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Enzymatic transformation of ginsenoside Rg_3 to Rh_2 using newly isolated *Fusarium proliferatum* ECU2042

Jin-Huan Su [a], Jian-He Xu [a,*], Wen-Ya Lu [b], Guo-Qiang Lin [b,*]

[a] *Laboratory of Biocatalysis and Bioprocessing, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China*
[b] *Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai 200032, China*

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Abstract

A new soil isolate, *Fusarium proliferatum* ECU2042, was found capable of producing a ginsenoside Rg_3 hydrolase (a special β-glucosidase) which is useful for production of a very potent antitumor agent Rh_2. By investigating its fermentation progress, the optimal time for β-glucosidase biosynthesis was determined as 72 h. The crude β-glucosidase was partially purified by 49.4-fold from the cell-free extract with an activity yield of 13%. The catalytic performance of the partially purified β-glucosidase was examined, giving optima of pH and temperature at pH 5.0–6.0 and 50 °C. It was found that the *F. proliferatum* β-glucosidase can also hydrolyze cellobiose, but not active towards a frequently used artificial substrate *p*-nitrophenyl β-D-glucopyranoside (pNPG). In a lab-scale preparation, the bio-hydrolytic reaction was carried out at 50 °C for 24 h in 50 ml NaAc/HAc buffer (100 mM, pH 5.0) containing 50 mg Rg_3 and 12 mg lyophilized crude enzyme extract, giving the ginsenoside Rh_2 in 60.3% conversion.
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Keywords: Ginseng saponin; Ginsenoside Rg_3; Ginsenoside Rh_2; β-Glucosidase; *Fusarium proliferatum*

1. Introduction

Saponins, glycosides with steroids or triterpenes as aglycons, are an important class of physiologically active compounds occurring in many herbs. In recent years, the sugar chains of the saponins were found to be closely related to the biological activity of the saponins, and modification of the sugar chains may markedly change the biological activity of the saponins [1–5]. For example, the protopanaxdiol type saponins in the ginseng, such as ginsenosides Ra, Rb, Rc, Rd, Rg_3 and Rh_2, have the same aglycon, but different sugar moieties at C-3 and C-20. As a result, their physiological functions differ much from each other [6–9].

Ginsenoside Rh_2, with a triterpenoid of dammarane skeleton as the aglycon and one glucose molecule moiety at C-3, has a strong antineoplastic capability. It can inhibit the growth of cancer cells even if isolated [10,11]. However, the production of ginsenoside Rh_2 is very difficult, because the contents of naturally occurring ginsenoside Rh_2 in red ginseng and wild ginseng are only 1/100 000 and 3/100 000, respectively. No ginsenoside Rh_2 is detected in the white ginseng [12]. Compared with ginsenoside Rh_2, ginsenoside Rg_3 has one more glucose molecule at C-2′ position, but it can be obtained easily. Therefore, a great interest exists in the preparation of ginsenoside Rh_2 by limited hydrolysis of Rg_3. Various chemical and biological methods have been reported so far for preparation of ginsenoside Rh_2 [13–28]. Among these attempts, biocatalytic approaches have remarkable predominance due to their high selectivity, mild reaction conditions and environmental compatibility. One of the most promising ways is the selective enzymatic hydrolysis to remove only one of two glucose residues of ginsenoside Rg_3 by using a specific β-glucosidase (Scheme 1). To the best of our knowledge, although there are several vended papers dealing with this reaction [13,14,27,28], most of them using bacteria from human, and only one simple report using an Aspegillus niger β-glucosidase, which gave a limited conversion of 43.2% at 18 h for a very low concentration (0.075 mg/ml) of substrate

* Corresponding author at: Laboratory of Biocatalysis and Bioprocessing, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, PR China. Tel.: +86 21 6425 2498; fax: +86 21 6425 2250.
E-mail addresses: jianhexu@ecust.edu.cn (J.-H. Xu), lingq@pub.sioc.ac.cn (G.-Q. Lin).

