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Purification, Molecular Cloning and Expression of Three Key Saponin Hydrolases from *Trichoderma reesei*, *Trichoderma viride* and *Aspergillus fumigatus*

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ABSTRACT

Microbial transformation is an environment-friendly approach to produce diosgenin from natural plant tubers. However, improving the bioconversion efficiency of this process remains a challenge. Saponin hydrolase plays a major role during the transformation. α-rhamnase, β-glycosidase and C3 β-glycosidase have been identified as three key enzymes in the stepwise conversion of saponins to diosgenin. The three enzymes have not been molecularly characterized. Here, α-rhamnase, β-glycosidase and C3 β-glycosidase were purified from *Trichoderma reesei*, *Trichoderma viride* and *Aspergillus fumigatus*, respectively. SDS-polyacrylamide gel electrophoresis showed that the molecular masses of the three enzymes were approximately 60, 36 and 56 kDa, respectively. α-rhamnase could efficiently hydrolyse the terminal rhamnoside on the C3 site in saponins. β-glycosidase could cleave the terminal β-glycoside at the C3 position, while C3 β-glycosidase demonstrated significant affinity to the β-glycoside at the C3 position. The amino acid sequences of the three saponin hydrolases were determined. Reverse transcription-PCR was used to clone the three plasmids with lengths of approximately 1035, 1416 and 1752 bp from the strains and expressed in *Escherichia coli*. The recombinant saponin hydrolases from the recombinant strains also demonstrated the hydrolysing of rhamnoside and glycosides of saponins similar to those of the wild-type hydrolases from *T. reesei*, *T. viride*, and *A. fumigatus*. The findings could facilitate the construction of recombinant cells by gene fusion to produce diosgenin from plant tubers.

INTRODUCTION

As the important intermediate for synthesizing steroid hormone drugs, diosgenin is widely used to produce drugs for antiphlogosis, cerebrovascular, and contraception (Saunders et al. 1986, Oncina et al. 2000). Approximately 60% of available steroid hormone drugs in the world are produced using diosgenin as precursor. Thus, this compound has high market demands. Diosgenin exists in the rhizome as saponins in plants such as *Dioscorea zingiberensis* (DZW). Moreover, diosgenin can be obtained from the hydrolysis of the carbohydrate chains at the C26 and C3 positions in saponins (Qian et al. 2006). The rhizome of DZW is traditionally hydrolysed directly with high-concentration strong acid to acquire diosgenin. Given the effect of strong acids, other components in the raw materials, such as starch, cellulose, and pectin, can also be hydrolysed into small-molecule substances and then enter into the processed wastewater. This process results in very high concentrations of chemical oxygen demand (COD), biochemical oxygen demand (BOD), SO_4^{2-} and H^+ in wastewater (Zhang et al. 2018).

China is one of the major exporters of diosgenin, and the manufacturing enterprises are mainly located in the Danjiangkou catchment area in the middle line of the south-to-north water transfer project in the Hanjiang River Basin (Yang et al. 2016). Sewage emission significantly destroys the local ecological environment and one of the main hidden risks of water quality safety in the south-to-north water transfer project. Compared with acid hydrolysis, biological transformation is superior because of its mild condition and strong specificity. Therefore, replacement of acid hydrolysis with biotransformation is an essential way to solve the environmental pollution problems caused by the production of diosgenin (Qi et al. 2009, Liu et al. 2010a, Dong et al. 2010).

Previous biotransformation studies mainly focused on the hydrolysis of saponins into diosgenin with pure strain using abundant microorganism screening experiment and the optimization of the fermentation conditions to improve the hydrolysis efficiency. However, this method could only increase the reaction efficiency to a limited extent (Zhu et al. 2010a,b, Chen et al. 2018). Only 70%-90% of acid hydrolysis yield was obtained after the fermentation of saponins with microorganisms for 6-7 days (Liu et al. 2010a, Zhu et

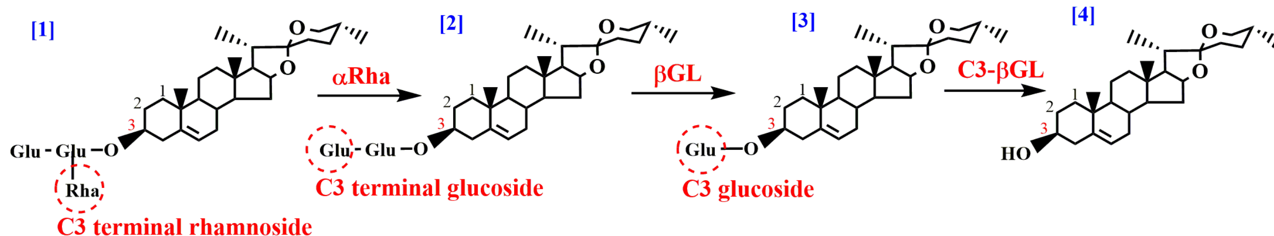

Fig. 1: Effects of α-rhamnase (αRha), β-glycosidase (βGL) and β-glycosidase on the C3 site (C3-βGL).

al. 2010c). The low yield and long fermentation time restrict the application and development of biological hydrolysis for diosgenin production. Saponin hydrolases, which are secreted by strains, take the dominant role in microbial hydrolysis of saponins to diosgenin. Studies on saponin hydrolases have just commenced, and systematic reports are scarce (Fu et al. 2010, Lei et al. 2012, Cui et al. 2013). The biological hydrolysis of saponins in DZW involves the effects of three enzymes, namely, α-rhamnosidase (αRha), β-glycosidase on the terminal of carbohydrate chain (βGL), and β-glycosidase on the C3 site (C3-βGL) (Liu et al. 2010b) (Fig. 1). However, one strain can hardly secrete these three enzymes simultaneously with high activity (Zhu et al. 2014). This limitation causes the low biological hydrolysis efficiency of single microorganism.

