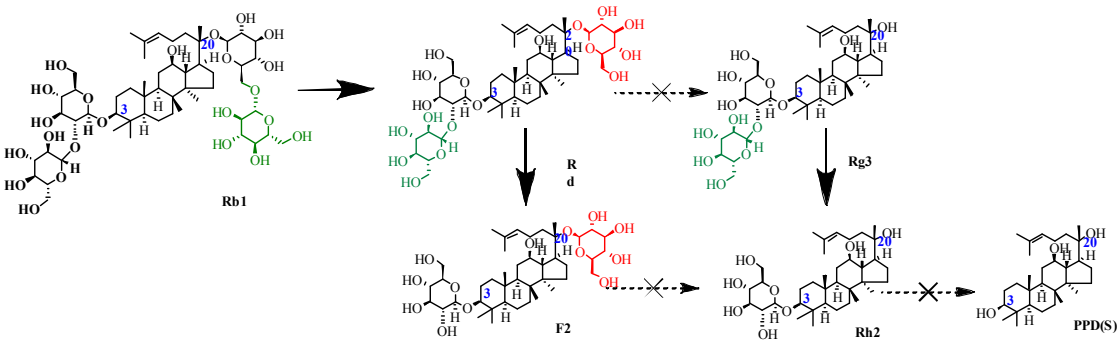

The *β*-galactosidase from *Aspergillus* sp. displayed *β*-glucosidase activity that was responsible for its ability to transform major ginsenoside Rb1 to rare ginsenoside F2 via ginsenoside Rd. Ginsenoside Rg3 can be selectively hydrolyzed with this *β*-galactosidase as well. *β*-1,2-Glucosidic linkage at C3 of ginsenoside Rg3 was cleaved selectively and only Rh2 was got eventually.

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ARTICLE

Highly efficient biotransformation of ginsenoside Rb1 and Rg3 using a *β*-galactosidase from *Aspergillus* sp.

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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A preliminary study on enzymatic biotransformation of ginsenosides was evaluated. A *β*-galactosidase from *Aspergillus* sp. displayed *β*-glucosidase activity that was responsible for its ability to transform major ginsenoside Rb1 to rare ginsenoside F2 via ginsenoside Rd. The Rb1 conversion, Rd and F2 yields reached 100%, 80.7% and 14.3% after 60 h at 60 °C, respectively. Ginsenoside Rg3 can be selectively hydrolyzed and only Rh2 was got with this *β*-galactosidase as well. Before hydrolysis, Rg3 inclusion complex was prepared with hydroxypropyl-*β*-cyclodextrin (HP-*β*-CD) for improving the aqueous solubility. The solubility of Rg3 increased 74.6 folds, and the phase solubility curve displayed a typical A_L-type indicating the formation of 1:1 inclusion complex. Using an enzyme loading of 500 U/g Rg3, the highest Rg3 conversion of 90.6% and Rh2 yield of 88.5% were obtained after 24 h at 60 °C. These results indicate that the *β*-galactosidase from *Aspergillus* sp. could be useful for the mass production of rare ginsenosides.

1. Introduction

Ginsenosides (*ginseng saponins*) are regarded as the principal ingredients responsible for immune-modulating, anti-fatigue and anti-tumor activities of ginseng, and more than 100 ginsenosides have been isolated and identified [1-3]. Basing on structure-activity relationship, some rare ginsenosides (F2, Rh2, etc.) possess higher bioavailability more active and easily absorbed by human body [4]. However, they are generally expensive for low content from natural. Therefore, numerous strategies have been proposed for preparation, such as chemosynthesis, enzymatic conversion and microbial transformation [5-7]. For high selectivity, mild condition and environmental compatibility, enzymatic conversion is a main way for rare ginsenosides preparation from major ginsenosides [8, 9].

