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Transcriptome Profiling, Cloning, and Characterization of *An*Glu04478, a Ginsenoside Hydrolyzing β-Glucosidase from *Aspergillus niger* NG1306

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Abstract

β-Glucosidase plays a pivotal role in transforming ginsenosides into specific minor ginsenosides. In this study, total ginsenosides from *Panax notoginseng* leaves were used as substrates to stimulate the growth of *Aspergillus niger* NG1306. Transcriptome analysis identified a β-glucosidase gene, *Anglu*04478 (1455 bp, 484 amino acids, 54.5 kDa, pI = 5.1), as a participant in the ginsenosides biotransformation process. This gene was cloned and expressed in *Escherichia coli* BL21 *Transetta* (DE3). The *An*Glu04478 protein was purified using a Ni^{2+} column, and its enzymatic properties were characterized. Purified *An*Glu04478 exhibited a specific activity of 32.97 U/mg when assayed against *p*NPG. Under optimal conditions (pH 4.5, temperature 40 °C), the kinetic parameters, K_m and V_{max}, for *p*NPG were 1.55 mmol/L and 0.014 mmol/min, respectively. Cu^{2+} displayed an inhibitory effect on *An*Glu04478, whereas Ca^{2+}, Co^{2+}, and Ni^{2+} ions had minimal impact. The enzyme showed tolerance to ethanol and was largely unaffected by glucose feedback inhibition. Testing with ginsenosides as substrates revealed selective hydrolysis at the C3 position of ginsenosides Rb1, Rb2, Rb3, and Rc, with the metabolic pathway delineated as Rb1 → GypXVII, Rb2 → C–O, Rb3 → C-Mx1 → C-Mx, and Rc → C-Mc1. The conversion rates of ginsenosides Rb1, Rb2, Rb3, and Rc varied from 2.58 to 20.63%. With 0.5 U purified enzyme and 0.5 mg total ginsenosides, incubated at 40 °C for 12 h, the conversion rates were 42.6% for GypXVII, 10.4% for C–O, 6.27% for C-Mx1, 26.96% for C-Mx, and 90% for Rc. These results suggest that *An*Glu04478 displays substrate promiscuity as a β-glucosidase, thus broadening the potential for ginsenoside biotransformation.

Introduction

Panax notoginseng (Burkill) and *Panax ginseng* (Meyer), both members of the *Panax* genus within the Araliaceae family [1], contain the bioactive component known as ginsenosides [2]. *Panax notoginseng*, an exclusive Chinese medicinal herb, is widely cultivated in Wenshan, Yunnan province. To date, over 70 ginsenosides have been isolated from this plant, including notable types such as Rg1, R1, Re, Rb1, Rb2, Rb3, Rd, C-Mx1, Rc, Fa, and Fc [3]. These compounds are primarily derived from the roots, leaves, flowers, and fruits. Ginsenosides are categorized based on the skeleton structure of their sapogenins into dammarane type, octilon type, oleanolic acid type, C17 side chain variant type, and miscellaneous subtypes [4]. According to the number and position of hydroxyl groups, dammarane type ginsenosides are divided into protopanaxadiol (PPD) and protopanaxatriol (PPT) types [5]. Notably, Rb1, Rb2, and Rc are of the PPD type, whereas Re and Rg1 pertain to the PPT type [6]. Despite their consistent aglycone skeleton structures, they differ in the sugar moieties attached at the C3, C6, and C20 positions [4]. The biological activities of different ginsenoside monomers vary due to differences in glycosylation sites and amounts [7–9]. Studies have demonstrated that minor ginsenosides, which are typically produced by the removal of glycosides from major ginsenosides [10–12], exhibit enhanced pharmacological activities [13–15]. The specific substrate recognition by enzymes provides significant advantages in producing diverse minor ginsenosides [15, 16].

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Directional catalysis of ginsenosides into minor forms is well-documented, particularly through specific glycoside hydrolases such as β-glucosidases from *Thermotoga thermarum* DSM 5069^T and *Burkholderia* sp. GE 17-7, which convert ginsenoside Rb1 to Rg3 [17, 18]. A β-glucosidase from *Aspergillus versicolor* LFJ1403 converts ginsenoside Rb1 to Rd, achieving a conversion rate of 96% [19]. Furthermore, the efficient production of minor ginsenosides can be achieved through the utilization of diverse glycoside hydrolases or their combined application. For instance, using various β-glucosidases or their combinations has increased the yield of the rare ginsenoside Rh2 by 7%, reaching a peak of 51.17 mg/L [13]. A synergistic combination of α-L-arabinofuranosidase and β-galactosidase from *Caldicellulosiruptor saccharolyticus*, along with β-glucosidase from *Sulfolobus acidocaldarius*, results in the complete conversion of major PPD ginsenosides into compound K (C–K) [20]. Additionally, specific glycoside hydrolases catalyze the transformation of certain ginsenosides into various minor counterparts. For example, β-glycosidase from *Armillaria mellea* mycelia effectively converts Rb2 into C–Y and C–K [21]. β-glucosidase from *Terrabacter ginsenosidimutans* selectively and sequentially converts ginsenoside Rb1 to minor gypenoside XVII, gypenoside LXXV, and ultimately C–K [22]. Similarly, β-glucosidase from *Flavobacterium johnsoniae*, overexpressed in *Escherichia coli* BL21 (DE3), hydrolyzes the outer glucose moiety of ginsenoside Rb1 and gypenoside XVII at the C-20 position, resulting in the formation of ginsenosides Rd and F2, respectively [23]. However, only a few β-glucosidases that can recognize ginsenosides Rb1, Rb2, Rb3, and Rc as substrates simultaneously.

In this study, to investigate the glycoside hydrolase gene catalyzing ginsenoside biotransformation in *Aspergillus niger* NG1306, total ginsenosides from *P. notoginseng* leaves were used as substrates to stimulate the growth of *A. niger*. Transcriptome analysis was conducted, identifying the β-glucosidase gene *Anglu*04478 as a potential participant in ginsenoside biotransformation. *Anglu*04478 was cloned, expressed heterologously, and characterized enzymatically. It showed selective hydrolysis toward the glucose at position C3 in ginsenosides Rb1, Rb2, Rb3, and Rc, providing a theoretical foundation for potential industrial applications of β-glucosidase *An*Glu04478.

