

Transcriptome profiling, cloning, and characterization of AnGlu04478, a Ginsenoside Hydrolyzing β -glucosidase from *Aspergillus niger* NG1306

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Abstract

Minor ginsenosides exhibit superior pharmacological activity compared to major ginsenosides, yet their presence in plants is limited. Therefore, it is crucial to efficiently obtain minor ginsenosides. Specific glycoside hydrolases offer the advantage of converting major ginsenosides into specific minor counterparts under mild reaction conditions while minimizing structural damage. In this study, we utilized total ginsenosides extracted from *Panax notoginseng* leaves as substrates to stimulate the growth of *Aspergillus niger* NG1306. Transcriptome analysis revealed that *AnGlu04478* potentially participates in the biotransformation process of ginsenosides. Subsequently, it was cloned and expressed in *Transetta* (DE3). The *AnGlu04478* protein was purified by Ni^{2+} column and its enzymatic properties were characterized. The results show that the optimum pH was 4.5 and the optimum temperature was 40°C, Cu^{2+} had a certain inhibitory effect on *AnGlu04478*, while other metal ions had little effect on it. *AnGlu04478* had a certain tolerance to ethanol, and it was not significantly affected by product (glucose) feedback inhibition. Using *p*NPG as a substrate, the kinetic parameter K_m of *AnGlu04478* was 1.55 mmol/L, the $V_{\max}$ was 0.014 mmol/min. The test with ginsenosides as substrate showed that it could selectively hydrolyze glucose of ginsenoside Rb1, Rb2, Rb3 and Rc at C3, and the putative metabolic pathway was $\text{Rb1} \rightarrow \text{GypXVII}$, $\text{Rb2} \rightarrow \text{C-O}$, $\text{Rb3} \rightarrow \text{C-Mx1} \rightarrow \text{C-Mx}$, $\text{Rc} \rightarrow \text{C-Mc1}$. These findings indicate that *AnGlu04478* exhibits substrate promiscuity as a β -glucosidase, thereby expanding the options for ginsenosides biotransformation.

Introduction

Panax notoginseng (Burkill) and *Panax ginseng* (Meyer) both belonging to the *Panax* genus, family Araliaceae (Shim et al. 2021), they both contain the bioactive ingredient known as ginsenosides (Kim 2012). *Panax notoginseng*, a distinctive Chinese medicinal herb cultivated extensively in Wenshan, Yunnan province. Now, more than 70 ginsenosides have been isolated from *Panax notoginseng*, mainly including Rg1, R1, Re, Rb1, Rb2, Rb3, Rd, C-Mx1, Rc, Fa, and Fc (Fuzzati 2004). These compounds are primarily found in the roots, stems leaves, flowers and fruits of the plant. Based on the difference of the skeleton structure of sapogenins, ginsenosides can be divided into dammarane type (PPD, PPT), octilon type (OT), oleanolic acid type (OA), C17 side chain variant type and miscellaneous subtypes (Yang et al. 2014). According to the position and quantity of hydroxyl groups, dammarane type ginsenosides are primarily categorized into two types, namely protopanaxadiol (PPD) type and protopanaxatriol (PPT) type (Attele et al. 1999). Among these, five main ginsenosides Rb1, Rb2 and Rc belong to protopanaxadiol (PPD) type, while Re and Rg1 belong to protopanaxatriol (PPT) type (Sun et al. 2009). Although their aglycone skeleton structures remain consistent, there are differences in the sugar groups connected at C3, C6, and C20 positions (Yang et al. 2014). Different monomers of ginsenosides often exhibit different biological activities attributed to variations in glycosylation sites and quantities (Ru et al. 2015; Shi et al. 2019). Research has shown that minor ginsenosides possess superior pharmacological activity compared to major ginsenosides (Choi and Kim 2023; Huang et al. 2023; Li et al. 2023), which are typically obtained by eliminating glycosides from ginsenosides (Cao et al. 2020). In recent years, enzyme

conversion technology has emerged as a prominent research focus for obtaining minor ginsenosides due to its advantages of mild reaction conditions and minimal structural damage inflicted upon the ginsenoside molecules (Murugesan et al. 2022; Wang et al. 2023; Yang et al. 2023).