Thus, three microorganisms with strong hydrolysis ability to C3 β-glycoside, β-glycoside, and α-rhamnaside were screened. Three types of saponin hydrolases were separated from the three strains, and their characteristics were studied. Relevant genes were cloned and expressed. The results may provide data for constructing reasonable hydrolase system based on molecular biological technique.

MATERIALS AND METHODS

Experimental materials: The rhizome of DZW was bought from the Yunxi County in Shiyan City, Hubei Province. The rhizome was cleaned, sliced, dried under 60°C, ground, and finally extracted by 80% ethanol for 12 h. The obtained liquid was volatilized by rotary evaporation, and the residues were the total saponins.

Strains: *Trichoderma reesei*, *Trichoderma viride* and *Aspergillus fumigatus* were supplied by the China Agricultural Microorganism Strain Preservation Center, with corresponding numbers ACCC30590, ACCC30552 and ACCC31551.

Standard samples: The standard sample of diosgenin was bought from Sigma-Aldrich. Standard samples of protodioscin, protogracillin, dioscin, gracillin and diosgenin-triglycoside were purchased from the Hefei Bomei Biotech Co. Ltd.

Reagents: The experimental kit, *Escherichia coli* competent cell JM109 and competent cell BL21 (DE3) were obtained from the Japan Takara Company.

Screening of saponin hydrolysis strains: The strains were stored in a PDA slant after being activated. Culture media were prepared using 5% total saponins (containing protodioscin, protogracillin, dioscin, gracillin and diosgenin-triglucose) as the sole carbon source, on which 2% mycete seed solution was inoculated and cultured for 7 days. The culture solution with the highest diosgenin content was used as the seed solution for continuous culture by 3 cycles. Then, the solution was inoculated into the liquid culture medium containing 10% total saponins for continuous accumulation. The transformation pathway of total saponins to diosgenin was analysed with HPLC.

Separation and purification of saponin hydrolases: The selected strains were cultured in 10% of total saponins and incubated for 96 h. The fermentation liquid was centrifuged at 12000 rpm for 10 min, and the supernatant was filtered by 0.22 μm membrane. Then, 90% saturated ammonium sulphate powder was added, and the sediment was dissolved, dialysed, and filtered before being stored in a 4°C refrigerator. The crude enzyme was separated and purified by DEAE-cellulose DE-52 anion exchange column (4.0 cm × 11.0 cm). The collected highest eluting peak was frozen, dried, and concentrated. The solution was further separated and purified by the Sephadex G-100 column (2.0 cm × 24.0 cm).

Properties of saponin hydrolases: Gracillin (1 mL, 0.05 mg L^{-1}) and 1 mL of purified enzyme solution were mixed in several 10 mL test tubes and processed by oscillation water bath for 168 h under the most appropriate temperature. One group of test tubes was collected every 24 h and extracted with butanol. The extraction solution was supplied with N_2 to volatize the butanol, and the saponins were analysed through TLC after concentration. The molecular weight of the purified saponin hydrolase was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with the method proposed by Laemmli (1970) with 5% (w/v) stacking poly-

acrylamide gel and 12.5% (w/v) separating gel. The purification enzyme was hydrolysed by trypsin, extracted and dried.

Gene cloning of saponin hydrolases: The total RNAs were extracted from the objective strain by the TakaRa RNAiso Plus Kit (9108Q) and named as CTG0330-RNA, CTG0331-RNA and CTG0332-RNA. PCR amplification was accomplished by the primers listed in Table 1.

The cDNA was synthesized from reverse transcription using TakaRa PrimeScript TM RT-PCR kit. TakaRa Tks Gflex DNA Polymerase, TakaRa Mini BEST and Agarose Gel DNA Extraction Kit Ver. 4.0 were used for PCR amplification, followed by gel cutting and recovery of the target fragments. DNA was connected according to the operation steps of In-Fusion HD Cloning Kit. Several connection products (1 μL) were treated by thermal transition to the *E. coli* competent cell JM109. The recombinant *E. coli* was coated on the plate and cultured overnight under 37°C. The positive clone bacteria were selected, and the plasmid was extracted and sequenced (Table 2).

Gene expression of saponin hydrolases: The target plasmid was inserted into the competent cell BL21(DE3) T1R to induce the expression for positive clones. The expression primers are given in Table 3. 1.0 μL of the plasmids was collected and put into the DE3 T1R. Then, 30 μL of the transformation solution was coated on a piece of cloth, which was cultured at 37°C. Then, 5 mL of LB/Kan (50 μg.mL^{-1}) medium was added into the glass tube followed by 100 μL of the seed culture solution. The mixture was cultured under 37°C for 4 h, and the absorbance was tested. After bacteria collection, bacteria equivalent to 2.0 OD were added into 320 μL of PBS suspension, followed by ultrasonic grinding. The bacterial ground solution was centrifuged and separated. Different leaching liquors (whole protein, supernatant, and precipitate) were collected for SDS-PAGE and stained by CBB-R250.