Ginsenoside Rb1 is present in greater abundance than any other ginsenosides in ginseng, and various rare ginsenosides could be prepared from Rb1. Aiming to prepared specific rare ginsenosides, appropriate catalysts should be considered firstly, Because Rb1 harbors four *β*-glucosidic linkages including *β*-sophorose and *β*-gentiobiose residues in the C3 and C20 positions, respectively, of 20(S)-protopanaxadiol (Scheme 1). The highly substrate-tolerant mutant *Paecilomyces bainier* 229-7 can selectively transform ginsenoside Rb1 to Rd with a high bioconversion rate [10]. Yang et al [5] reviewed various biotransformation of rare ginsenoside compound K (20-O-beta-D-glucopyranosyl-20(S)-protopanaxadiol, CK) from Rb1. The thermostable *β*-glucosidase BGL3T from T. thermarumDSM 5069^T catalyzed the conversion of ginsenoside Rb1 to Rg3[11, 12]. *S*- Aglycone protopanaxadiol (PPD(S)) also can be produced with *Sphingobacterium multivorum* GIN723 [13] and a thermostable *β*-glycosidase from *Pyrococcus furiosus* [14]. Nevertheless, there are few literatures on F2 preparation from Rb1 [15].

Moreover, some ginsenoside's poor water solubility is another bottleneck for enzymatic reaction, such as Rg3 is almost insoluble in water (<0.02 mg/mL). However, most of studies ignored this factor.

In this report, Rb1 and Rg3 were chosen as substrates for rare ginsenosides F2 and Rh2 preparation using a *β*-galactosidase from *Aspergillus* sp. (Scheme 1). *β*-*Galactosidases* (EC 3.2.1.23) are mainly employed to hydrolyze lactose and synthesize galacto-oligosaccharides (GOS) [16, 17]. However, most of *β*-glycosidases have both *β*-galactosidase and *β*-glucosidase activities, even if they are referred on the basis of their dominant activity, as either *β*-glucosidases or *β*-galactosidase [18]. Before biotransformation, Rg3 inclusion complex was prepared for improving water solubility while Rb1 needed no treatment.

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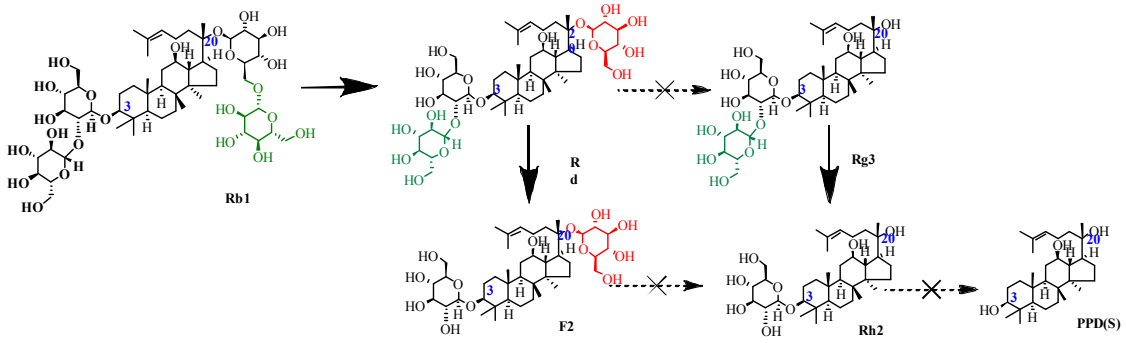

Scheme 1 Transformation pathway from ginsenoside Rb1 to F2 via Rd, Rg3 to Rh2 using the *β*-galactosidase from *Aspergillus* sp.

2. Materials and methods

2.1 Chemicals

β-Galactosidase from *Aspergillus sp.* was provided by Dr. Wu Jing at the State Key Laboratory of Food Science and Technology at Jiangnan University. Ginsenoside Rb1 (≥90%, HPLC), ginsenoside Rg3 (≥90%, HPLC) and ginsenoside standards (ginsenoside Rb1, Rd, Rg3, Rh2, XVII ≥98%, HPLC) were purchased from Zelang Medical Technology Co., Ltd (Jiangsu, PR China). HP-*β*-CD (1522.6 g/mol) was purchased from Zibo Qianhui Biological Technology Co., Ltd (Shandong, PR China). *o*-Nitrophenol (*o*NP, 99%) and *p*-Nitrophenol (*p*NP, 99%GC) were purchased from Aladdin (Shanghai, PR China). 4-Nitrophenyl *β*-D-glucopyranoside (*p*NPG, 98%) and 2-Nitrophenyl *β*-D-galactopyranoside (*o*NPG, 99%) were purchased from Bioman Biotechnology Co. Ltd (shanghai, PR China). Acetonitrile was purchased from Oceanpak Alexative chemical Co., Ltd; other reagents were analytical grades were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, PR China).