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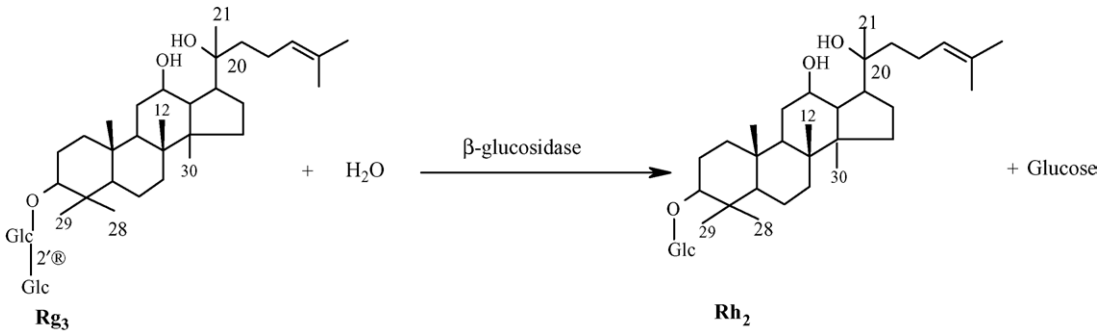

Scheme 1. The enzymatic hydrolysis of ginsenside Rg_3 to Rh_2.

Rg_3 [14]. This prompted us to screen from soil samples, the most abundant resource of diverse microorganisms, for new β-glucosidase producers with high specificity towards the outer glucose residue of ginsenoside Rg_3.

In this paper, we describe the isolation and characterization of a new strain of fungus, *Fusarium proliferatum* ECU2042, for the limited hydrolysis of ginsenoside Rg_3, as well as the lab-scale preparation of ginsenoside Rh_2 using cell-free enzyme extract of the new isolate.

2. Experimental

2.1. Materials and basal medium

Both ginsenosides Rg_3 and Rh_2 were purchased from Hongjiu Biotech Co. Ltd., Jilin, China. *p*-Nitrophenyl β-D-glucopyranoside (pNPG) was obtained from Sigma Chemical Co., USA. *p*-Nitrobenzyl β-D-glucopyranoside (pNBG) and *p*-hydroxyphenethyl β-D-glucopyranside (salidroside) were prepared as described by Tong et al. [29]. All other chemicals were obtained from local suppliers and of reagent grade. More than 200 soil samples were collected from different locations in several regions of China in 2003.

Fermentation medium (FM): sucrose 30.0 g/l, $NaNO_3$ 3.0 g/l, K_2HPO_4 1.0 g/l, KCl 0.5 g/l, $MgSO_4{\cdot}7H_2O$ 0.5 g/l, $FeSO_4{\cdot}7H_2O$ 0.01 g/l.

2.2. Isolation of β-glucosidase-producing microorganisms with high activity towards ginsenoside Rg_3

Microorganisms with high hydrolytic activity on ginsenoside Rg_3 were isolated through two rounds of screening: the first screening from the soil samples for strains with high β-glucosidase activity on artificial substrates and the second screening for strains with specific activity towards ginsenoside Rg_3 among those strains obtained.

The first round of screening employed different screening procedures, including that with total saponins of ginseng as sole carbon source and that based on the color alteration during the hydrolytic reaction of an artificial substrate pNPG. The microorganisms producing β-glucosidases were submitted to secondary screening with other two glucosides (pNBG and salidroside), and then those strains with high activity on these two substrates were subjected to further selections in the next step.

In the second round of screening, the cells of the microbial strains obtained from above steps were harvested by centrifugation (10,000 × *g*, 10 min) and resuspended in 0.5 ml of 50 mM potassium phosphate buffer (KPB, pH 7.0) to which a final concentration of 10 mM ginsenoside Rg_3 was added. After 24 h of bioconversion (at 30 °C and 160 rpm), the reactants were extracted with 0.25 ml of *n*-butanol and analyzed by high-performance liquid chromatography (HPLC) for determination of the percent conversion.

2.3. Culture conditions

The isolated strain, *F. proliferatum* ECU2042 which was identified by DSMZ (Germany), was shaken aerobically at 160 rpm and 30 °C for 96 h in 100 ml Erlenmeyer flasks with 20 ml of FM. The FM was inoculated with an 18 h preculture. At each time interval, two flasks were withdrawn for determination of enzyme activity and dry mass of cells (DCW). The enzyme activity was measured by HPLC analysis as described in the enzyme assay. The dry mass of cells was measured after drying the wet cells at 50 °C until a constant weight.

