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Research Article

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Characterization of the saponin hydrolases involved in the biotransformation of saponins with *Trichoderma reesei*

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ABSTRACT

To increase the efficiency of biotransforming saponins in Dioscorea zingiberensis by Trichoderma reesei, four saponin hydrolases (E1, E2, E3 and E4) were purified and characterized. The molecular weights of E1, E2, E3 and E4 determined by SDS-PAGE were 50, 83, 67 and 46 kDa, respectively. E2 and E3 retained high activities at 20-60 °C. Based on HPLC-MS analysis, the substrate preferences of E1, E2, E3 and E4 were carefully studied. Both E2 and E3 could cleave C-26 glycoside, C-3 terminal glycoside, C-3 terminal rhamnoside and C-3 glycoside attached to aglycone in saponins. Four parameters (pH, T, Ca^{2+} and K^{+}) which influenced the performances of E2 and E3 significantly were optimized for high biotransformation efficiency. After optimization, saponins in Dioscorea zingiberensis were efficiently hydrolyzed by the crude enzyme from Trichoderma reesei, with a diosgenin yield of 94.9%.

Keywords: saponin hydrolase; sponins; diosgenin; *T. reesei*

INTRODUCTION

Diosgenin, an important drug in pharmaceutical industry, is widely used in the production of oral contraceptives, sex hormones, and other steroids [1,2]. It was usually found in the plant tubers in glycosidal forms called saponins, attaching sugar chains to aglycon with glycosidic bonds at C-3 and C-26 [3]. Release diosgenin from saponins mainly depends on cleaving the sugars at the two positions. In traditional process, strong acid (H_2SO_4 or HCl) hydrolyzation is usually used for this purpose. This method, however, caused many environmental problems. A great volume of acid wastewater with high concentrations of organic matters and SO_4^{2-} (or Cl^-) was produced during the hydrolysis process [4,5].

Compared with chemical hydrolysis, biotransformation is a well-known environmental friendly process for its high selectivity and mild reaction condition [6]. Previous study indicated that the multiple enzymes (cellulase + β-glycosidase+ pectinase) could hydrolyze saponins to diosgenin effectively [7]. However, the high price of commercial enzyme limited the industrial application [8]. Microbial transformation is an economic alternative, but the low diosgenin yield and long reaction time restrict its application [9]. In order to increase the efficiency of the microbial transformation, researches mainly focused on the new strain selection and the transformation process optimization [10,11]. With these two methods, about 70-90% of diosgenin could be released from saponins, and the reaction time was 6-7 days. Its application is still restricted [12].

Dioscorea zingiberensis C.H. Wright (DZW) is the main raw material for diosgenin production in China because of the high concentration of saponins in its tubers [7]. In previous work, a strain of *T. reesei* which can convert saponins in DZW effectively was selected from available fungal cultures and soil isolates [13-15]. It was found that during the hydrolyzation of DZW by *T. reesei*, diosgenin can be produced from saponins. However, the target

product yield was generally low if the intermediate products were accompanied in the bioprocess [13]. Thus, in order to improve the diosgenin yield, revealing the mechanism of the transformation process is essential. Enzymes play key roles in this biotransformation system [16-18]. Study of the multiple enzymes in the biotransformation process is important for the efficiency improvement. Nevertheless, few studies have focused on this field. Thus, the objective of this research is to analyze the multiple enzymes (saponin hydrolases) involved in the biotransformation of saponins with *T. reesei* for high diosgenin yield.

EXPERIMENTAL SECTION

Materials

The DZW tubers were from Tianhe Pharmaceutical Co., Ltd. (China). The standard diosgenin, p-nitrophenyl-β-D-glucopyranoside (pNPG) and p-nitrophenyl-α-L- rhamnopyranoside (pNPR) were obtained from Sigma Company. *T. reesei* (ACCC 30597) was from Agricultural Culture Collection of China and acclimated in the sterilized substrate of 50% of saponins [19].