2.2 Determination of enzyme activity of *β*-galactosidase [18]

*o*NPG activity assay: *o*NPG was used as substrate. The increase in the absorbance at 420 nm caused by the release of *o*-nitrophenol (*o*NP) was measured to calculate the hydrolytic activity. The reaction mixture containing 1.8 mL phosphate buffer (50 mM, pH 5.6), 100 μL enzyme solution (dissolved and diluted using the aforementioned buffer) and 100 μL oNPG (20 mM) was shaken at 37 °C for 10 min, and then quenched by 1 mL Na_2CO_3 (1 M).

*p*NPG activity assay: *β*-glucosidase activity was assayed by measuring the amount of *p*NP liberated from pNPG as substrate according to the absorbance at 405 nm. This method is similar to the above *β*-galactosidase activity assay except the substrate.

One unit (U) of activity (both of *p*NPG activity and *o*NPG activity) is defined as the amount (g) of enzyme required to release 1 μmol *o*NP or *p*NP per min under the above reaction conditions.

2.3 Phase solubility curve of Rg3 [19]

Different concentrations of HP-*β*-CD solutions (0-300 mg/mL) were prepared and excess Rg3 standard was added, respectively. Then, the above solutions were shaken for 24 h at 45 °C, 55 °C or 65 °C. After centrifugation, Rg3 concentration in supernatant fluid was determined by HPLC (Waters 2695, United States) equipped a C18 column (Lichrospher C18, 4.6×250 mm, 5 μm) and a photodiode array detector (waters 996, 205 nm). The column temperature was 35 °C and the injection volume was 20 μL. A mixture of acetonitrile and water was used as mobile phase at 1 mL/min: 75:25 (*v/v*, 0 min), 50:50 (*v/v*, 20 min), 75:25 (*v/v*, 21-30 min). The retention times of ginsenoside Rb1, Rd, F2, Rg3 and Rh2 were 10.86 min, 14.06 min, 15.93 min, 16.68 min and 22.60 min, respectively.

2.4 Preparation of the inclusion complex of Rg3 with HP-*β*-CD

The methanol solution of Rg3 (1 mL, 20 mg/mL) was added to the aqueous solution of HP-*β*-CD (200 mg/mL) by dropping, the mixture was stirred thoroughly for 2 h at 60 °C and then went on for 3 h at 25 °C. The resulting solution was dried at 70 °C. The solubility of complex was analyzed by HPLC (refer to 2.3, Rg3 retention time was 16.7 min). Morphology of the samples was observed using SEM (1.0 KV, gold sputtering treated for 1 min).

2.5 Enzymatic biotransformation of ginsenoside

Because of high water solubility (> 150 g/L, 25 °C), Rb1 was enzymatic hydrolyzed directly using β-galactosidase from *Aspergillus* sp. and the reaction conditions as follows: Rb1 water solution (5 g/L) and β-galactosidase from *Aspergillus* sp. (750 U/g Rb1) were mixed in an Erlenmeyer flask, and then shaken at 60 °C for 96 h. The reaction mixture and the final product were analyzed with HPLC (refer to 2.3, Rb1, Rd and F2 retention times were 10.9 min, 14.2 min and 16.0 min). Ginsenosides were identified by HPLC using standards and liquid chromatography-mass spectrometry according to their molecular weights. UPLC-MS profile was taken from Waters Acquity UPLC system (BEH HILIC column; mobile phase: acetonitrile and water (30:70, 0 min; 100:0, 11 min-13 min); 0.3 mL/min; column temperature: 30 °C; injection volume: 2 μL; collision energy: 6 eV; 200-2000; polarity: ES^-). Concentration of glucose in the reaction mixture was determined with SBA-50 Glucose Biosensor (Biology Institute of Shandong Academy of Sciences, Shandong, PR China).