Materials and Methods

Materials

4-Nitrophenyl β-D-glucopyranoside (*p*NPG) was obtained from Sigma-Aldrich Chimie S.a.r.l. (Lyon, France). Ginsenosides Rb1, Rb2, Rb3, and Rc were sourced from Yunnan Yunuo Bioengineering Co., Ltd. (Yunnan, China). Restriction endonucleases and the BCA protein assay kit were purchased from Takara Biomedical Technology Co., Ltd. (Dalian, China). TransTaq DNA Polymerase High Fidelity and a Ni^{2+}-affinity column for chromatography were acquired from Transgen Biotech Co., Ltd. (Beijing, China). Protein Marker was purchased from Cofitt Life Sciences Co., Ltd. (Kunming, China). IPTG and Kanamycin were obtained from BioFroxx Biotech Co., Ltd. (Kunming, China).

Strains, Plasmids, and Culture Conditions

Aspergillus niger NG1306 was screened by our laboratory in the previous work [24]. *Escherichia coli* DH5α for routine cloning, and *E. coli* BL21 *Transetta* (DE3) (Transgen Biotech) for protein expression. The T-cloning vector pEASYT1 (Transgen Biotech) and the expression vector pET29a (Novagen, Madison, WI, USA) were utilized to express the His-tag fused recombinant *Anglu*04478. *Escherichia coli* strains were cultured at 37 °C in Luria–Bertani (LB) medium or on LB agar plates, supplemented with kanamycin (50 μg/mL) as necessary. *A. niger* NG1306 was cultured at 28 °C on Potato Dextrose Agar (PDA) medium.

Transcriptome Sequencing and Differential Expression Analysis of Unigenes

Panax notoginseng leaves, which predominantly contain ginsenosides Rb1, Rb3, Rg1, and Rd [25], were added to the fermentation medium (comprising 0.2% ginsenosides, $NaNO_3$ 0.3 g, K_2HPO_4 0.1 g, $MgSO_4{\cdot}7H_2O$ 0.05 g, KCl 0.05 g, $FeSO_4$ 0.001 g, sucrose 2 g, ddH_2O 1000 mL) without control ginsenosides. *Aspergillus niger* was induced to ferment at 28 °C until the fifth day. The RNA sample protector (Takara, Dalian, China) was added to the harvested cells. Transcriptome sequencing was conducted by Wuhan Huada Medical Research Institute Co., Ltd. The transcriptome data for *A. niger* NG1306 were referenced against its own genome and annotated using Rfam, Pfam, Reactome, COG, EggNOG, and InterPro databases. Differential expression of Unigenes was analyzed using the Dr. Tom interactive multi-omics data mining system at BGI.

GO and KEGG Pathway Classification

Gene Ontology (GO) categories were divided into three functional categories: molecular function, cellular component, and biological process. Following differential gene analysis, functional classification was performed.

The KEGG metabolic pathway analysis classified transcription-related genes into 7 branches: Cellular Processes, Environmental Information Processing, Genetic Information Processing, Human Disease (animals only), Metabolism,

Organic Systems, and Drug Development. Each branch was further subdivided and analyzed statistically.

Identification of Candidate Genes in the Ginsenoside Hydrolyzing Process

Based on GO annotations, differentially expressed Unigenes were functionally categorized, with a focus on catalytic activity under molecular function. Enrichment analysis was performed using the phyper function in R software, *P* value were calculated and subsequently adjusted using FDR correction. Functions with a *Q* value ≤ 0.05 were considered significantly enriched. Additionally, KEGG enrichment analysis targeted genes in catalytic activity, focusing on Carbohydrate metabolism under Metabolish, and identifying key genes involved in hydrolase activity within the secondary metabolic process.

Aspergillus niger Fermentation Induced by Ginsenosides from *P. notoginseng* Leaves and RNA Extraction

0.2% ginsenosides were added to the fermentation medium to induce the growth of *A. niger* until the fifth day. Cells were harvested, and total RNA was extracted using a Geneode Fungus RNA Kit following the manufacturer's protocol (Geneode bio-Tek, Wuhan, China). RNA quality was assessed by measuring its concentration and purity with a UV–Vis spectrophotometer (BiodropuLite, Holliston, USA) and evaluating its integrity on a 1.2% agarose gel.

Cloning, Expression, and Purification of β-Glucosidase *Anglu*04478

cDNA was synthesized from RNA using the PrimeScript RT kit with gDNA Eraser (Takara, DaLian). The *Anglu*04478 gene was amplified from cDNA using primers *Anglu*04478-F: 5′ ATGGGTTCAGCAACAACCTCAAC 3′ and *Anglu*04478-R: 5′ TAATGTCTTCTCGATATACTG GCTGAA 3′. The PCR protocol was as follows: initial denaturation at 94 °C for 2 min, followed by 31 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The amplified products were cloned into pEASY-T1 and sequenced by Sangon Biotech (shanghai, China). The primers included *Kpn* I and *Hin*d III restriction sites (underlined): *Anglu*04478 (*Kpn* I)-F: 5′ GGGGTACCATG GGTTCAGCAACAACCTCAAC 3′ and *Anglu*04478 (*Hin*d III)-R: 5′ CCCAAGCTTTAATGTCTTCTCGATATACTG GCTGAA 3′. The fragments were digested with *Kpn* I and *Hin*d III, purified using a TIANgel Purification Kit (TIANGEN Bio-Tek, Beijing, China), and ligated into the pET29a expression vector, following the same protocol.