Estimation of hydrolysis ability of saponin hydrolases: Gracillin was used as the substrate, and a degeneration model was constructed to analyse the hydrolysis mechanism of the expressed proteins. The degeneration of gracillin to diosgenin might induce eight steps of reaction (Fig. 2).

The crude enzymes generated by engineered bacteria were mixed with gracillin and then processed by water bath under the most appropriate temperature. The samples were collected every 4 h, and the hydrolysis products were quantitatively analysed using HPLC-MS. The Michaelis constants of the different reactions were calculated from the experimental data by fitting the Michaelis-Menten equation as follows (Zhu et al. 2014):

$$\frac{d[S_i]}{d_t} = \sum_{\text{sythesis}} \frac{V_{\max,l}[S_j]}{K_{m,l} + [S_j]} - \sum_{\text{degradation}} \frac{V_{\max,m}[S_j]}{K_{m,m} + [S_j]} \quad ...(1)$$

Where, $[S_i]$ is the molecular concentration of saponin *i*; $V_{max,l}$ and $K_{m,l}$ are the maximum reaction rate and Michaelis constant of the synthesis reaction, respectively; $V_{max,m}$ and $K_{m,m}$ are the maximum reaction rate and Michaelis constant of the decomposition reaction, respectively; and $[S_j]$ is the molecular concentration of the upstream substrate saponin *j* during the synthesis of saponin *i*.

Data were analysed using Matlab 7.0. The K_m and K_{cat}/K_m of the expressed saponin hydrolase were determined by the hydrolysis reaction.

RESULTS

Screening of saponin hydrolysis strains: During the microbial transformation test, three strains, namely, *T. reesei*, *T. viride* and *A. fumigatus*, could transform total saponins into diosgenin, but their hydrolysis processes differed (Fig. 3). Protodioscin and protogracillin were degraded in the first 12 h. Dioscin and gracillin presented the same variation trends. These two saponins were not detected in the hydrolysis solution after 5 days. *T. reesei* showed distinct hydrolysis ability to diosgenin-triglycoside. At 6.5 days, diosgenin-triglycoside of 5.6 μmol L^{-1} was detected in the

Table 1: Primers applied for PCR amplifications.

Gene	Primer	Sequence (5'-3')	Length
αRha	TR INF	5' -TCGCGGATCCGAATTCATGATCCAGAAGCTTTCCAA-32	36 mers
	TR INR	5' -TGCTCGAGTGCGGCCGCCTAGTTAAGGCACTGGGCGT-32	37 mers
	TR Fw	5' -CTGAGACTCGCTTTGTTTCT-32	20 mers
	TR Rw	5' -ACTACCTCAGTTCTAGGAAG-32	20 mers
βGL	TV INF	5' -GTCGCGGATCCGAATTCATGATTGTCGGCATTCT-32	34 mers
	TR INR	5' -GTGCTCGAGTGCGGCCGCTTACAGGAACGATGGGT-32	35 mers
C3-βGL	AF INF	5' -TCGCGGATCCGAATTCATGAGACAGTGCGGTGAGTT-32	36 mers
	AF INR	5' -TGCTCGAGTGCGGCCGCCTACTTGGACATCCTCGATG-32	37 mers
	AF P1	5' -CAACGGACTGCTATAGCTTC-32	20 mers
	AF P2	5' -GAATGGAGAAGCTGTAGTAC-32	20 mers

Table 2: Primers applied for PCR of positive plasmid.

Primers	Sequence (5’-3’)	Length
TR P1 (αRha)	5’ -CAAGATCGATGGCGTTGGTC-3’	20mers
TR P2 (αRha)	5’ -AGACGGCAATGTTGAAGCAC-3’	20mers
TV P1 (βGL)	5’ - GAAGCGACATTGTAGGACCA-3’	20mers
TV P2 (βGL)	5’ - CAAGATCGATGGCGTTGGTC-3’	20mers
AF P1 (C3-βGL)	5’ -CGATCTGGGTCACCTTCAAC-3’	20mers
AF P2 (C3-βGL)	5’ -CCGTACTGCGTGGTCCAGGA-3’	20mers

hydrolysis solution. When *T. viride* was applied to hydrolyse the total saponins, the protodioscin and protogracillin were not detected after 12 h. *T. viride* could more effectively transform gracillin than dioscin. In the hydrolysis system, diosgenin-triglycoside was degraded effectively in 4 days. In the hydrolysis of total saponins with *A. fumigatus*, protodioscin and protogracillin were not detected in the hydrolysis solution after 1 day. The degradation effect of *A. fumigatus* to dioscin and gracillin was weaker than the previous two fungi, but it could degrade diosgenin-triglycoside to some extent. The three strains demonstrated diverse hydrolysis characteristics to the five saponins, which contained different glycosidic bonds. The biotransformation of the five saponins involves many enzymatic reactions. The enzymes in the three strains were assumed to have high activity toward C26 glycoside. The enzymes in *T. reesei* demonstrated higher activity to the terminal α-rhamnoside at the C3 position in the saponins, while the enzymes in *T. viride* showed higher activity to the terminal-β-glucoside at the C3 position in the saponins. The enzymes in *A. fumigatus* exhibited higher activity to β-glucoside at the C3 site. These findings suggested that the composition and function of the glycosidase system in the three strains differed during the hydrolysis of saponins and should be further studied.