Rg3 inclusion complex solution (25 g/L) and the β-galactosidase from *Aspergillus* sp. (250 U/g Rg3, 500 U/g Rg3) were mixed in an Erlenmeyer flask, and then shaken at 60 °C for 48 h. The reaction mixture and the final product were analyzed with a HPLC system (refer to 2.3, Rh2 retention time was 22.60 min). The conversion of Rg3 was calculated as following: Rg3 conversion = $(C_0 - C_{t1})/C_0 \times 100\%$, here C_0 is the initial Rg3 concentration (g/L), C_{t1} is the real time Rg3 concentration in the reaction mixture (g/L). The Rh2 yield was calculated as following: Rh2 yield $= C_{t2}/C_2 \times 100\%$, here C_2 is the theoretical Rh2 concentration when Rg3 conversion is 100% (g/L), C_{t2} is the real time Rh2 concentration in the reaction mixture (g/L). The Rg3 and Rh2 concentrations were determined with each standard calibration curves. Each test was carried out in triplicate.

3. Results and discussion

3.1 Transformation of Rb1

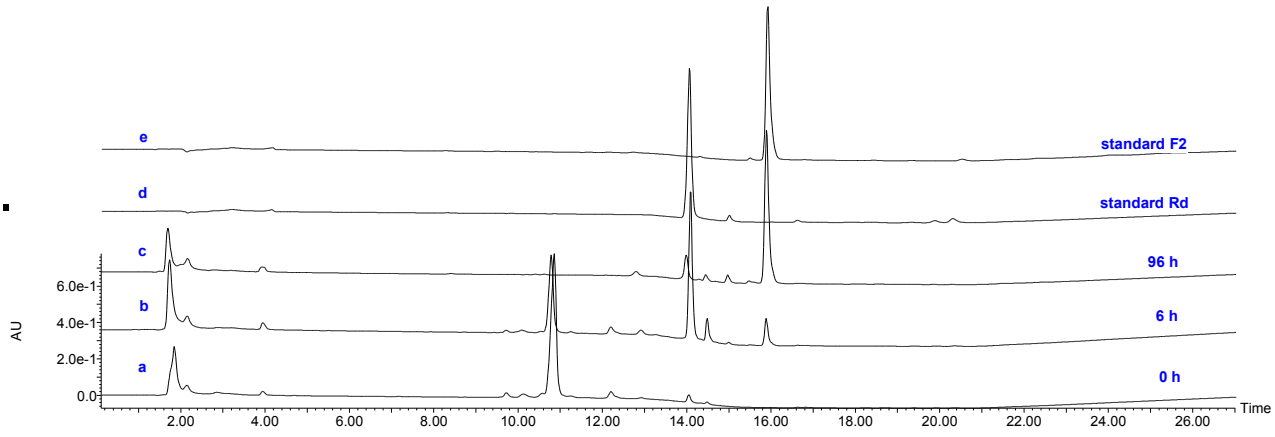

Fig. 1 Time-course HPLC profiles of Rb1 hydrolysis reaction.

a-c represent 0 h, 6 h and 96 h; d and e represent Rd and F2 standards (1 mg/mL, methanol); 60 °C, Rb1 5 mg/mL, 750 U/g Rb1, 25 mL.

*p*NPG and *o*NPG activities of the β-galactosidase from *Aspergillus* sp. were 14 U/g and 6540 U/g, respectively. The *p*NPG activity was responsible for its ability to carry out transforming major ginsenosides into rare ginsenosides. Therefore, enzyme loading of all tests was based on the *p*NPG activity.

Firstly, the enzymatic conversion of Rb1 over a time-course was conducted using the β-galactosidase from *Aspergillus* sp. (Fig. 1). The retention times (RT) and characteristic Collision-Induced Dissociation (CID) fragment ions of the ginsenoside standards and Rb1 hydrolysis products were summarized in Table 1. These results shows that Rb1 was converted to Rd at the initial reaction stage by the loss of a glucose moiety at the β-(1, 6)-glucosidic linkage of the C20 position of Rb1; Rd continued to be converted by the loss of a glucose moiety at the β-(1, 2)-glucosidic linkage of C3 position of Rd and F2 was obtained finally.