2.4. Enzyme assay

The activity of β-glucosidase in the culture broth was determined by HPLC as follows. The cells harvested from the culture broth were washed with a physiological saline solution (0.85% NaCl). Then 50 mg of the resting cells were taken out and resuspended in 0.45 ml of 50 mM buffer (NaAc/HAc, pH 5.0). After 0.05 ml of ginsenoside Rg_3 solution (5 mg/ml, in water) was added, giving a final concentration of 0.5 mg/ml, the mixture was vortexed and incubated at 40 °C on a shaker (160 rpm) for 18 h. The reaction mixture was subsequently extracted by 0.25 ml of *n*-butanol. After centrifugation, the supernatant was directly subjected to HPLC analysis to determine the quantity of the ginsenoside Rh_2 generated. One unit of β-glucosidase activity (U) was defined as the amount of enzyme catalyzing the formation of 1.0 nmol ginsenoside Rh_2 per hour under above conditions.

The condition for determining the glucosidase activity of the cell-free extract and the partially purified enzyme was slightly changed. The standard reaction mixture consisted of 100 μl enzyme solution, 80 μl of NaAc/HAc buffer (100 mM, pH 5.0) and 20 μl of ginsenoside Rg_3 solution (5 mg/ml, in water). After incubated (at 50 °C and 160 rpm) for 2 h, the reaction mixture was extracted by 200 μl of *n*-butanol. The analysis and the definition of enzyme activity were same as above.

2.5. Enzyme purification

All purification operations were conducted at 4 °C, and centrifugation was usually conducted at 15,000 × *g* for 30 min. The protein concentration was routinely estimated by measuring the absorbance at 280 nm or precisely measured by the method of Bradford [30] with bovine serum albumin as a standard protein. The enzyme activity was analyzed as described in the enzyme assay.

Acetone-dried cells (3 g) of *F. proliferatum* ECU2042 were suspended in 40 ml of NaAc/HAc buffer (20 mM, pH 5.0), then an equal volume of quartz sand was added and the cells were grinded. After centrifugation, the supernatant was precipitated with ammonium sulfate (80% saturation) to remove inactive proteins, and then the supernatant was loaded onto Butyl-Toyopearl column (Ø1.2 cm × 15 cm, bed volume: 20 ml). The enzyme was washed with 200 ml of NaAc/HAc buffer (20 mM, pH 5.0) and then eluted with a linear gradient of $(NH_4)_2SO_4$ solution (60%—0 saturation, 200 ml). The active fractions were pooled, dialyzed, and then submitted to DEAE-Cellulose column (Ø1.2 cm × 15 cm, bed volume: 20 ml). The enzyme was washed with 100 ml of Tris–HCl buffer (10 mM, pH 8.0) and then eluted with a linear gradient of NaCl solution (0–1.0 M in the buffer, 200 ml). The active fractions were combined, concentrated and submitted to Sephadex G-150 column (Ø2.0 cm × 95 cm, bed volume: 80 ml). After elution with NaAc/HAc buffer (20 mM, pH 5.0), the active fractions were pooled and used for subsequent characterization.

2.6. Catalytic characteristics of isolated enzyme

The optimum pH of the partially purified enzyme was measured directly in buffers at various pHs. The pH stability (residual activity) of the enzyme was determined after preincubation at 4 °C for 16 h in various buffers. The optimum temperature of the partially purified enzyme was measured at temperatures ranging from 30 to 60 °C after incubation for 10 min. The thermal stability of the enzyme (residual activity) was determined at 50 °C after preincubation at different temperatures for 16 h in NaAc/HAc buffer (20 mM, pH 5.0).

2.7. Preparative hydrolysis of ginsenoside Rg_3 by cell-free extract of F. proliferatum ECU2042

Lyophilized crude enzyme (cell-free extract) of *F. proliferatum* ECU2042 (12 mg) were suspended in 50 ml NaAc/HAc buffer (100 mM, pH 5.0) and 50 mg of ginsenoside Rg_3 was added, giving a final concentration of 1 mg/ml. The mixture was shaken at 160 rpm and 50 °C. After incubation for 24 h, the mixture was extracted four times with 25 ml *n*-butanol, and the organic layers were combined and evaporated in vacuum. The crude product of ginsenoside Rh_2 was purified by silica gel column chromatography with chloroform–methanol (9:1, v/v) as eluent and recrystallized in a mixed solvent of methanol–water (70:30, v/v).