Separation and purification of saponin hydrolases: To study the key enzymes involved in the three fungi, three enzymes with the highest activities were isolated and purified from 90% ammonium sulphate precipitation, DEAE-cellulose DE-52 column chromatography and Sephadex G-100 gel filtration (Fig. 4A). Fragment 1 was separated from the hydrolysis solution of *T. viride* with apparent molecular weight of 36 kDa. Fragment 2 was isolated from *T. reesei* with molecular weight of 60 kDa. Fragment 3 was separated from *A. fumigatus* with molecular weight of 56 kDa.

Substrate specificities of saponin hydrolases: We analysed the substrate specificities of purified saponin hydrolases using gracillin as the substrate to test the affinity of three enzymes to different glucosidic bonds, because α-rhamnoside, β-glycoside, and C3β-glycoside were involved in gracillin. According to the standard samples, substrate structure and distance, the following deductions were formed as shown in Fig. 4B: (a) gracillin, (b) diosgenin-diglycoside, (c) diosgenin-glycoside-rhamnoside, (d) diosgenin-glycoside, and (e) diosgenin.

The results revealed that diosgenin-diglycoside was generated first in the *T. reesei* hydrolysis reaction. Thus, the most active enzyme in the *T. reesei* had strong affinity to α-rhamnoside. The most reactive enzyme in the *T. viride* system had strong affinity to β-glycoside, and diosgenin-glycoside-rhamnoside was generated first in the hydrolysis reaction. The most reactive enzyme in the *A. fumigatus* system had strong affinity to C3β-glycoside. During the hydrolysis, diosgenin was quickly detected in the substrate. αRha, βGL, and C3-βGL were the three most important enzymes involved in the hydrolysis of saponins into diosgenin. These enzymes had poor specificities. αRha also showed β-glycosidase activity to C3 terminal glycoside and C3 glycoside, while βGL owned the cleavage ability to C3 terminal rhamnoside and C3 glycoside. C_3-βGL could also hydrolyse the terminal glycoside and rhamnoside.

Amplification and sequence analysis of saponin hydrolases: The total RNA was extracted, and cDNA was acquired through reverse transcription. PCR amplification of the αRha, βGL and C3-βGL genes in *T. reesei*, *T. viride*, and *A. fumigatus*, respectively, was performed using cDNA as the template. Corresponding plasmids with lengths of approximately 1035, 1416 and 1752 bp were obtained and named as CTG0330, CTG0331 and CTG0332, respectively. The three nucleotide sequences were submitted to the GenBank database with accession numbers MH748522, MH748523, and MH748524. CTG0330 demonstrated high homology with the glycoside hydrolase family 61 from *T. reesei* CM6a (GenBank Accession No. XM_006961505.1). CTG0331 showed high similarity with the enzyme belonging to cellobiohydrolase II family. CTG0331 exhibited high similarity with *A. fumigatus* Af293 (AFUA_8G06970) with GeneBank Accession No. XM_742402.1. The three sequences were compared using the multiple sequences alignment using DNAMAN 8, and the results are shown in Fig. 5. The similarity among CTG0330, CTG0331 and CTG0332 was 39%, implying that the three sequences differed.

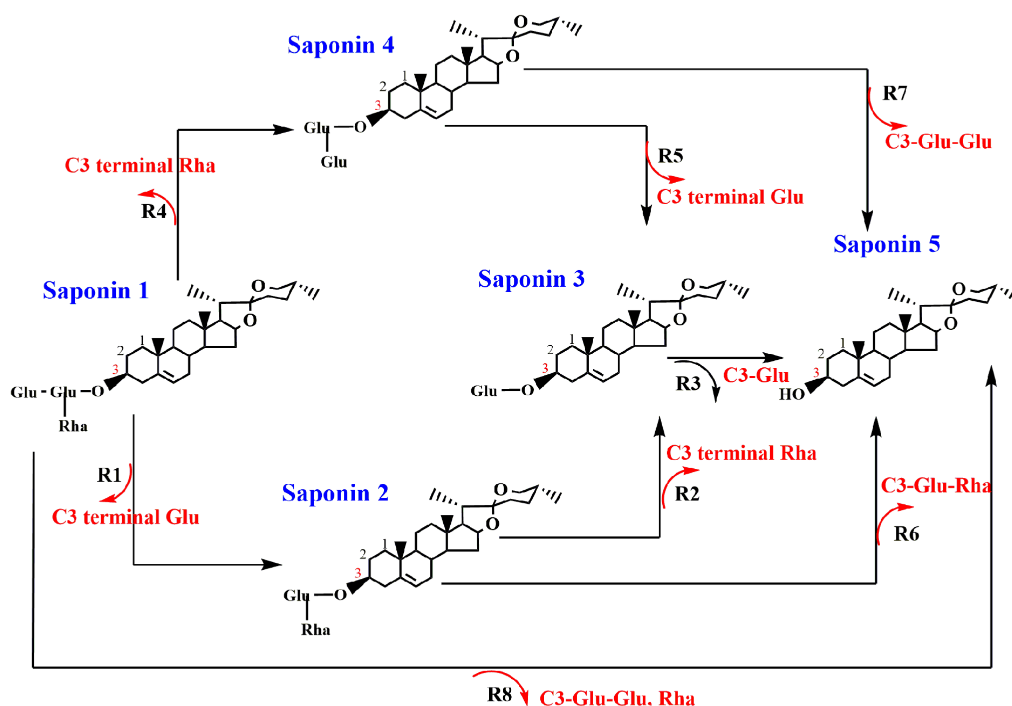

Fig. 2: Proposed transformation pathways of gracillin to diosgenin. R1–R8: Proposed reactions. Saponin 1: gracillin; Saponin 2: diosgenin-glucose-rhamnose; Saponin 3: diosgenin-glucose; Saponin 4: diosgenin-diglucose; Saponin 5: diosgenin