The expression constructs were transformed by heat shock into *E. coli* BL21 *Transetta* (DE3) expression cells (Transgen Biotech). The transformants were cultured on solid Lauria–Bertani (LB) medium containing 50 μg/mL of kanamycin. The verified recombinant strain was cultured overnight in 10 mL LB medium with 50 μg/mL kanamycin at 37 °C with shaking at 180 rpm. This pre-culture was used to inoculate 50 mL LB medium at 1% (v/v) for expression. Upon reaching an OD_{600} of approximately 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the culture was incubated for an additional 7 h under the same conditions. Cells were harvested by centrifugation at 5000 rpm for 10 min at 4 °C and resuspended in 10 mL PBS buffer (pH 7.4). Cell disruption was achieved through ultrasonication; cell debris was removed by centrifugation at 12,000 rpm for 20 min at 4 °C, and the supernatants were filtered using a 0.22 μm Millipore filter. Protein expression and molecular weight were assessed by 12% SDS–PAGE and Coomassie Blue staining.

For purification, the *An*Glu04478 protein was purified using a Ni^{2+}-affinity column (Transgen Biotech) as per the manufacturer's instructions. The purified protein was then desalted using a PD-10 desalting column (GE Healthcare, Chicago, USA) and concentrated using a centrifugal filter device. The concentrated *An*Glu04478 was resuspended in PBS buffer containing 10% glycerol (pH 7.4) for further analysis. Protein concentration was determined using a BCA protein assay kit (Takara, Dalian, China).

Characterization of the Recombinant *An*Glu04478

The enzymatic activity of *An*Glu04478 was assayed using *p*NPG as the substrate. One unit (U) of enzyme activity was defined as the amount required to release 1 μmol of *p*-nitrophenol per min under standard conditions. Specific activity was expressed as U/mg of protein (μmol/min/mg protein). The enzyme activity assay comprised 1 μL of the purified enzyme solution, 50 μL of *p*NPG, and PBS buffer to achieve a final volume of 100 μL (*p*NPG final concentration: 5 mmol/L). The reaction was incubated at 30 °C for 10 min and terminated by adding 100 μL 0.5 mmol/L Na_2CO_3 solution. Absorbance was measured at 405 nm.

To assess the pH stability of recombinant *An*Glu04478, the enzyme was incubated at 30 °C for 2 h in PBS buffer ranging from pH 3.0 to 8.0. After incubation, residual activities were measured [26]. The enzyme's temperature stability was evaluated by incubating samples at temperatures from 25 to 50 °C, with 5 °C intervals, under optimal pH conditions for 2 h. The enzyme's peak activity was designated as 100%.

The impact of various metal ions (Na^+, K^+, Ca^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+}, and Cu^{2+}) and chemical agents (SDS and EDTA-Na_2) on *An*Glu04478's activity was analyzed [26].

Additionally, the effects of glucose and ethanol concentrations on the enzyme's activity were investigated [27, 28].

Enzyme Kinetics

The kinetic parameters of *An*Glu04478 for *p*NPG were determined by measuring the initial reaction rates at different *p*NPG concentrations (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mmol/L). The kinetic parameters (K_m, mmol/L) and maximum reaction rate (V_{max}, mmol/L) of *An*Glu04478 for *p*NPG were calculated by double reciprocal mapping [29].

Enzyme Assays and Product Analysis

A reaction system consisting of 0.2% ginsenosides Rb1, Rb2, Rb3, Rc, 0.5 U *An*Glu04478, and PBS buffer (50 mmol/L, pH 4.5) was assembled in a 500 µL volume. After a 60 min reaction at 40 °C, 500 µL of n-butanol was added for extraction. The mixture was vortexed for 2 min at 9000 r/min and centrifuged for 10 min. The n-butanol phase was analyzed using thin-layer chromatography (TLC, GF254, Qingdao Marine Chemical Co., Ltd, China) with a developing solvent of $CHCl_3$–CH_3OH (85:15, v/v). TLC spots were detected by spraying with CH_3OH–$C_4H_8O_2$–H_2SO_4 (85:10:5, v/v/v, low phase) and heated with a blower.

The remaining n-butanol phase was evaporated in a water bath, redissolved in chromatographic pure methanol, filtered through a 0.45 µm organic membrane, and further analyzed by HPLC. Chromatographic conditions were as follows: Column C18, 4.6 × 250 mm; flow rate: 1 mL/min; column temperature: 30 °C; detection wavelength: 203 nm. The mobile phase consisted of acetonitrile (A) and distilled water (B) with a gradient program: 31:69 to 65:35 A/B ratio from 0 to 53 min, 65:35 to 100:0 A/B ratio from 54 to 69 min, and a final 10 min at a 31:69 A/B ratio.

Sequence Information

The gene sequence referenced in this study is *Anglu*04478 (GenBank Accession No. PQ041204.1).

Results

Transcriptome Sequencing

A total of six samples were analyzed using Huada's DNBSEQ platform (three replicates each for control and treatment), generating 38.22 GB of data. The average genome alignment rate was 89.91%, and the gene set alignment rate was 77.48%. A total of 10,391 expressed genes were identified, consisting of 10,346 known genes and 45 predicted new genes. Analysis revealed 5174 new transcripts, including 5129 novel alternative splicing variants of known protein-coding genes and 45 transcripts for newly predicted protein-coding genes.

Unigenes Differential Expression Analysis

The heat map (Fig. S1) displayed 2830 differentially expressed genes, with 1452 upregulated and 1378 downregulated. GO annotation (Fig. S2) categorized 1769 genes under biological processes, 1851 under cellular components, and 2718 under molecular function. KEGG pathway analysis identified 379 genes related to Cellular Processes, 221 Environmental Information Processing, 371 to Genetic Information Processing, 2179 to Metabolism, and 18 to Organic Systems (Fig. S3).

Identification of Candidate Genes Involved in Ginsenoside Hydrolyzing Process by GO and KEGG Analysis

GO analysis highlighted catalytic activity within molecular functions, enriching 1236 genes, comprising 664 upregulated and 572 downregulated. KEGG pathway analysis (Fig. S4) included these genes, with distributions of 148 in Cellular Processes, 85 in Environmental Information Processing, 181 in Genetic Information Processing and Human Diseases, 1464 in Metabolism, and 10 in Organic Systems. The focus was on the carbohydrate metabolic process, where 254 genes were enriched, including 86 related to secondary metabolites. Further GO annotation revealed nine of these genes involved in glycoside hydrolase activity (Table S1), with seven upregulated and two downregulated. Among the upregulated genes, four had a log2 Fold Change > 1, specifically *Anglu*01436, *Anglu*04358, *Anglu*04478, and *Anglu*07314, all annotated as β-glucosidase in the Gene Ontology database.