Enzyme purification

Saponin hydrolases were precipitated by ammonium sulfate at 90% saturation and then centrifugation at 10,000 rpm for 10 min. The pellet was re-suspended in 0.02 mol l^{-1} HAc-NaAc buffer (pH 5.0) and dialyzed using 40 kDa dialysis bag, then freeze-dried and dissolved in 10 ml distilled water. The enzyme solution in the filtrate were fractioned on a DEAE-cellulose DE52 column (4.0 cm×11.0 cm) and then separated with a Sephadex G-100 column (2.0 cm×24.0 cm). Some fractions were further purified using Preparative Native-PAGE separation following the method described by Schagger and Von (1991) with 5% (*w/v*) stacking polyacrylamide gel and 10% (*w/v*) separating gel (3 mm).

Characteristics

Substrate specificity

Progracillin was used as the substrate to determine the substrate specificities of the purified enzymes with the method we described previously [12].

Optimal pH and pH stability, optimal temperature and thermal stability

The optimal pH for the saponin hydrolases was analyzed by incubating the enzyme at 50 °C using 5 mM pNPG or pNPR as substrates respectively in different buffers: Gly-HCl (50 mM, pH 3.0) Na_2HPO_4-citrate (50 mM, pH 4.0-7.0), Gly-NaOH (50 mM, pH 8.0-11.0) within the pH range of 3.0-11.0. The pH stability was determined by pre-incubating the enzyme solution at different pH buffers at 25 °C for 1 h prior to standard enzyme assay.

The optimal temperature for each purified enzyme was estimated by measuring the enzyme activity at different temperatures (20-80 °C). The thermal stability was evaluated by standard enzyme assay after incubating the enzyme at different temperature for 120 h in the absence of the substrate.

Effect of metal ions on the enzyme activity

To evaluate the effects of metal ions on the saponin hydrolases, the purified enzyme was incubated for 30 min at optimal temperature in optimized buffer solution containing the following metal ions (5mM) Fe^{2+}, Ca^{2+}, Mg^{2+}, Cd^{2+}, Ag^{+}, Co^{2+}, Ba^{2+}, Mn^{2+}, Cu^{2+}, K^{+}, Cr^{3+} respectively. The residual enzyme activity was assayed by standard method using pNPG or pNPR as substrate.

Process optimization for production of diosgenin from saponins with crude enzyme

Saponins were prepared from DZW as the method described by Liu et al [19], crude enzyme was obtained from *T. reesei* following the above procedur. Four factors, pH (5.0, 5.5, 6.0), Temperature (50, 55, 60 °C), Ca^{2+} (0, 5, 10 mM) and K^{+} (5, 10, 15 mM) were optimized for highest diosgenin yield, successively. The parameters were determined through series of experiments in 50 ml Erlenmeyer flasks containing 0.5 g of saponins, 1 ml of crude enzyme and 9 ml of designed buffer, fermented for 5 days. After fermentation, sample media in each flask was dried, extracted with 10.0 ml $CHCl_3$ for 30 min by ultrasonic treating and then filtrated. The filtrate was dried and dissolved in 1 ml methanol (HPLC grade) and analyzed by HPLC. The diosgenin yieid was calculated as described previously [13].

RESULTS AND DISCUSSION

Purification of saponin hydrolase

With DEAE-Cellulose column, Sephadex G-100 gel filtration and preparative Native-PAGE separation, four saponin hydrolases (E1, E2, E3 and E4) were purified from *T. reesei*, with molecular masses of 50, 83, 67, 46 kDa, respectively (Fig. 1, [12]).

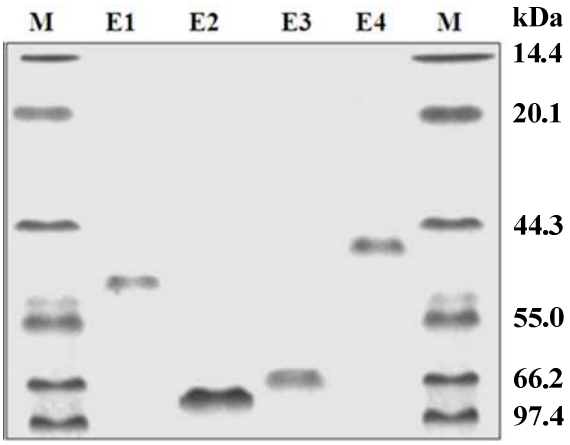

Fig.1 SDS-PAGE (15%) of the purified enzymes E1-E4 (Coomassie Blue stained)