2.8. Analytical methods

The concentrations of enzymatic substrate and product were determined by HPLC using a reverse phase column (RP-18, Ø5.0 mm × 200 mm, 10 μm). The mobile phase was methanol–water (85/15, v/v) and its flow rate was 0.8 ml/min. Detection was made at 203 nm. The retention times for ginsenosides Rg_3 and Rh_2 were 12.5 min and 20.3 min at 20 °C, respectively.

3. Results and discussion

3.1. Isolation of microorganisms for hydrolysis of ginsenoside Rg_3

In the first screening, we found that many microorganisms could produce β-glucosidase. Through different screening procedures, more than 160 strains were obtained, and about 70% of them were fungi. After reacted with pNBG at a concentration of 3.2 mM, 30 strains were selected and submitted to react with pNBG and salidroside, respectively, and the concentration of both substrates was 10 mM. At last 10 strains were subjected to secondary examination with respect to their high activity in bioconversion of both the two substrates.

The second screening was carried out as described in Section 2. It was found that a high activity on pNBG and salidroside did not imply that it was also effective for the hydrolysis of ginsenoside Rg_3, and some of the strains could not react with Rg_3 at all (data not shown). We chose the best five strains of them and compared their hydrolytic activity towards pNPG and Rg_3 using their cell-free extract. As shown in Table 1, the initial rates for pNPG and Rg_3 did not act as one-to-one correspondence, and this indicates a subclass may exist in glucosidase family.

Among these active strains, a strain of fungus, originally designated as T42, was selected for further study due to its high and specific activity towards the terminal glucose linkage in the hydrolysis of ginsenoside Rg_3. This newly isolated strain has been identified as *F. proliferatum* (Matsushima) Nirenberg and presently deposited in both the culture collection center of East China University of Science and Technology (ECUST) with an accession number of ECU2042 and China General Microbiogical Culture Collection Center (CGMCC) with an accession number of CGMCC No. 1495.

3.2. Growth and glucosidase biosynthesis of F. proliferatum ECU2042 on fermentation medium

To further characterize the new isolate, the profile of glucosidase production was monitored by cultivating *F. proliferatum*

Table 1
The hydrolytic activity of glucosidase from several selected strains measured using different substrates

Substrate	Initial rate (nmol h^{-1} mg $protein^{-1}$)				
	T42	P5-13	MA2-2	MA3-30	X2-15
pNPG	23.9×10^3	16.9×10^3	6.24×10^3	5.34×10^3	2.52×10^3
Rg_3	39.7	ND	28.7	0.071	ND

ND: not detected.

ECU2042 in 100 ml flasks using FM, as shown in Fig. 1. The enzyme activity was measured with ginsenoside Rg_3 as substrate, as described in enzyme assay. The glucosidase activity increased slowly during the first 24 h, so this time point was considered suitable for adding the enzyme inducers. The maximum activity of 48.8 U/ml was reached at 72 h when the dry mass of cells (DCW) was 13.3 mg/ml. Then the cell growth continued, whereas the enzyme activity began to decline. The reasons are not yet clear, probably because of the cease of glucosidase synthesis in the aged cells and the degradation of the glucosidase by the in vivo protease during the later phase of growth. The glucosidase activity of the cells was doubled when 0.5 mg/ml of ginseng saponins was added at the 24 h of the cultivation (Fig. 2). However, when the concentration of saponins was more than 1 mg/ml, the glucosidase activity fell down rapidly, and little activity could be detected at a concentration of 2 mg/ml. These facts suggest that the glucosidase is an inducible enzyme though a high concentration of ginseng saponins has a strong inhibitory effect on biosynthesis of the enzyme.