Blast analysis of the four genes showed that *Anglu*01436 had 100% similarity with the exo-β-1,3-glucanase gene (XM_035499218.1) from *Aspergillus tubingensis*, indicating potential plant cell wall polysaccharide-degrading enzymes activity [30]. *Anglu*04358 showed 100% similarity with a glycoside hydrolase gene (XM_035505577.1) from *A. tubingensis,* suggesting β-1,3-endoglucanase activity [31]. *Anglu*07314 exhibited 98.54% similarity with the β-glucosidase gene (KJ739789.1) from *A. niger* strain B2, indicating potential activity in hydrolyzing ONP-β-D-glucopyranoside [32]. *Anglu*04478 showed 99.79% similarity with another glycoside hydrolase gene (XM_035505698.1) from *A. tubingensis* WU-2223L, however, its function has not been characterized. Based on amino acid sequence homology and structural similarity, β-glucosidases are classified into seven families: GH1 (glucoside hydrolase 1), GH3, GH5, GH9, GH17, GH30, and

GH116, with GH1 and GH3 being particularly prominent. The phylogenetic tree was constructed using the Neighbor-Joining and Maximum Likelihood methods (Fig. 1a). Clustering β-glucosidase (XM_035505698.1) from *A. tubingensis* WU-2223L and β-glucosidase (MH648000.1) from *A. niger* strain B2 with *An*Glu04478 in the GH1 family. These genes are annotated as β-glucosidases but have unreported functions. The full-length *Anglu*04478 gene is 1455 bp, encoding a protein of 484 amino acids with a predicted molecular weight of 54.54 kDa, and a pI of 5.1 (https://web.expasy.org/protparam). Aligning *An*Glu04478 with several GH1 β-glucosidase showed conserved motifs: KVKHWITFNE (residues: 164–173) [33], and predicted the presence of a conserved glutamate residue at the fourth β fold (Fig. S5).

Cloning of Full Length β-Glucosidase Gene *Anglu04478*, Expression, and Purification

Total RNA from *A. niger* was successfully extracted (Fig. S6a), and used as a template for reverse transcription to generate cDNA. This cDNA was then employed to amplify the *Anglu*04478 gene (Fig. S6b). Subsequently, the recombinant expression strain *Transetta*/pET29a-*Anglu*04478 was developed for enzyme expression and verification.

Following 1 mM IPTG induction, the recombinant strain *Transetta*/pET29a-*Anglu*04478 exhibited significant protein expression, with a distinct band appearing near the 50 kDa Marker (Fig. 1b, lane 2). The enzyme was purified using Ni^{2+} chromatography, resulting in a single band of *An*Glu04478 on SDS-PAGE (Fig. 1b, lane 3). The specific activity of the purified *An*Glu04478 against *p*NPG was 32.97 U/mg, which was 1.7-fold higher than that of the crude enzyme solution, with a purification yield of approximately 41.9%. Additionally, the specific activities toward natural substrates Rb1, Rb2, Rb3, and Rc were 10.62 U/mg, 8.51 U/mg, 20.18 U/mg, and 12.28 U/mg, sequentially. These activities were lower than those against the synthetic substrate *p*NPG, suggesting a stronger affinity for *p*NPG.

Characterization of the Recombinant *An*Glu04478

The effects of pH and temperature on *An*Glu04478 activity were investigated. The enzyme exhibited optimum activity at pH 4.5, with activity decreasing gradually at pH levels above 5.5, indicating higher activity under acidic conditions (Fig. 2a). Under the optimum pH condition, the optimum temperature of the enzyme was determined. The optimum temperature for activity was 40 °C; activity decreased significantly above 45 °C, indicating limited high-temperature tolerance (Fig. 2b).

The impact of metal ions and chemical reagents on *An*Glu04478 activity was also evaluated. Cu^{2+} exhibited an inhibitory effect, whereas high concentrations of Na^{+}, K^{+}, and Mg^{2+} enhanced enzyme activity. Ca^{2+}, Co^{2+}, and Ni^{2+} had minimal effects on activity with increasing concentrations. *An*Glu04478 was inhibited by high concentrations of disodium ethylenediamine tetraacetate (EDTA-Na_2) and sodium dodecyl sulfate (SDS) (Table S2).

The conversion products of ginsenosides are minor ginsenosides and glucose. Due to the poor water solubility of minor ginsenosides, ethanol is often required to aid their dissolution. To evaluate the effects of ethanol and glucose on the activity of *An*Glu04478, various concentrations were tested. At ethanol concentrations greater than 10%, enzyme