Table 1 UPLC-MS and corresponding CID data of ginsenoside standards and enzymatic hydrolysis products using the β-galactosidase from *Aspergillus* sp.

RT (min)	MS (m/z)			CID (m/z)	Identity
	$[M-H]^-$	$[M+Cl]^-$	$[M+HCOO]^-$		
1.60	1108	-	-	946$[M-H-Glc]^-$, 784	Rb1

				[M-H-2Glc]$^-$	
2.25	946	982	992	784[M-H-Glc]$^-$	Rd
2.48*	946	982	992	784[M-H-Glc]$^-$	XVII
3.43	784	820	830	622[M-H-Glc]$^-$	F2

*minor composition

The time course of Rb1 hydrolysis was studied at 60 °C and 750 U/g Rb1 enzyme loading. Fig. 2 indicated that the hydrolysis reaction reached an endpoint at about 60 h, and Rd yield reached the maximum of 58.9% at 6 h. In addition, the glucose concentration in the reaction mixture was 1.0 g/L when Rb1 conversion reached 100%. This is equivalent to the calculated value when the F2 and Rd yield reached 80.7% and 15.3% respectively. In contrast with previous research [15], *Aspergillus*, the source of *β*-galactosidase, is common and safe. More work is needed for optimization reaction conditions.

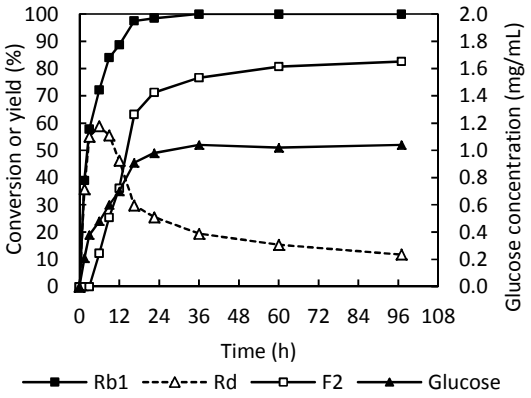

Fig. 2 Time course of enzymatic transformation of Rb1 with *β*-galactosidase. 60 °C, Rb1 5 mg/mL, 750 U/g Rb1, 25 mL

3.2 Preparation of Rg3 inclusion complex

No products were detected when Rg3 was used as substrate directly for its low water solubility (less than 0.017 mg/mL at 25 °C). The *β*-galactosidase will lose activity with additional methanol, although it can improve solubility. Therefore, Rg3 inclusion complex with HP-*β*-CD was prepared before enzymatic reaction. Lots of hydrophobic substances water solubility can be improved through inclusion method with hydroxypropyl-*β*-cyclodextrin (HP-*β*-CD), which has been approved obviously by FDA due to its superior water-solubility and safety profiles [20]. The yield of inclusion was 88.2% with 2.45% Rg3 concentration. HP-*β*-CD presented a globular hole aspect, based on SEM micrograph (Fig. 3b). It is easy to distinguish Rg3 and HP-*β*-CD from their physical mixture (Fig. 3c). However, the original morphology of the HP-*β*-CD disappeared after complex (Fig. 3d). This drastic change in particle shape and aspect indicated the presence of a new solid phase. The Rg3 water solubility was significantly enhanced from 0.017 mg/mL to 1.2 mg/mL (about 74.6 folds).

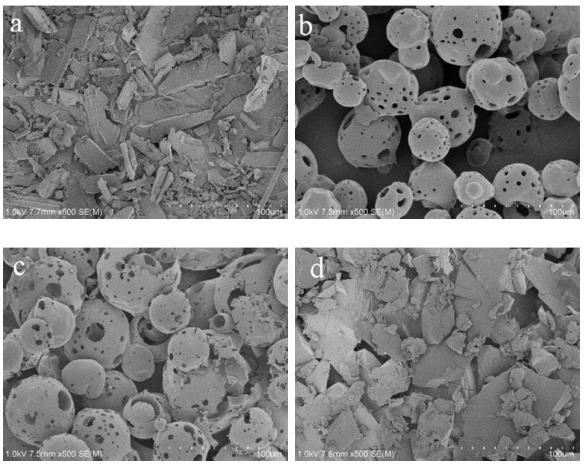

Fig. 3 SEM micrographs of Rg3 (a), HP-*β*-CD (b), HP-*β*-CD and Rg3 physical mixture (50:1 mass ratio) (c) and Rg3/HP-*β*-CD complex (d)

A linear relationship was obtained between the amount of Rg3 and the concentration of HP-*β*-CD at all tested temperatures, which classified as a typical A_L-type (Fig. 4). According to Higuchi and Connors's theory [19], this may be attributed to the formation of a 1:1 inclusion complex between Rg3 and HP-*β*-CD.