3.3. Catalytic performance of partially purified *F. proliferatum* β-glucosidase

To further optimize the catalytic conditions for ginsenoside Rg_3 hydrolysis, the enzyme was partially purified from a cell-free extract of *F. proliferatum* ECU2042 by precipitation with ammonium sulfate (80% saturation) and column chromatography with Butyl-Toyopearl, DEAE-Cellulose and Sephadex G-150. The glucosidase was purified by about 49.4-fold with an overall yield of 13%. The isolated enzyme showed hydrolytic activities on ginsenoside Rg_3 and cellobiose, but almost no activity on the artificial substrate pNPG.

As shown in Fig. 3(A) and (B), the partially purified enzyme showed the maximal activity between pH 5.0 and 6.0 and the enzyme was stable over a wide pH range from 4.5 to 7.0. The optimum temperature of the enzyme was 50 °C. The enzyme was quite stable at temperatures below 50 °C with nearly 80% of the activity remained after preincubation for 16 h, and over 50% of the activity still remained after preincubation at 60 °C for 16 h. Therefore, the optimal conditions for Rg_3 hydrolysis with this enzyme were considered to be pH 5.0 and 50 °C.

Other properties of the partially purified enzyme were also examined. Using Lineweaver–Burk plots ($1/V_0$–$1/[S]$) [31], kinetic constants (K_m and V_{max}) of the partially purified enzyme towards ginsenoside Rg_3 were evaluated as 1.17 mM and 2.60 μmol/h mg protein, respectively. Heavy metal ions, e.g., 10 mM of Cu^{2+}, Mn^{2+} $Fe^{2+}$and Fe^{3+}, strongly inhibited the enzyme (data not shown), suggesting that the hydrosulfide group is probably essential for the enzyme activity. Whereas, chelating reagents such as EDTA did not inhibit the enzyme obviously at a concentration up to 10 mM, indicating that metal ions are not essential for the manifestation of activity and the enzyme may not be a metalloenzyme.

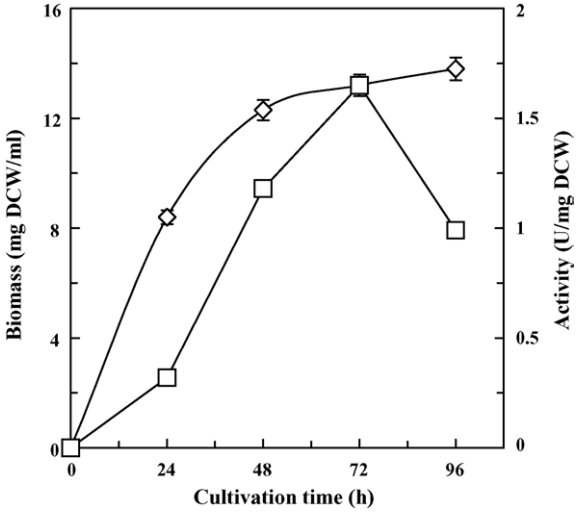

Fig. 1. Time course of *Fusarium proliferatum* ECU2042 growth and glucosidase biosynthesis: (◊) biomass (mg DCW/ml); (□) glucosidase activity (U/ml). The glucosidase activities were determined by HPLC as described in Section 2.

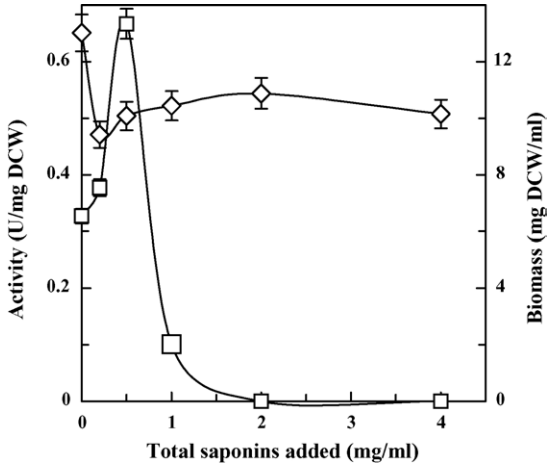

Fig. 2. The effect of ginseng saponin as an enzyme inducer on the cell growth and glucosidase biosynthesis of *F. proliferatum* ECU2042: (◊) biomass (mg DCW/ml); (□) relative activity (%).