Substrate specificities of E1, E2, E3 and E4

The four purified enzymes were incubated with progracillin respectively to analyze the substrate specificities. The fermentation broth was determined by HPLC-ESI-MS every 12 h. Five compounds (peaks 1-5) were determined in the biotransformation process (Fig.2). Major product ions at m/z 1077.7, m/z 883.7, m/z 721.7, m/z 575.6, m/z 423.4 of peaks 1-5 were presented in the average mass spectra. According to a reported work [12, 13], the five peaks were identified as progracillin (Peak 1, Compound **1**), gracillin (Peak 2, Compound **2**), diosgenin-3-O-β-D-glucopyranoside-α-L-rhamnopyranosyl (Peak 3, Compound **3**), trillin (Peak 4, Compound **4**) and diosgenin (Peak 5, Compound **5**).

Incubated with E1 (Fig. 2 E1), progracillin was converted to gracillin (Compound **2**) in the first 24 h, and then to Compound 3. Trillin (Compound **4**) and diosgenin (Compound **5**) were not detected in the medium. Both E1 and E4 can hydrolyze the glycoside at C-26 or C-3 terminal position, but can't hydrolyzeC-3 terminal rhamnoside or C-3 glycoside.

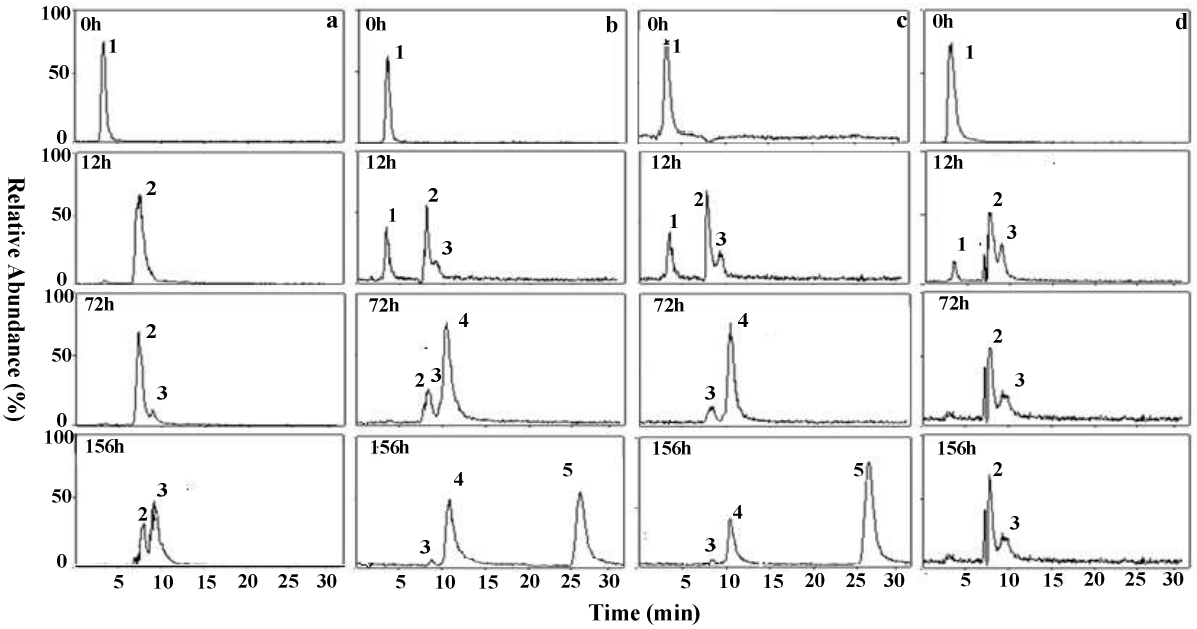

Fig.2 HPLC profile of hydrolyzed products of progracillin by E1 (a), E2 (b), E3 (c), E4 (d) for 0h, 12h, 72h and 156h respectively. The peaks 1-5 correspond to progracillin, gracillin, diosgenin-3-O-β-D-glucopyranoside-α-L-rhamnopyranosyl, trillin, and diosgenin. The peaks were identified with standards and LC-MS

As shown in Fig. 2E2 and Fig. 2E3, hydrolyzed by E2 or E3, progracillin was transformed to gracillin and Compound **3** in the first 24 h. After 72 h, only Compound **3** and trillin were detected in the system, while diosgenin appeared after further incubation. Both E2 and E3 can hydrolyze the four kinds of glycosides in progracillin. The possible hydrolyzing reactions occurred in the process of transforming progracillin to diosgenin were presented in Fig. 3.