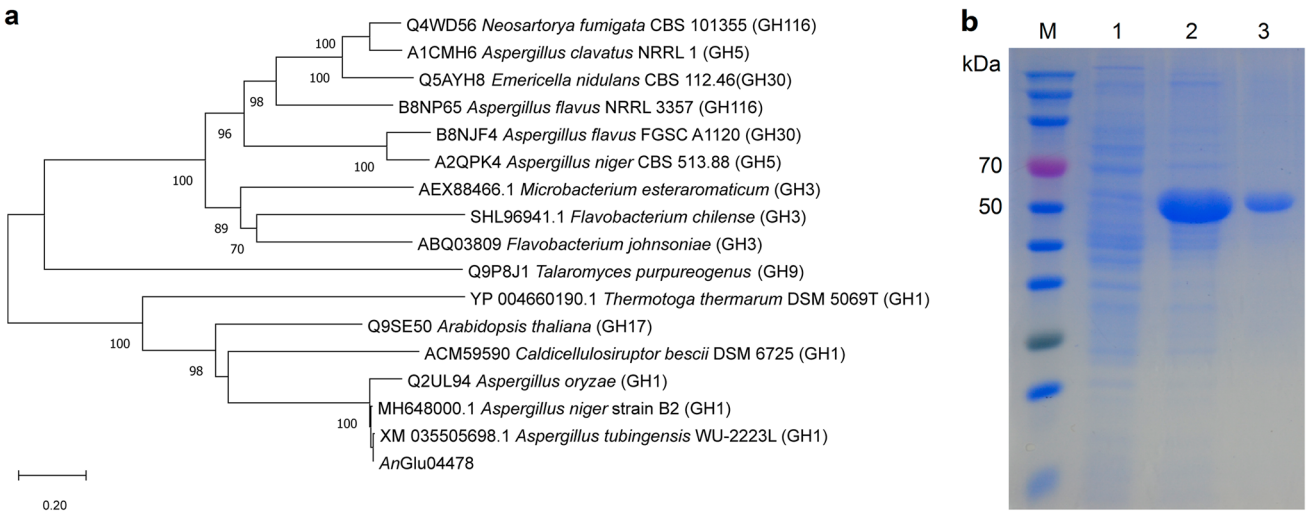

Fig. 1 **a** The phylogenetic tree results from analysis of β-glucosidase of amino acid sequences using Neighbor-Joining (NJ) method. Numbers on nodes correspond to percentage bootstrap values for 1000 replicates. **b** SDS-PAGE of *An*Glu04478 protein. (1: control; 2: crude enzyme solution; 3: purified *An*Glu04478)

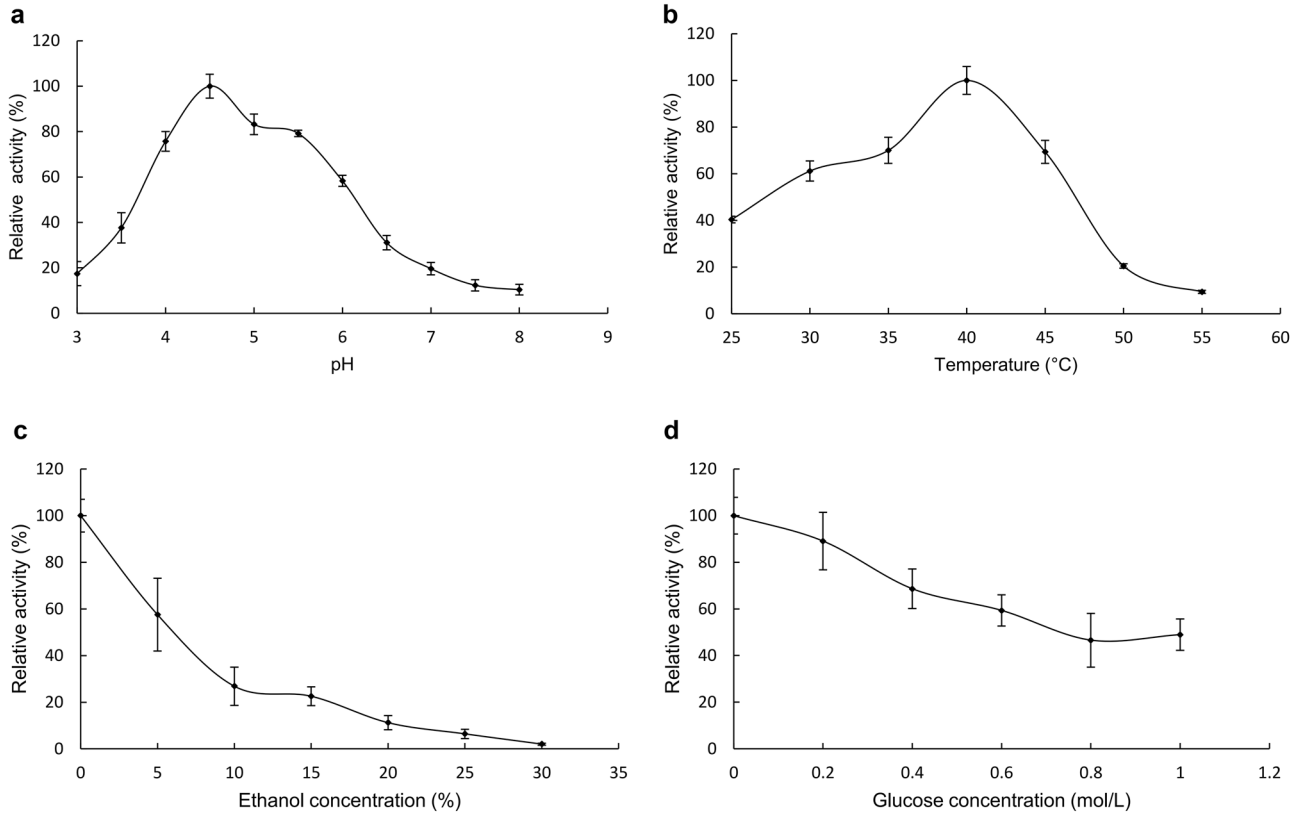

Fig. 2 Effects of pH (**a**) and temperature (**b**) on the activity of recombinant *An*Glu04478 determined using *p*NPG as a substrate, *An*Glu04478 tolerance to ethanol (**c**) and glucose (**d**). The maximum activity obtained was defined as 100%. Results are presented as means ± standard deviations ($n = 3$)

activity fell below 50%, indicating a significant influence of ethanol on *An*Glu04478 activity (Fig. 2c). In contrast, the impact of glucose on enzyme activity was minimal. At a glucose concentration of 1 mol/L, residual enzyme activity was nearly 50%, suggesting high glucose tolerance by *An*Glu04478 (Fig. 2d).

Analysis of Enzyme Kinetic Parameters Using *p*NPG as a Substrate

*An*Glu04478 was assayed under optimum conditions with *p*NPG as the substrate using the double reciprocal method. The kinetic parameters (K_m, mmol/L) and maximum reaction rate (V_{max}, mmol/min) were determined to be 1.55 mmol/L and 0.014 mmol/min, respectively (Fig. S7).