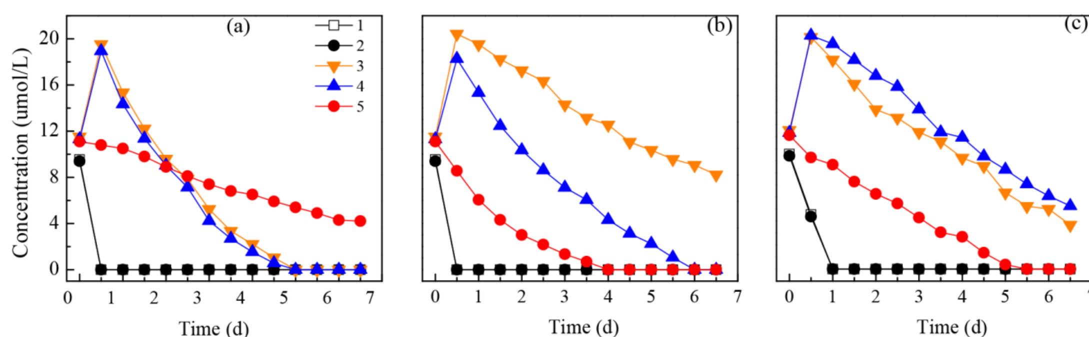

Fig. 3: Changes in the saponins during the hydrolysis of protodioscin (1), protogracillin (2), dioscin (3), gracillin (4), and diosgenin-triglycoside (5)

Gene expression of saponin hydrolases: CTG0330 was transformed into the Rosetta2(DE3)pLysS competent cell, while CTG0331 and CTG0332 were transformed into the competent cell BL21(DE3)T1R. The positive clones were expressed by inducing with 0.5% methanol. After conversion and culturing for 24 h, the recombinant *E. coli* cells were harvested and named as EXP0278-1(CTG0331), EXP0263-2(CTG0331) and EXP0263-3(CTG0332). The enzymes induced by the recombinant *E. coli* were verified by SDS-PAGE. An insoluble protein with molecular mass of 60 kDa was produced by EXP0278-1 with the target gene of CTG0330. This result was in accordance with the molecular mass of αRha. The target gene of CTG0331 was transformed into the competent cell BL21(DE3)T1R and expressed in pET-28a(+). The target protein was generated after induction, and the molecular weight was approximately 36 kDa, which basically conformed to βGL. An expressed protein with molecular mass of 56 kDa appeared in the SDS-PAGE of EXP 0263-3, indicating that the target gene (CTG0332) was successfully expressed in pET-28a(+).

Hydrolysis performance test of expressed enzymes: The crude enzyme extractions of EXP0278-1, EXP0263-2 and

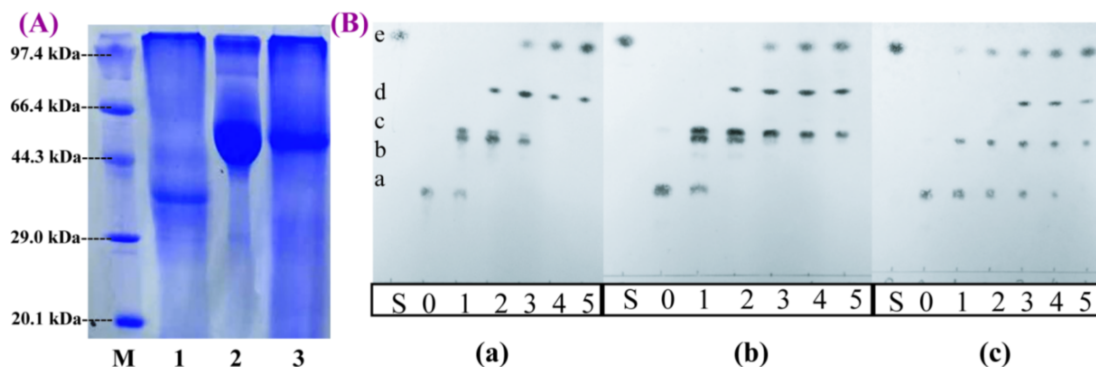

Fig. 4: (A) SDS-PAGE electrophoretogram of saponin hydrolase; M: Marker; 1. βGL from *T.viride*; 2. αRha from *T. reesei*; *3.* C3-βGL from *A. fumigatus*. (B) TLC of the time course of saponins in the cultivation of gracillin by αRha (a), βGL(b), and C3-βGL(c). S: diosgenin; 0–5: hydrolyzing time (d). (a: gracillin, b: diosgenin-diglucoside, c: diosgenin-glucoside-rhamnoside, d: diosgenin-glucoside, and e: diosgenin).

EXP0263-3 were named as Y1, Y2, and Y3, respectively. An enzyme assay of Y1, Y2 and Y3 was conducted with the solution from EXP0278-1, EXP0263-2 and EXP0263-3. The dynamic analysis results are given in Table 4. Four steady-state kinetic constants (V_{max}, K_m, K_{cat}, and K_{cat}/K_m) to different sugar moieties were calculated with the equations proposed previously. V_{max} indicates the maximum velocity of an enzyme reaction, and K_m represents the substrate concentration with the reaction rate of half of V_{max}. K_{cat} coincides with the V_{max} per unit concentration of enzyme, while the value of K_{cat}/K_m could be used to assess the transformation efficiency of enzymatic catalysis. High K_{cat}/K_m implied higher catalytic efficiency.