The enzymatic conversion of ginsenosides into minor ginsenosides through glycoside hydrolase is a widely employed method for their production. It has been widely reported that the directional catalysis of ginsenosides towards minor forms by specific glycoside hydrolases, such as β -glucosidase from *Thermotoga thermarum* DSM 5069T and *Burkholderia sp.* GE 17 – 7, which can convert ginsenoside Rb1 to Rg3 (Fu et al. 2017; Quan et al. 2012). β -glucosidase isolated from *Aspergillus versicolor* LFJ1403 can convert ginsenoside Rb1 to ginsenoside Rd, and the conversion rate could reach 96% (Lin et al. 2015). Furthermore, efficient production of minor ginsenosides can be achieved through the utilization of diverse glycoside hydrolases or their combined application. For example, by using different β -glucosidases or their combinations, the yield of rare ginsenoside Rh2 is further increased by 7%, and the highest yield reached 51.17mg/L (Cao et al. 2020). By employing a synergistic combination of α -L-arabinofuranosidase and β -galactosidase derived from *Caldicellulosiruptor saccharolyticus*, along with β -glucosidase sourced from *Sulfolobus acidocaldarius*, the complete conversion of major protopanaxadiol ginsenosides into compound K is achieved (Shin et al. 2013). Additionally, certain glycoside hydrolases catalyze the transformation of specific ginsenosides to generate various minor counterparts. For instance, *A. mellea* mycelium derived β -glycosidase effectively converts Rb2 into C-Y and C-K (Kim et al. 2018). β -glucosidase from *Terrabacter ginsenosidimutans* could selectively and sequentially converts ginsenoside Rb1 to the minor ginsenosides gypenoside XVII, gypenoside LXXV, ultimately leading to C-K (An et al. 2010). β -glucosidase from *Flavobacterium johnsoniae*, which has been overexpressed in *Escherichia coli* BL21(DE3), could hydrolyze the outer glucose moiety of ginsenoside Rb1 and gypenoside XVII at the C-20 position of aglycon, resulting in the formation of ginsenosides Rd and F2, respectively (Hong et al. 2012). However, there is a scarcity of literature reporting on β -glucosidase's ability to simultaneously convert PPD type ginsenosides Rb1, Rb2, Rb3 and Rc into minor ginsenosides.

The leaves of *Panax notoginseng* mainly contain ginsenosides Rg1, Re, Rb1, Rb3 and Rd. In this study, total ginsenosides from *Panax notoginseng* leaves were used as substrates to stimulate the growth of *Aspergillus niger* NG1306, and the up-regulated glycoside hydrolase gene was analyzed through transcriptome analysis. It was found that the β -glucosidase gene *Anglu04478* may be involved in the biotransformation of ginsenosides. Subsequently, *Anglu04478* was cloned and subjected to heterologous expression and enzymatic characterization. Notably, it exhibited selective hydrolysis activity towards glucose at position C3 in ginsenoside Rb1, Rb2, Rb3 and Rc. These findings provide a theoretical foundation for potential industrial applications of β -glucosidase *AnGlu04478*.

Results

Transcriptome sequencing

A total of Six samples were measured by using Huada's DNBSEQ platform (Three duplications in control and treatment) and 38.22GB data were generated. The average alignment rate of the sample to the genome is 89.91%, and the average alignment rate of the gene set is 77.48%. A total of 10,391 expressed genes were identified, comprising 10,346 known genes and predicting 45 new genes. A comprehensive analysis revealed a detection of 5,174 new transcripts, including 5,129 novel alternative splicing subtypes for known protein-coded genes and 45 new transcripts for new protein-coded genes.