Effect of pH on the α-rhamnase, β-glycosidase activities of E1, E2, E3 and E4

As shown in Fig. 4 and 5, the maximal β-glycosidase activity of E1 was obtained at pH 4.0, and E1 was retained activity in the pH range of 3.0-6.0 after 1h incubation. E2 showed optimum α-rhamnase activity at pH 5.0 and β-glycosidase activity at pH 4.0. The two activities were stable at pH 3.0-6.0 and 4.0-9.0 respectively. Although the optimum pH for α-rhamnase and β-glycosidase activities of E2 were different, but both the two activities were relatively high at pH 5.0. E3 showed optimum α-rhamnase activity and β-glycosidase activity at pH 5.0. Both the two activities were stable at pH 3.0-6.0. The maximal β-glycosidase activity of E4 was obtained at pH 4.0. E4 was stable in the pH range of 6.0-7.0.

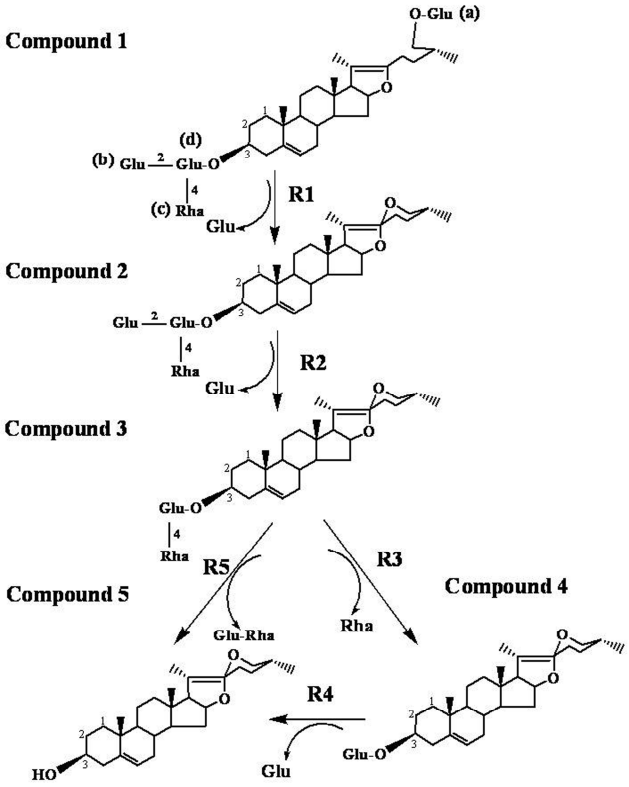

Fig. 3 Possible transformation pathways of progracillin by E1, E2, E3 and E4. R1-R5: Possible hydrolyzing reactions. (a): C-26 glycoside; (b): C-3 terminal glycoside; (c): C-3 terminal rhamnoside; (d): C-3 glycoside

Effect of temperature on the α-rhamnase, β-glycosidase activities of E1, E2, E3 and E4

The optimal temperature for β-glycosidase activity of E1 was 60°C. About 40% of the β-glycosidase activity was maintained at temperatures ranging from 20 to 50°C after 120 h incubation. The optimum temperature for both α-rhamnase and β-glycosidase activities of E2 was 50°C. E2 retained about 65% of its initial α-rhamnase activity and 75% of its initial β-glycosidase activity in the temperature range of 20-60°C after 120 h incubation. The temperature optimal for both α-rhamnase and β-glycosidase activities of E3 was 60°C. About 85% of the α-rhamnase activity and 80% of the β-glycosidase activity were maintained at temperatures ranging from 20 to 60°C after 120 h incubation. The β-glycosidase activity of E4 was highest at 60 °C. The residual activity maintained 60% of the initial after treatment at 20-50°C for 120 h. E2 and E3 were more stable than the other two saponin hydrolases. The thermostabilities of E2 and E3 are favorable in industrial operations for diosgenin production.