Five steps (R1-R5) occurred during the hydrolysis of gracillin with Y1. The highest K_{cat}/K_m was achieved in R4, indicating that this reaction has the highest probability of occurrence. R4 refers to the cleavage of the C3 terminal rhamnoside. R3 was the least reaction to occur because of its smallest K_{cat}/K_m, which reflects the hydrolysis of C3 glucose. The results demonstrated that Y1 had the highest affinity to the C3 terminal rhamnoside and the least affinity to C3 glycoside. When Y2 was incubated with gracillin, R1-R5 occurred, and the C3 terminal rhamnoside, C3 terminal glycoside and C3 glycoside were cleaved from gracillin. The K_{cat}/K_m value showed that Y2 demonstrated the highest preference for the C3 terminal rhamnoside and C3 terminal glycoside. This result indicated that these two reactions were the easiest to occur. R1-R8 mainly occurred during the transformation of gracillin to diosgenin by Y3. The highest K_{cat}/K_m of 1.29 mM h^{-1} was obtained in R3, indicating that Y3 was highly active to C3 glycoside. The K_{cat}/K_m value of R7 was 0.126 mM h^{-1}, indicating that this reaction was hard to happen. Hence, the crude enzyme extractions generated by recombinant *E. coli* had strong affinity to αRha, βGL, and C3-βGL.

DISCUSSION

Complex enzyme reactions are involved in the microbial transformation of saponins to diosgenin (Dong et al. 2010). Saponin hydrolases played important roles in this process. Numerous studies have been conducted to purify saponin hydrolases from microorganisms. In this study, the three key enzymes for the cleavage of saponins to diosgenin were investigated systematically for the first time. The three enzymes, αRha, βGL, and C3-βGL purified from *T. reesei*, *T. viride*, and *A. fumigatus* exhibited high hydrolysis ability to C3 terminal rhamnoside, C3 terminal glycoside, and C3 glycoside, respectively.

These reaction characteristics of the three purified enzymes are different from the previously reported saponin hydrolases. For example, a novel protodioscin-glycosidase-1 (PGase-1) was purified from *Aspergillus oryzae*. PGase-1 could cleave the terminal 26-O-β-D-glucopyranoside and terminal 3-O-(1→4) -α-L-rhamnopyranoside of protodioscin to yield dioscin. Meanwhile, PGase-1 can hardly cleave the 3-O-(1→2)-α-L-rhamnopyranoside belonging to progenin III, 3-O-β-D-glycoside belonging to trillin, and the 1-O-glycosides belonging to ophiopogoninD (Liu et al. 2013). Feng et al. (2007) purified and characterized a glucoamylase because of the steroidal saponin rhamnosidase activity from *Curvularia lunata*. The enzyme could cleave the terminal 1,2-linked rhamnosyl residues in the sugar chains at the C-3 position. Lei et al. (2010) reported *A. fumigatus*, which can hydrolyse saponins in DZW to diosgenin, was separated from soils where DZW grew. Glycosidase with strong specificity to dioscorein bonds on the C3 site were purified from *A. fumigatus*. However, it showed poor hydrolysis efficiency to βGL and α-rhamnoside. Four electrophoresis-purified glycosidases (E1-E4) were isolated from the fermen-