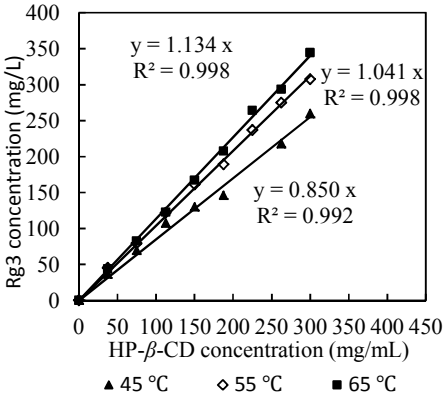

Fig. 4 Phase solubility curve of Rg3.

3.3 Transformation of Rg3

When Rg3 inclusion complex was used as substrate, only one product was produced and its RT was the same as that of Rh2 standard based on total ion chromatograms (Figs. 5a1-c1). Moreover, association of MS spectrums (m/z: 829.6, $[M_{Rg3}+HCOO]^-$; m/z: 783.6, $[M_{Rg3}-H]^-$; m/z: 1268.2, $[2M_{Rg3}-H]^-$; m/z: 667.5, $[M_{Rh2}+HCOO]^-$; m/z: 1290.0, $[2M_{Rh2}+HCOO-2H]^-$), Rh2 was confirmed as a single hydrolysis product. This indicated that inclusion with HP-*β*-CD did not block the *β*-galactosidase from selective cleaving *β*-1, 2 glucosidic linkage of Rg3, without attacking on other glucosidic linkages.

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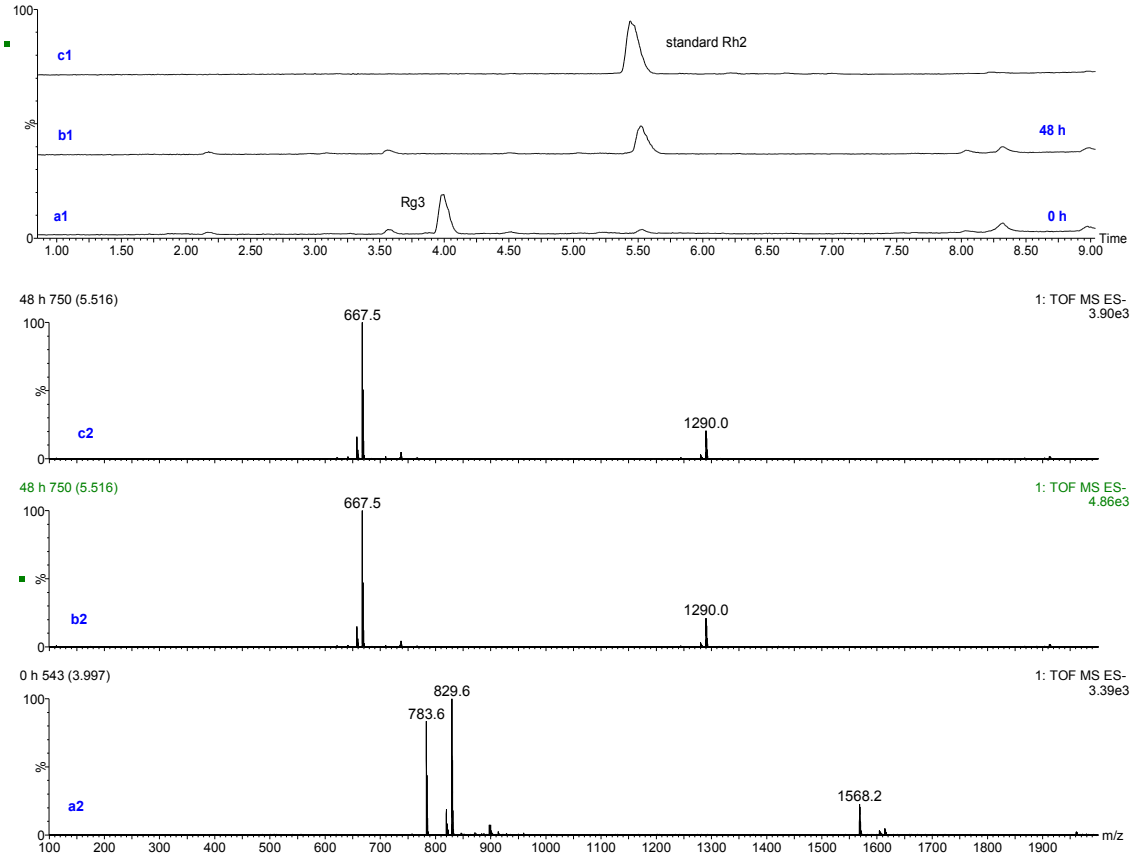
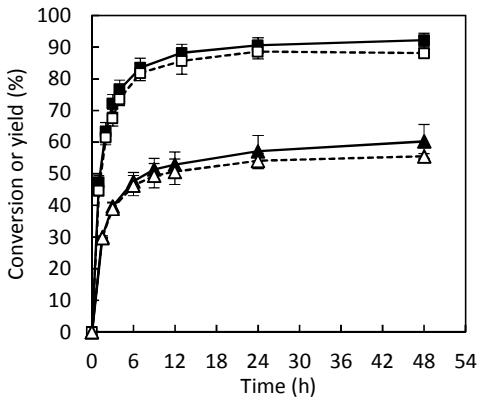