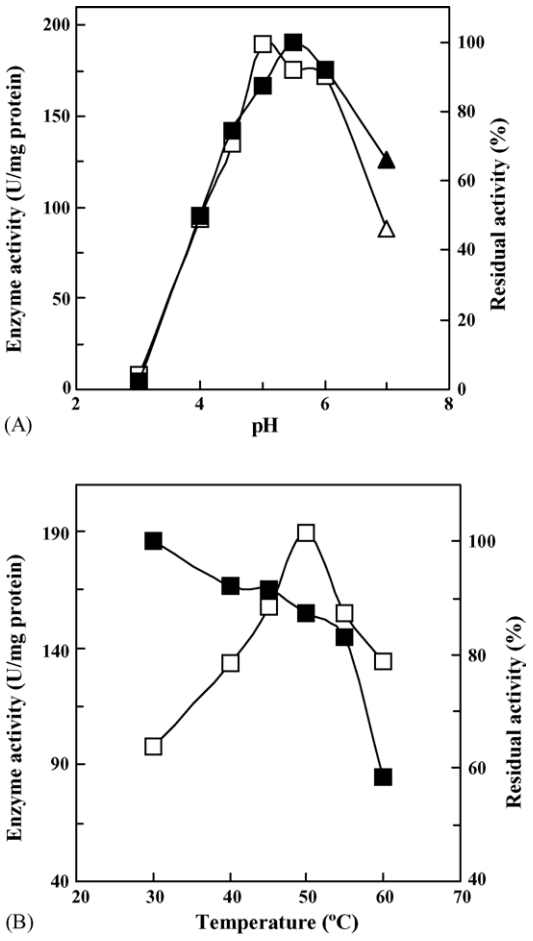

Fig. 3. Catalytic performance of the partially purified enzyme from *F. proliferatum* ECU2042. (A) Effect of pH on the activity and stability of the enzyme. Empty symbols (□, △) activity, measured at various pHs; filled symbols (■, ▲) stability, expressed by residual activity measured under standard conditions after incubation at various pHs for 16 h. Buffers used (■, □) 50 mM citrate (pH 3.0–6.0); (▲, △) 50 mM sodium phosphate (pH 7.0). (B) Effect of temperature on activity and stability of the enzyme. (□) Relative activity (%), measured at various temperatures; (■) residual activity (%), measured under standard conditions after incubation at various temperatures for 16 h.

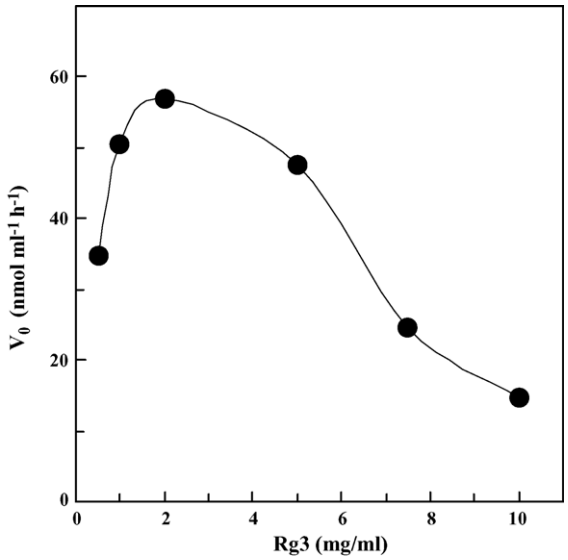

Fig. 4. Effect of substrate concentration on the hydrolysis of Rg_3 by cell-free extract of *F. proliferatum* ECU2042. Reactions were carried out in 200 μl NaAc/HAc buffer (100 mM, pH 5.0) at 40 °C containing 100 μl of cell-free extract.

3.4. Hydrolysis of ginsenoside Rg_3 for preparation of Rh_2 at the optimal substrate concentration by cell-free extract of *F. proliferatum* ECU2042

As shown in Fig. 4, the highest initial rate observed for Rg_3 hydrolysis was at 2 mg/ml of substrate, and this was considered as the optimal concentration for this enzyme reaction. It was obvious that ginsenoside Rg_3 had inhibitory effect on the glucosidase. The inhibition became extraordinarily remarkable when the concentration was above 7.5 mg/ml.