Unigenes differential expression analysis

The volcanic map (Fig. 1) showed a total of 2,830 differentially expressed genes, with 1,452 genes exhibiting up-regulation and 1,378 genes displaying down-regulation. The differentially expressed genes were analyzed by GO annotation (Fig. 2), 1,769 genes were attributed to biological process, 1,851 genes to cellular component and 2,718 genes to molecular function. KEGG pathway analysis revealed that 379 genes attributed to Cellular Processes, 221 genes to Environmental Information Processing, 371 genes to Genetic Information Processing, 2,179 genes to Metabolism and 18 genes to Organic Systems (Fig. 3).

Identification of candidate genes involved in Ginsenosidehydrolyzing process by GO and KEGG analysis.

Among the differentially expressed genes, GO analysis focused on catalytic activity under molecular function. A total of 1,236 genes were enriched in this process, including 664 up-regulated genes and 572 down-regulated genes. 1,236 genes in this process were enriched by KEGG (Fig. 4), including 148 Cellular Processes genes, 85 Environmental Information Processing genes, 181 Genetic Information Processing and Human Disease genes, 1,464 Metabolism genes and 10 Organic Systems genes. Subsequently, the Carbohydrate metabolic process in which KEGG Pathway was enriched into the Metabolic process was focused. Totally 254 genes were found to be enriched in this process, with 86 genes associated with secondary metabolites. Further analysis through GO annotation revealed that among these 86 genes, 9 were implicated in glycoside hydrolase activity (Table 1). It was found that 7 genes were up-regulated and 2 genes were down-regulated. Among these 7 up-regulated genes, 4 genes had an absolute value of log2 Fold Change > 1, namely *Anglu01436*, *Anglu04358*, *Anglu04478* and *Anglu07314*, and all annotated as β -glucosidase in the Gene Ontology database.

Table 1
Glycoside hydrolase activity genes

Gene ID	log2(NTS_T/NTS_CK)	Family
<i>Anglu01436</i>	2.936163245	GH55
<i>Anglu01438</i>	0.688832007	Unknown
<i>Anglu02903</i>	0.880203453	Unknown
<i>Anglu04358</i>	1.825311194	GH17
<i>Anglu04478</i>	1.171940036	GH1
<i>Anglu06391</i>	-0.792758485	Unknown
<i>Anglu07314</i>	1.557747073	Unknown
<i>Anglu08771</i>	0.570207492	GH63
<i>Anglu10365</i>	-0.695254656	Unknown

Table 2
Effect of metal ions and reagents on the activity of *AnGlu04478*

Metal or reagents	Relative activity (%)		
	10 mmol/L	50 mmol/L	100 mmol/L
NaCl	150 ± 0.12	132 ± 0.28	151 ± 0.26
KCl	121 ± 0.21	120 ± 0.12	116 ± 0.30
MgCl ₂	106 ± 0.20	135 ± 0.8	124 ± 0.27
CuCl ₂	63 ± 0.11	89 ± 0.06	55 ± 0.08
CaCl ₂	102 ± 0.02	95 ± 0.13	118 ± 0.02
CoCl ₂	112 ± 0.36	105 ± 0.21	96 ± 0.03
NiCl ₂	98 ± 0.02	98 ± 0.17	106 ± 0.17
SDS	100 ± 0.07	63 ± 0.04	38 ± 0.15
EDTA-Na ₂	123 ± 0.18	106 ± 0.11	53 ± 0.06
These data were calculated for triplicate experiments and shown as mean ± SD			

Then the 4 genes were analyzed by blast, and the similarity between *Anglu01436* and exo-β-1,3-glucanase gene (XM_035499218.1) from *Aspergillus tubingensis* was 100%, which may have plant cell walls polysaccharide-degrading enzymes activity (Bauer et al. 2006). The similarity between *Anglu04358* and glycoside hydrolase gene (XM_035505577.1) derived from *Aspergillus tubingensis* was 100%, and it