Analysis of the Transformation Products of Ginsenoside Rb1, Rb2, Rb3, and Rc by *An*Glu04478

Under optimal conditions, *An*Glu04478 was added to solutions of ginsenosides (Rb1, Rb2, Rb3, or Rc) and analyzed qualitatively by thin-layer chromatography (TLC). The enzyme converted Rb1 into GypXVII, Rb2 into C–O, Rb3 into C-Mx1 and C-Mx, and Rc into C-Mc1 (Fig. 3).

To confirm these products, samples were analyzed by HPLC. The retention times matched those of reference substances: GypXVII at 29.13 min for Rb1, C–O at 32.526 min for Rb2, C-Mx1 and C-Mx at 43.047 min and 43.9 min for Rb3, and C-Mc1 at 30.97 min for Rc (Fig. 4). The conversion rates were 15.38% for Rb1 into GypXVII, 2.58% for Rb2 into C–O, 17.62% and 13.01% for Rb3 into C-Mx1 and C-Mx, respectively, and 20.63% for Rc into C-Mc1. Quantitative conversion of total ginsenosides extracted from *P. notoginseng* leaves involved reactions with 0.5 mg of total ginsenosides, 0.5 U of purified enzyme, incubated at 40 °C for 12 h. The conversion rates were 42.6% for GypXVII, 10.4% for C–O, 6.27% for C-Mx1, 26.96% for C-Mx, and 90% for Rc.

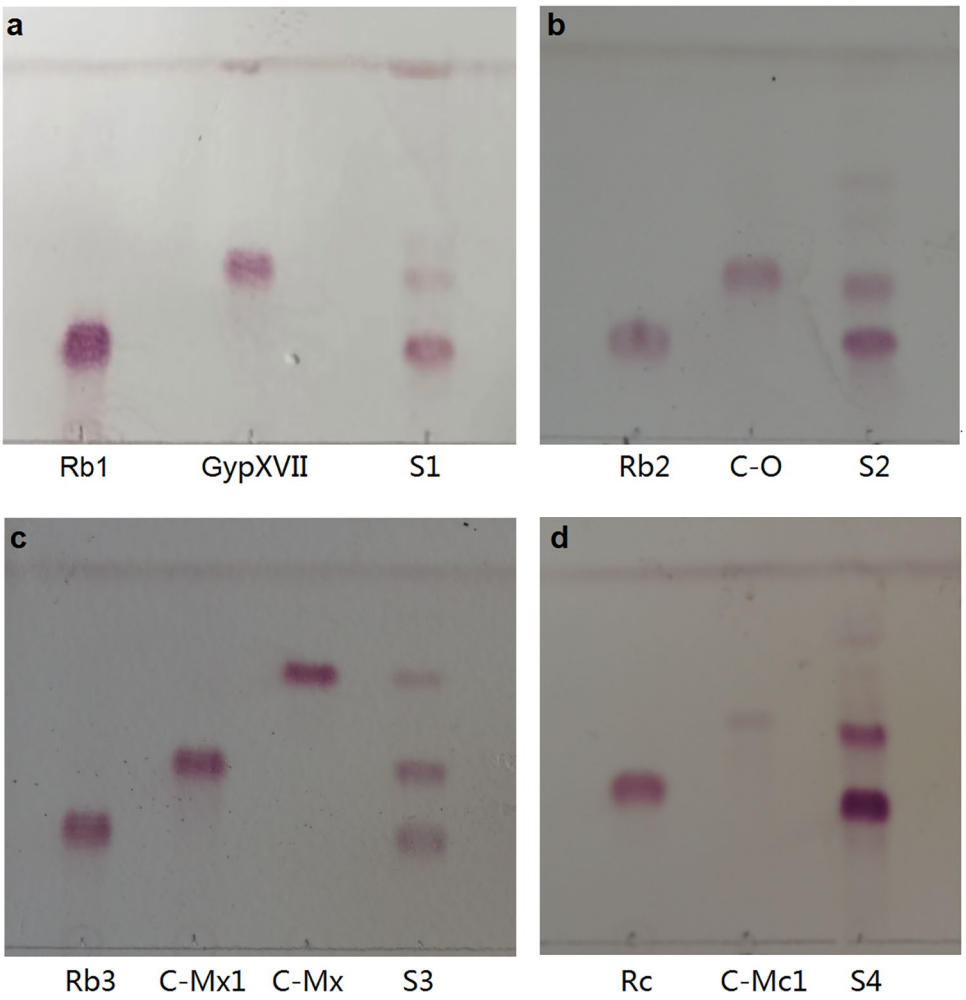

Fig. 3 TLC detection of different ginsenoside conversion products. Ginsenoside reference substances: Rb1, GypX-VII, Rb2, CO, Rb3, C-Mx1, C-Mx, Rc, C-Mc1. **a** S1: Rb1 conversion product detection, **b** S2: Rb2 conversion product detection, **c** S3: detection of Rb3 conversion products, **d** S4: detection of Rc conversion products

Discussion

Currently, glycosidases from bacteria and fungi are utilized in the biotransformation of ginsenosides [34–36]. Major ginsenosides can be transformed into various minor ginsenosides using crude enzymes extracted from *A. niger* after fermentation [37–39], indicating an abundance of glycosidases suitable for ginsenoside biotransformation in *A. niger*. However, crude enzyme transformations do not yield specific minor ginsenosides, which are often required for the activity research of natural drugs or industrial production, necessitating specific glycosidases for targeted ginsenoside transformation [40].

In this study, *Anglu*04478 was cloned from *A. niger* NG1306 to obtain a more efficient β-glucosidase for the specific transformation of ginsenosides. Through heterologous expression, recombinant β-glucosidase *An*Glu04478 was successfully produced in vitro. Despite high expression levels, the enzyme's activity was comparatively low, potentially due to the absence of eukaryotic post-translational modifications in the prokaryotic expression system [41]. Enzyme activity was influenced by reaction conditions including temperature, pH, substrate concentration, and product concentration. The optimal conditions were identified as 40 °C and pH 4.5, suggesting suitability for enzymatic ginsenoside conversion under acidic and moderate temperature conditions. An outstanding feature of *An*Glu04478 is its excellent tolerance to high concentrations of glucose, indicating no feedback inhibition from glucose, which could benefit industrial applications of ginsenoside biotransformation. The structural conservation of aromatic Trp168 and aliphatic Leu173 within the GH1 family β-glucosidase contributes to this glucose tolerance [42]. *An*Glu04478 also features an aromatic amino acid Trp at position 168, but aliphatic Leu at position 180.