Based on the results above, the hydrolytic reaction of Rg_3 by cell-free extract of *F. proliferatum* ECU2042 was performed under its optimal conditions. As shown in Fig. 5, a conversion of 57% was obtained at 30 h.

Nevertheless, in the lab-scale preparation of ginsenoside Rh_2 using cell-free extract of *F. proliferatum* ECU2042, the concentration of Rg_3 was still set at 1 mg/ml to save the substrate and for a higher conversion. As a result, after 24 h of bioconversation and a simple workup as described in Section 2.7, 11 mg of ginsenoside Rh_2 was finally obtained from 50 mg of Rg_3, with an overall isolation molar yield of 29%. ESI-MS *m/z*: 1245 $(2M + H)^+$, gave a molecular weight of 622; ^{1}H NMR (500 MHz, d^4-methanol), δ (ppm): 0.85 (s, 3H, CH_3-19), 0.91 (s, 3H, CH_3-30), 0.92 (s, 3H, CH_3-18), 1.00 (s, 3H, CH_3-29), 1.04 (s, 3H, CH_3-28), 1.14 (s, 3H, CH_3-21), 1.61 (s, 3H, CH_3-27), 1.68 (s, 3H, CH_3-26), 4.30 (d, 1H, J = 7.8Hz, Glc-C_1-H), 5.13 (m, 1H, C_{24}-H); ^{13}C NMR: Aglycon moiety C_1–C_{30}: 40.75, 27.87, 91.13, 41.50, 58.10, 19.76, 36.46, 38.49, 50.14, 40.86, 32.52, 72.24, 49.08, 51.91, 32.56, 27.06, 58.10, 17.29, 16.70, 74.92, 27.69, 36.84, 23.80, 126.70, 132.47, 26.38, 18.19, 28.94, 16.70, 17.65; sugar moiety C'_1–C'_6: 107.21, 76.20, 78.83, 72.66, 78.21, 63.37.

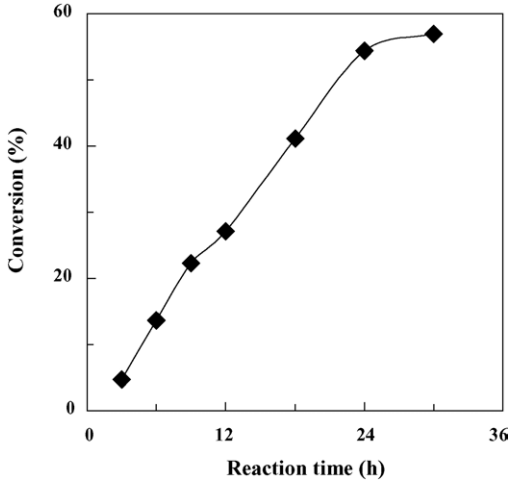

Fig. 5. Time course of Rg_3 hydrolysis catalyzed by cell-free extract of *F. proliferatum* ECU2042 at 50 °C. The initial concentration of Rg_3 was 2 mg/ml.

4. Conclusions

Through two steps of screening, a strain of fungus (ECU2042) capable of selectively transforming ginsenoside Rg_3 into ginsenoside Rh_2 was isolated from soil samples and identified as *F. proliferatum*. The maximum production (48.8 U/ml) of the β-glucosidase by *F. proliferatum* ECU2042 was reached at 72 h of cultivation, when the biomass (DCW) was 13.3 mg/ml. The β-glucosidase was partially purified by 49.4-fold with an activity recovery of 13%. The catalytic performance of the partially purified β-glucosidase was examined, giving optima of temperature and pH at 50 °C and pH 5.0. We found that the β-glucosidase can hydrolyze both ginsenoside Rg_3 and cellobiose, but almost no activity towards pNPG could be detected, which implies the occurrence of a new subclass of glucosidase (gensenoside Rg_3 glycosyl hydrolase) in the *Fusarium* species. In the preparation of ginsenoside Rh_2 using the crude cell-free extract of *F. proliferatum* ECU2042, a higher conversion of 60.3% was obtained as compared with that reported previously [14]. Therefore, the method is considered to be potentially useful for the practical preparation of ginsenoside Rh_2.

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