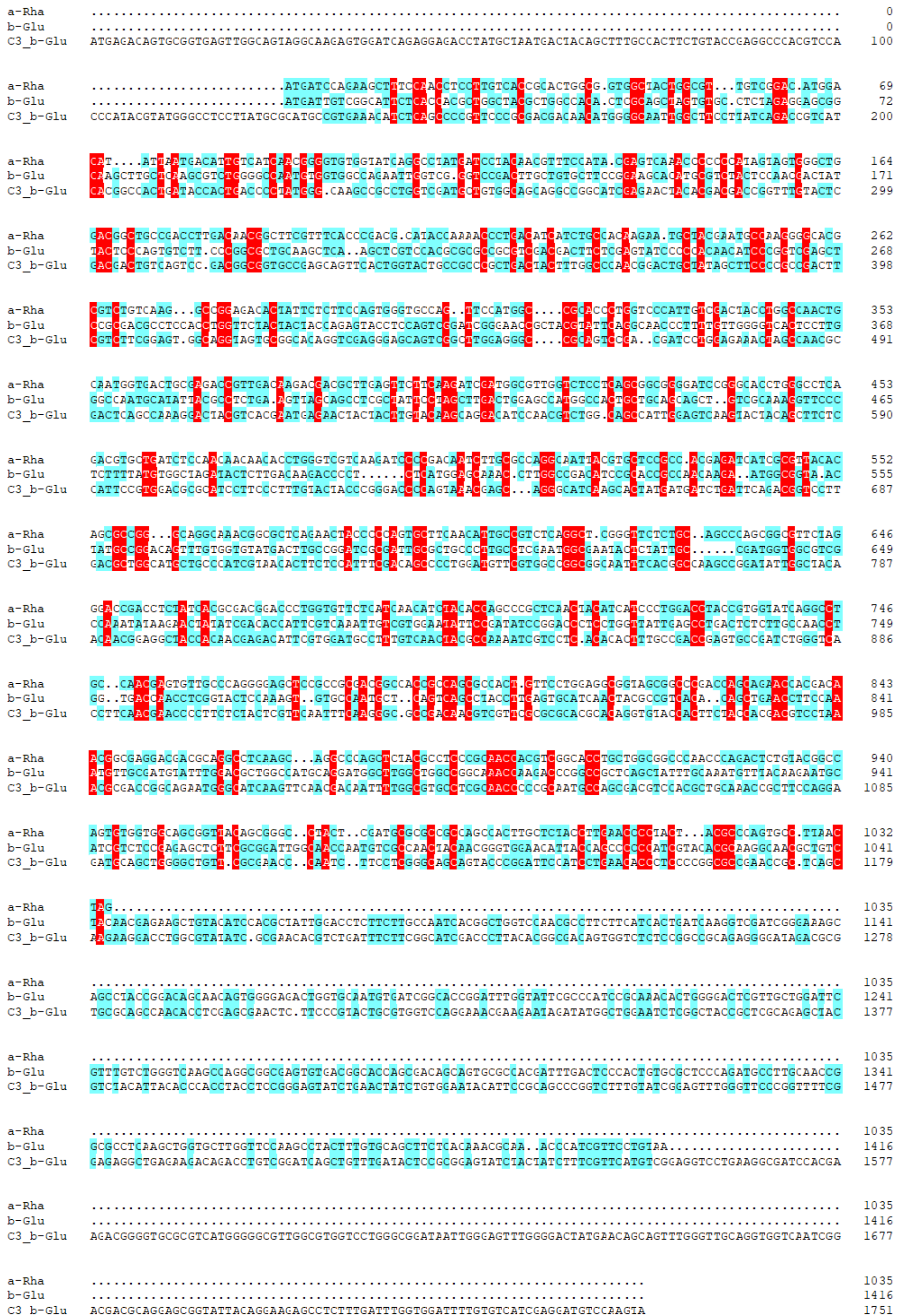

Fig. 5: Alignment of nucleotide sequences of αRha, βGL, and C3-βGL from *T. reesei*, *T. viride* and *A. fumigatus*, respectively. The same nucleotides shared in the three sequences were indicated by red boxes, whereas same nucleotides shared in two sequences were denoted by green boxes.

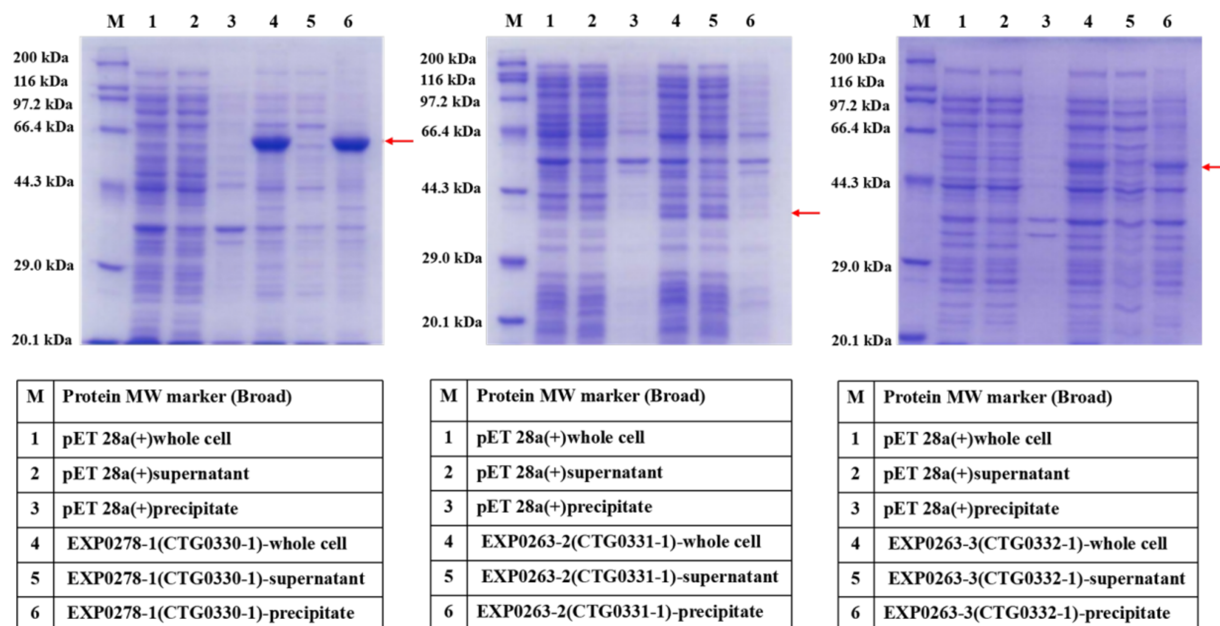

Fig. 6: SDS-PAGE analysis of EXP0278-1(CTG0330-1), EXP0263-2(CTG0331-1) and EXP0263-3(CTG0332-1) in *E. coli*.

tation hydrolysis solution of *T. reesei* through Native-Page purification in our laboratory (Zhu et al. 2014). Among these glycosidases, E1 and E4 only had β-glycosidase activity and could hydrolyse β-glycoside on the C26 site and β-glycoside at the carbohydrate chain terminal of C3 site of saponin. E2 and E3 had β-glycosidase and β-glycosidase activities. They could hydrolyse β-glycoside at the C26 site, β-glycoside at the carbohydrate chain terminal of the C3 site of saponin, α-rhamnoside, and β-glycoside on C3 site. They played important roles in the transformation of saponins, and E2 and E3 were the crucial enzymes in this process for cleavage of glycoside at the C3 site, which was a rate-limiting process. However, the compositions of E1-E4 in this system were not appropriate, leading to the accumulation of diosgenin-glucose. Thus, optimization of the enzyme system is especially important, and cloning the gene and overexpression of the target gene is an efficient strategy to increase the key enzyme in the transformation process.