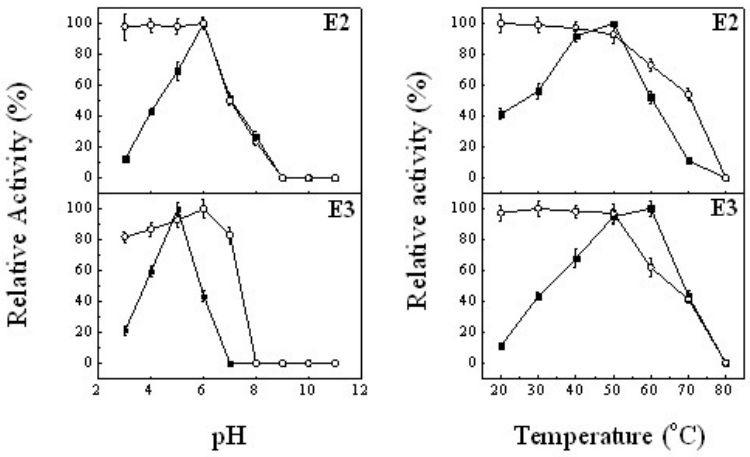

Fig. 4 Effects of pH and temperature on the *α*-rhanmase activity (■) and stability (○) of E2 and E3.

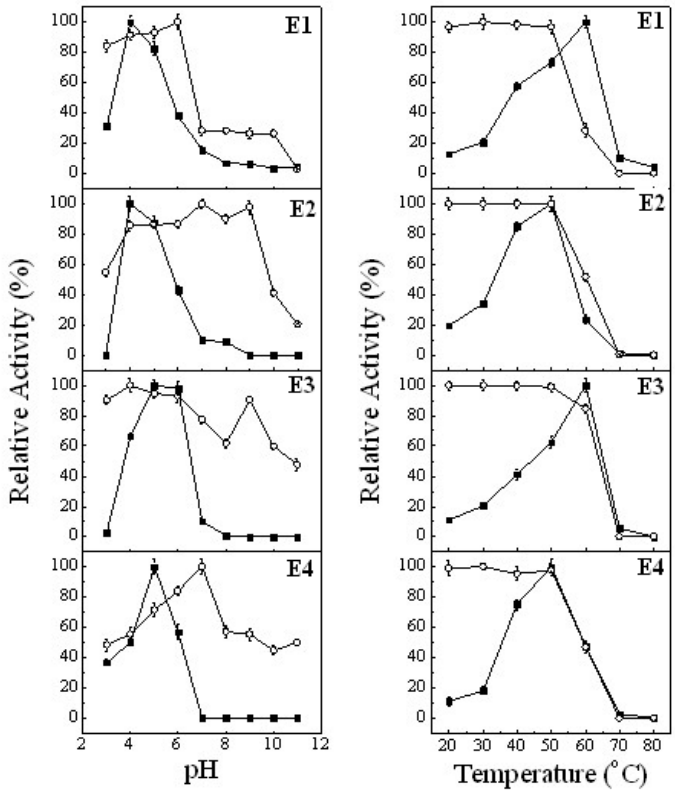

Fig. 5 Effects of pH and temperature on the *β*-glycosidase activity (■) and stability (○) of E1, E2, E3 and E4.

Influences of metal ions on the α-rhamnase, β-glycosidase activities of E1, E2, E3 and E4

The effects of various metal ions on the purified enzymes were investigated and presented in Table 1. For E1, the β-glycosidase activity was completely inactive in the presence of Cd^{2+}, Ag^{+} and Cr^{3+}. The enzyme activity was significantly inhibited by the addition of Fe^{2+}, Co^{2+} and Cu^{2+}. Significant increases in β-glycosidase activity were observed by adding Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+} and K^{+}. For E2, the α-rhamnase activity was inactived by Cd^{2+}, Ag^{+}, Cr^{3+} and Cu^{2+}, and significantly inhibited by Fe^{2+}, Co^{2+}, Ba^{2+} and partially increased by adding Mg^{2+} and Mn^{2+}. While Cd^{2+}, Ag^{+}, Cr^{3+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Ca^{2+}, Ba^{2+}, K^{+}, Mg^{2+} and Mn^{2+} showed inhabitant effects on the β-glycosidase activity of E2. For E3, K^{+} had a stimulative effect on the α-rhamnase activity, which was found to be 142.9% in the presence of K^{+}. While Ca^{2+} had a stimulative influence on the β-glycosidase activity, which was increased to 171.5% when Ca^{2+} was in the medium. Inhibitory effects were observed in the presence of other metal ions. For E4, The addition of Mg^{2+} was observed to slightly enhance the β-glycosidase activity of the enzyme. Little effect was observed by adding Co^{2+} and Ba^{2+}. While Fe^{2+}, Ca^{2+}, Cd^{2+}, Ag^{+}, Mn^{2+}, Cu^{2+}, K^{+} and Cr^{3+} showed inhibitory influences on E4.