Fig. 5 UPLC-MS profiles of Rg3 hydrolysis reaction.

a1, b1 and c1 represent total ion chromatograms (TIC) of 0 h, 48 h and Rh2 standard (1 mg/mL, methanol); a2, b2 and c2 represent the MS spectrum of Rg3 hydrolysis product and Rh2 standard, respectively.

Effects of enzyme loading and reaction time on Rg3 hydrolysis were tested. Fig. 6 shows that the reaction reached equilibrium after 24 h. Rg3 conversion and Rh2 yield increased by a factor of 1.6 since doubling the enzyme loading, reached 90.6% and 88.5%, respectively.

The route of Rh2 chemosynthesis from PPD is extreme tedious with special glycosyl donor and group protection/deprotection [21]. The *β*-glucosidase from *Fusarium proliferatum* ECU2042 (Rg3 conversion 60%) and the crude enzyme from *Esteya vermicola* CNU 120806 (Rg3 conversion 90%) can also be used in Rh2 preparation from Rg3, however, both of microorganisms employed in these experiments were uncommon and water solubility of Rg3 was ignored [22, 23].



▲, ■ represent Rg3 conversion with 250 and 500 U/g Rg3; △, □ represent Rh2 yield with 250 and 500 U/g Rg3;

Fig. 6 Time course of enzymatic transformation of Rg3. 60 °C, Rg3 complex concentration 25 mg/mL, 500 U/g Rg3, 150 rpm

4. Conclusions

In summary, rare ginsenoside F2 and Rh2 can be prepared from Rb1 and Rg3 using the *β*-galactosidase from *Aspergillus* sp. The solubility of Rg3 increased 74.6 folds through inclusion with HP-*β*-CD, and inclusion did not block the *β*-galactosidase from selective hydrolysis of Rg3. As a result, these processes would allow a specific bioconversion process possible to obtain specific rare ginsenosides in pharmaceutical industry. Further work will be carried out to optimize reaction conditions and investigate the activity of this *β*-galactosidase on other ginsenosides.

Acknowledgements

The authors are grateful to the financial supports from National Natural Science Foundation of China (31171752, 31371837), The Project of State Key Laboratory of Food Science and Technology, Jiangnan University (SKLF-ZBB-201404) and The Youth Science Foundation of Jiangnan University (1042050205141410).

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