Only a few studies have been conducted on the gene cloning of saponin hydrolases which could effectively hydrolyse saponins to diosgenin. One kind of saponin hydrolase (PGase-1) was purified from *A. oryzae* (Liu et al. 2013). Diosgenase 1 could hydrolyse the original dioscin26-O-β-D-glucosyl groups and 3-O-α-L-(14)-rhamnose in saponins. The gene of diosgenase 1 was obtained by combined RT-PCR and RACE strategy. The gene was 1725bp long and successfully expressed in *Pichia pastoris* GS115. Wang et al. (2011) extracted dioscin glycosidase gene with pronucleus and eukaryotic expression. The results demonstrated that when the gene was expressed in *E. coli*, the yielded enzyme did not show glycosidase activity, but the enzyme protein from the expressed *P. pastoris* showed dioscin glycosidase activity. The optimal incubation time for *P. pastoris* was 144 h, which was too long. In this paper, all three cloned saponin hydrolase genes were successfully expressed in *E. coli*. After 24 h of culture, glycosidase could be acquired, which significantly increased the enzyme production efficiency. The hydrolysis efficiency of the three expressed enzymes to different sugar moieties were evaluated with a mathematical model. The activities of Y1, Y2 and Y3 were quantified with the model. The results illustrated that the K_{cat}/K_m values for R4 (Y1), R1 (Y2) and R3 (Y3) were similar, which indicates that their activities to terminal rhamnoside, terminal glycoside and C3 glycoside were not significantly different. Previous studies showed that the cleavage of C3 glycoside was the rate limiting step in the transformation of saponins to diosgenin. To solve this problem, CTG0330, CTG0331 and CTG0332 were expressed in an engineered strain to achieve a proper enzyme system. The strategy has been widely used in the hydrolysis of cellulose and biological synthesis of lycopenes. For example, production of miltiradiene from (E,E,E)-geranylgeranyl diphosphate was catalysed by three key enzymes and diterpene synthases SmCPS and SmKSL. Zhou et al. (2012) present a modular pathway engineering strategy to rapidly assemble the synthetic miltiradiene pathways in *Saccharomyces cerevisiae* with the gene fusion technology. The obtained diploid strain YJ2X involving multiple genes could pro-

Table 3: Primers applied for gene expression.

Primer name	Sequence of nucleotide acid (5'®3')
R1-	ACGGAATTCATGAAACGAGTGTTGCTAATTCTTGT
F2-	TTGCCAGTAGTCTGTGCTAGCTTTTTTTGTATAGCGCAC
R2-	GCTAGCACAGACTACTGGCA
F3-	ATTCTCGAGTTACCACACTGTTACGTTAGAAC

Table 4: Calculated kinetic rate parameters of hydrolysis reactions for Y1, Y2 and Y3.

	Reaction/Enzymes	R1	R2	R3	R4	R5	R6	R7	R8
Y1	Vmax (mM h^{-1})	0.0421	0.0359	0.0103	0.0259	0.0387	-	-	-
	*K*m (mM)	2.14	2.03	4.33	0.43	1.18	-	-	-
	*K*cat (h^{-1})	1.00	0.855	0.245	0.62	0.92	-	-	-
	*K*cat/*K*m (h^{-1} mM^{-1})	0.468	0.42	0.0566	1.43	0.78	-	-	-
Y2	*V*max (mM h^{-1})	0.0214	0.0597	0.0829	0.0324	0.0452	-	-	-
	*K*m (mM)	0.526	2.425	2.056	1.354	2.014	-	-	-
	*K*cat (h^{-1})	0.563	1.57	2.18	0.853	1.19	-	-	-
	*K*cat/*K*m (h^{-1} mM^{-1})	1.07	0.65	1.06	0.631	0.591	-	-	-
Y3	*V*max (mM h^{-1})	0.0452	0.0543	0.0347	0.0949	0.0241	0.0911	0.0149	0.0121
	*K*m (mM)	2.047	2.478	0.691	7.087	4.33	6.025	3.024	2.332
	*K*cat (h^{-1})	1.16	1.39	0.889	2.43	0.618	2.33	0.382	0.310
	*K*cat/*K*m (h^{-1} mM^{-1})	0.567	0.562	1.29	0.343	0.143	0.388	0.126	0.133

duce miltiradiene titer of 365 mg L^{-1} in a 15-L bioreactor. This result suggested that the construction of microbial cell with multiple genes was an efficient way to improve the microbial transformation efficiency.

CONCLUSION

T. reesei, *T. viride* and *A. fumigatus,* which can transform total saponins into diosgenin, were screened. Analysis of the biotransformation pathway showed that the hydrolysis of the three strains differed. Three key saponin hydrolases (αRha, βGL, and C3-βGL) were separated and purified from *T. reesei*, *T. viride* and *A. fumigatus*, respectively. Among these enzymes, αRha had strong affinity to the terminal α-rhamnoside in saponins. βGL had strong affinity to saponin terminal-β-glycoside. C3-βGL showed strong affinity to β-glycoside on the saponin C_3 site. The molecular weights of the three saponin hydrolases were determined. The genes that encoded αRha, βGL, and C3-βGL were amplified and expressed in *E. coli*. The obtained expressed proteins could effectively transform terminal α-rhamnoside, terminal β-glycoside and C3-glycoside in saponins.

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