may have β -1,3-endoglucanase activity (Choi et al. 2005). The *Anglu04478* gene shares a 99.79% similarity with the glycoside hydrolase gene (XM_035505698.1) from *Aspergillus tubingensis*, which hydrolyze the β -O-glycosidic bond at the anomeric carbon of a glucose moiety at the non-reducing end of a carbohydrate or glycoside molecule (Opassiri et al. 2006). The *Anglu07314* gene exhibits a 98.54% similarity to the β -glucosidase gene (KJ739789.1) from *Aspergillus niger* strain B2, suggesting its potential activity as a β -glucosidase capable of hydrolyze ONP- β -d-glucopyranoside (Yang et al. 1996). Based on amino acid sequence homology and structural similarity, β -glucosidase was classified into seven families: GH1 (glucoside hydrolase 1), GH2, GH3, GH5, GH9, GH30 and GH116. Among these families, the distribution of β -glucosidases was more prominent in the GH1 and GH3 families. Therefore, the *Anglu04478* gene from the GH1 family was cloned and characterized.

Cloning of full length β -glucosidase gene *Anglu04478*, expression and purification

The total RNA of *Aspergillus niger* was successfully extracted (Fig. 5a), and used as a template for reverse transcription to generate cDNA, which was subsequently employed for amplification of the gene *Anglu04478* (Fig. 5b). Then the recombinant expression strain *Transetta/pET29a-Anglu04478* was obtained for subsequent enzyme expression and verification.

Compared to the control, the recombinant strain *Transetta/pET29a-Anglu04478* showed obvious protein expression after 1 mM IPTG induction, and the size corresponded to the expected outcome (Fig. 6). The crude enzyme was purified by Ni^{2+} chromatography and the protein concentration detected by BCA was 12.52 mg/mL.

Effect of pH and temperature on the activity of *AnGlu04478*

The effects of pH and temperature on enzymatic activity were investigated. The results showed that optimum pH of *AnGlu04478* was 4.5. When $\text{pH} > 6.0$, the relative enzyme activity gradually decreased, indicated that the enzyme activity was higher under acidic conditions (Fig. 7a). Under the optimum pH condition, the optimum temperature of the enzyme was determined. The enzyme activity was higher at 35 ~ 45 °C, and the enzyme activity decreased obviously after exceeding 45 °C, the maximum activity for the recombinant *AnGlu04478* was recorded at 40 °C (Fig. 7b).

Effect of metal ions and chemical reagents on the activity of *AnGlu04478*

The effects of metals ions and chemical reagents on the activity of *AnGlu04478* were also investigated. The results showed that the addition of Cu^{2+} lead to a sharp decline in enzyme activity, which indicated that Cu^{2+} had a certain inhibitory effect on *AnGlu04478*. The high concentrations of Na^+ , K^+ and Mg^{2+} have an effect of promoting enzyme activity. Ca^{2+} , Co^{2+} and Ni^{2+} had little effect on enzyme activity with the increase of concentration. *AnGlu04478* was inhibited by disodium ethylenediamine tetraacetate (EDTA-Na_2) and sodium dodecyl sulfate (SDS) at high concentration.

Tolerance of *AnGlu04478* to ethanol and glucose

To assess the effect of ethanol and glucose on the activity of *AnGlu04478*, different concentrations of ethanol and glucose were assayed. When the ethanol concentration was greater than 10%, the enzyme activity drops below 50%, indicated that ethanol concentration had a great influence on the activity of *AnGlu04478* (Fig. 8a). The effect of glucose concentration on *AnGlu04478* was relatively small. When glucose concentration reached 1 mol/L, the residual enzyme activity of *AnGlu04478* was close to 50%. It indicated that *AnGlu04478* was not significantly affected by product (glucose) feedback inhibition (Fig. 8b).

Analysis of enzyme kinetic parameters using *p*NPG as substrate

The *AnGlu04478* was reacted under the optimum conditions with *p*NPG as substrate by double reciprocal method, kinetic parameters (K_m , mmol/L) and maximum reaction rate (V_{max} , mmol/min) of *AnGlu04478* for *p*NPG was 1.55 mmol/L and 0.014 mmol/min (Fig. 9).