Table 1 Effects of metal ions on saponin hydrolases (relative activity, %)

ion	*β*-Glycosidase relative activity (%)				*α*-Rhamnase relative activity (%)	
	E1	E2	E3	E4	E2	E3
Fe^{2+}	46.07±2.14	53.28±3.02	61.34±0.42	33.71±1.24	21.70±2.12	43.91±2.28
Ca^{2+}	213.1±1.17	80.44±0.92	171.5±1.06	63.24±1.36	65.42±0.75	95.43±3.41
Mg^{2+}	167.8±1.65	45.72±1.82	93.09±2.14	115.6±0.85	112.7±2.54	96.44±4.33
Cd^{2+}	0	0	0	0	0	0
Ag^{+}	0	3.79±0.47	49.15±3.44	25.94±0.42	0	0
Co^{2+}	65±3.11	88.47±2.23	100.9±1.98	101.8±2.69	87.94±3.58	64.33±2.66
Ba^{2+}	153.6±2.08	50.26±4.76	102.9±2.03	99.45±1.84	64.78±4.62	98.76±5.25
Mn^{2+}	116.3±1.84	80.29±4.32	95.49±1.72	85.13±0.66	132.9±4.51	83.09±3.38
Cu^{2+}	54.76±1.77	17.54±1.07	79.01±4.03	32.02±2.52	0	0
K^{+}	155.9±3.06	59.55±0.84	101.1±2.83	56.75±2.98	65.44±2.64	142.9±4.62
Cr^{3+}	0	0	0	0	0	0

Process optimization for production of diosgenin from saponins with crude enzyme

As discussed above, E2 and E3 were key enzymes in transforming saponins to diosgenin. In industry, crude enzyme (including E2 and E3) play roles in this system. The impacts of pH, temperature and metal ions on the activities of the two enzymes, however, were different. In order to improve the biotransformation efficiency, the crude enzyme fermentation parameters, i.e. pH, T, Ca^{2+}, K^{+} were optimized based on the characteristics of E2 and E3. The highest diosgenin yield was achieved with 5 mM of Ca^{2+} and 10 mM of K^{+}, under condition of pH 5 and temperature 55 ℃, using saponins from DZW as substrate. After optimization, the diosgenin yield increased from 87.3% to 94.9% (Table 2). This value was only 76.09% when saponins were transformed by commercial cellulase [19]. So, E2 and E3 are of great potential significances for industry. Further study can focus on cloning and expression of the two enzymes.

Table 2 Effects of fermentation parameters on biotransformation efficiency

Conditions	pH			T (℃)		
	5	5.5	6	50	55	60
Diosgenin yield (%)	87.3±3.28	86.7±4.55	85.2±7.21	87.3±3.28	89.2±1.71	88.1±4.09
Conditions	Ca^{2+} (mM)			K^{+} (mM)		
	0	5	10	5	10	15
Diosgenin yield (%)	89.2±1.71	93.7±2.98	93.5±1.06	93.2±0.85	94.9±1.09	94.4±2.75

[1]*Data presented in table is mean value ± standard deviation.*

CONCLUSION

Four saponin hydrolases (E1, E2, E3 and E4) were purified from the culture filtrate of *T. reesei*. E2 and E3 were stable at 20-60 ℃. Substrate preference study showed that E2 and E3 were able to cleave C-26 glycoside, C-3 terminal glycoside, C-3 terminal ramnoside and C-3 glycoside attached to aglycone in saponins. They were key enzymes. The fermentation conditions of biotransformation saponins in DZW by crude enzyme from *T. reesei* were optimized for high diosgenin yield, providing a promising guide for the improvement of the biotransformation method in the future.

Acknowledgements

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