In the biotransformation process, it is often necessary to add ethanol to a final concentration of 10% or higher to aid in the dissolution of minor ginsenosides. The activity of *An*Glu04478 decreases significantly when ethanol concentrations exceed 10%, consistent with the tolerance levels of other β-glucosidase to ethanol [43]. Ethanol's strong permeability disrupts the internal structure and properties

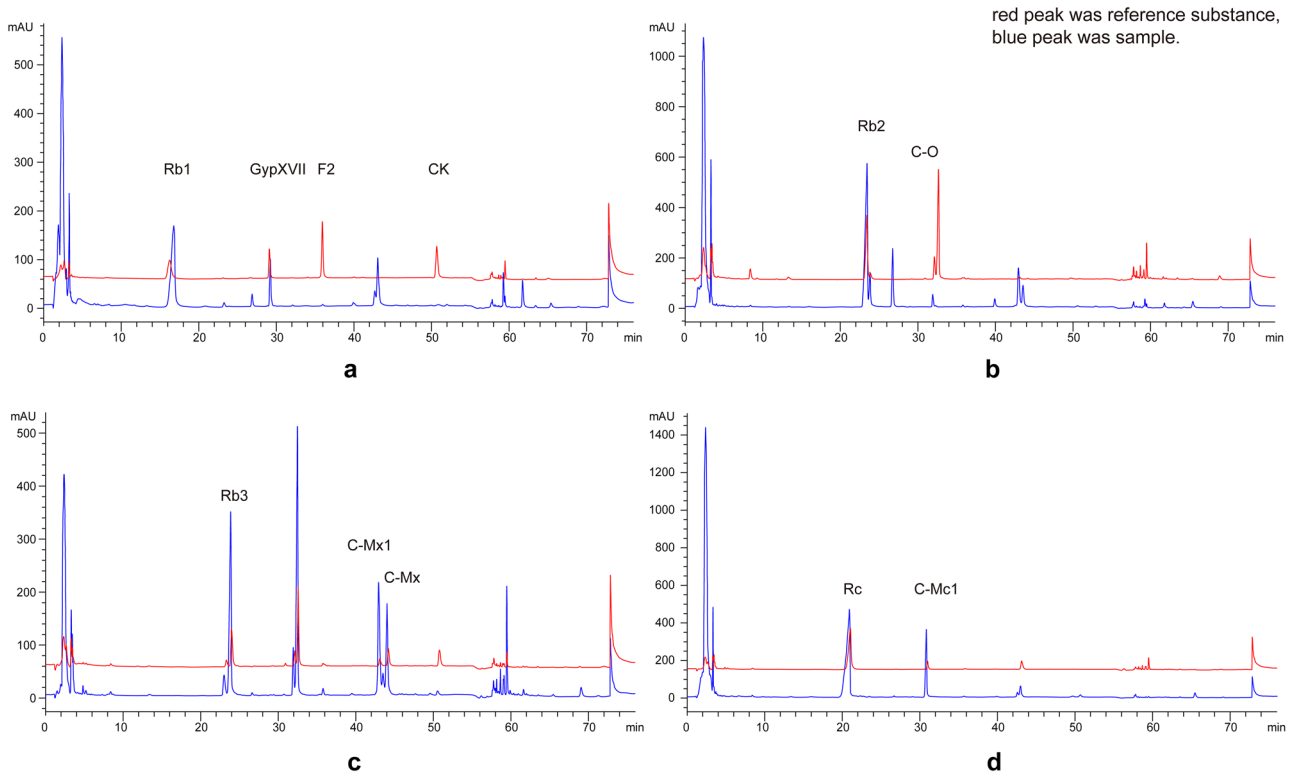

Fig. 4 Detection of ginsenoside conversion products by HPLC. Red peak was reference substance, blue peak was sample. **a** Detection of Rb1 conversion products, **b** detection of Rb2 conversion products, **c** detection of Rb3 conversion products, **d** Detection of Rc conversion products

of protein molecules, leading to denaturation and inactivation. To circumvent this, various deep eutectic solvents are employed to enhance the solubility of minor ginsenosides without compromising enzyme activity [44, 45]. Apart from Cu^{2+}, other metal ions have a minimal impact on the activity of *An*Glu04478, indicating its adaptability to a wide range of buffer solutions.

β-Glucosidase with the ability to simultaneously convert multiple ginsenosides has also been reported. β-glucosidase from *Sphingopyxis alaskensis* specifically hydrolyzes the outer glucose at the C3 position of ginsenosides, transforming ginsenoside Rb1, Rb2, Rc, Rd, and Rg3 into ginsenoside GypXVII, C–O, C-Mc1, F2, and Rh2, respectively. An alignment of the amino acid sequence with *An*Glu04478 shows only a 38.76% similarity, despite both being in the GH1 family due to similar domains [46]. In contrast, the novel endoglucanase BcelFp from *Fervidobaterium pennivorans* DSM9078 also hydrolyzes the outer glucose at the C-3 position in PPD-type ginsenosides, resulting in the conversion of ginsenoside Rb1, Rb2, Rc, and Rd into GypXVII, C–O, C-Mc1, and F2. However, BcelFp belongs to the GH5 family and shares only 9.92% amino acid sequence similarity with *An*Glu04478 and lacks similar domains [47]. *An*Glu04478 and the glucosidase (XM_035505698.1) from *A. tubingensis* WU-2223L show the highest similarity of 99.79% in amino acid sequence, but this sequence was submitted without protein expression and functional verification. Consequently, a β-glucosidase from *A. niger* that can simultaneously catalyze the hydrolysis of glucose at the C3 position of Rb1, Rb2, Rb3, and Rc has not been previously reported.

A conversion pathway has been proposed for *An*Glu04478 based on its selective hydrolysis of glucoside groups (Fig. 5). Although *An*Glu04478 exhibits substrate promiscuity, the enzymatic conversion yields for various substrates are low, suggesting low affinity for these substrates. Future efforts will involve homologous modeling, molecular docking, and directed evolution to enhance enzyme activity and biotransformation efficiency of ginsenosides [48, 49]. These advancements will potentially increase the industrial applicability of β-glucosidase *An*Glu04478.

Conclusion

In this study, the ginsenoside β-glucosidase *An*Glu04478 was successfully cloned from *A. niger* NG1306 and overexpressed in *E. coli* BL21 *Transetta* (DE3). The enzyme is composed of 484 amino acids, with a predicted molecular weight of 54.54 kDa and an isoelectric point (pI) of 5.1. Phylogenetic analysis indicates that *An*Glu04478 is

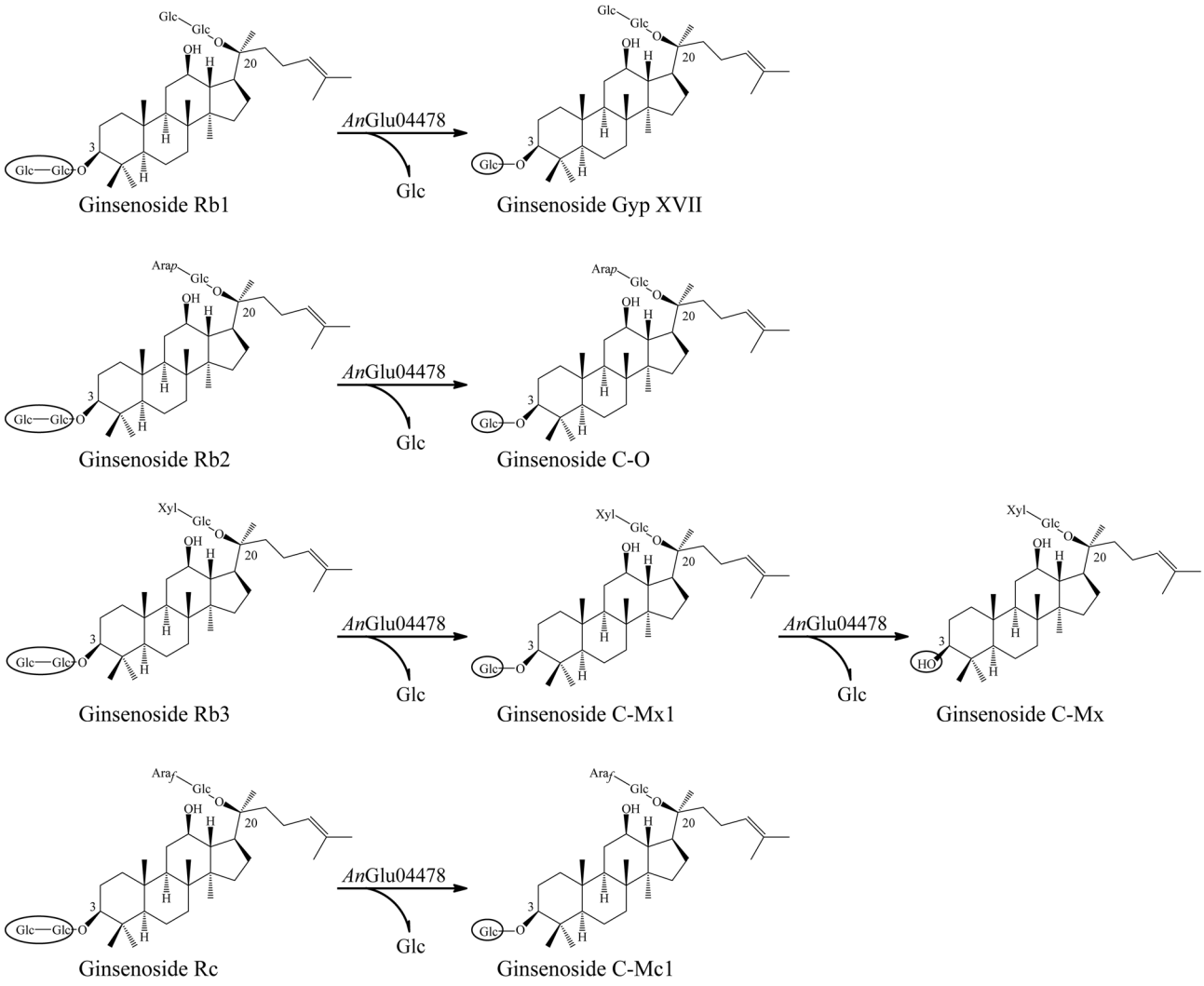

Fig. 5 Diagrammatic representation of the *An*Glu04478 to ginsenoside conversion pathway (Rb1 → GypXVII, Rb2 → C–O, Rb3 → C-Mx1 → C-Mx, Rc → C-Mc1)

a member of the glycoside hydrolase family 1. It exhibits optimal activity at pH 4.5 and a temperature of 40 °C. While Cu^{2+} exerts a notable inhibitory effect, most other metal ions have minimal impact on its activity. *An*Glu04478 demonstrates certain tolerance to ethanol, and feedback inhibition by conversion products, such as glucose, is negligible. Enzymatic assays reveal that *An*Glu04478 can catalyze the transformation of ginsenoside Rb1 into GypXVII, Rb2 into C–O, Rb3 into C-Mx1 and C-Mx, and Rc into C-Mc1. Although its catalytic efficiency is modest, *An*Glu04478 offers a viable option for the multifunctional enzymatic biotransformation of ginsenosides.

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Author Contributions MXJ and LZ: designed and performed the experiments. MXJ, LZ, SHX, ZR, XC, MJL and GSY: analyzed the data. MJL and GSY: conceived and supervised the project. MXJ and GSY: wrote the paper with contributions from all authors.

Declarations

Conflict of interest The authors have no financial conflicts of interest to declare.

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