Analysis of the Transformation Products of Ginsenoside Rb1, Rb2, Rb3 and Rc by *AnGlu04478*

Under optimal conditions, *AnGlu04478* was added to ginsenosides (Rb1, Rb2, Rb3 or Rc) solution, and then qualitatively detected by thin layer chromatography (TLC). The results showed that *AnGlu04478* can convert substrate Rb1 into GypXVII, Rb2 into C-O, Rb3 into C-Mx1 and C-Mx, Rc into C-Mc1 (Fig. 10).

In order to further confirm the products, the samples after enzyme conversion were detected by HPLC. The retention time of GypXVII in the transformation product of ginsenoside Rb1 was 29.13 min, C-O in the conversion product of Rb2 was 32.526 min, C-Mx1 and C-Mx in the conversion product of Rb3 was 43.047 min and 43.9 min, C-Mc1 in the transformation product of Rc was 30.97 min, all consistent with those of reference substance (Fig. 11).

Discussion

Minor ginsenosides have better pharmacological activities than major ginsenosides (Chu et al. 2023; Ji et al. 2023; Liu et al. 2022). Therefore, Minor ginsenosides are produced by hydrolyzing the glycosidic bonds of major ginsenosides. Compared with microbial transformation, enzymatic hydrolysis has the characteristics of good specificity, high efficiency and strong controllability. Glycosidases of different properties act on glycosidic bonds of different configurations and components, so as to achieve the purpose of directional hydrolysis of ginsenosides (Shin and Oh 2016).

At present, glycosidases used in the biotransformation of ginsenosides have been found in bacteria and fungi (An et al. 2022; Chang et al. 2012; Upadhyaya et al. 2016). Major ginsenosides can be converted into a variety of minor ginsenosides by using the crude enzyme extracted from *Aspergillus niger* after fermentation (Jeong et al. 2020; Li and Ji 2017; Xiao et al. 2019), suggested that *Aspergillus niger* was rich in glycosidases for the biotransformation of ginsenosides. However, the biotransformation of crude

enzymes cannot yield specific minor ginsenosides, but there is often a requirement for specific minor ginsenosides in activity research of natural drugs or industrial production, so specific glycosidases are needed for the biotransformation of ginsenosides.

In this study, *AnGlu04478* was cloned from *Aspergillus niger* NG1306 in order to obtain a more efficient β -glucosidase for specific transformation of ginsenosides. Based on the selective hydrolysis of glucoside groups by *AnGlu04478*, a conversion pathway was proposed (Fig. 12). Various diol ginsenosides can be converted into minor ginsenosides by single β -glucosidase. Different from other β -glucosidase, *AnGlu04478* can recognize different substrates and hydrolyze C3 glucose in ginsenosides. Therefore, multiple ginsenosides can be converted only by using this single enzyme.

β -glucosidase with the ability to simultaneously convert multiple ginsenosides has also been reported. β -glucosidase gene *bglAch* cloned from *Arthrobacter chlorophenolicus* and expressed in *E. coli* BL21 (DE3). BglAch could hydrolyzed the lateral glucose and arabinofuranose groups at C20 position of ginsenosides Rb1 and Rb2 to form Rd, and then hydrolyzed the lateral glucose groups at C3 position to form F2. In addition, BglAch can transform Rc into C-Mc1 and F2 (Park et al. 2014). β -glucosidase BgpA from *Terrabacter ginsenosidimutans* can transform Rb1 into GypXVII, GypLXXV and C-K, but cannot transform Rb2, Rb3 and Rc (An et al. 2010). β -glucosidase from *Sphingopyxis alaskensis* exhibits specific hydrolysis of the outer glucose at position C3 of ginsenosides, resulting in the conversion of ginsenoside Rb1, Rb2, Rc, Rd and Rg3 into ginsenoside GypXVII, C-O, C-Mc1, F2 and Rh2 respectively. Amino acid sequence alignment analysis with *AnGlu04478* reveals a similarity of only 38.76%, despite their membership in the GH1 family due to shared similar domains (Shin and Oh 2014). In contrast, a novel endoglucanase BcelFp from *Fervidobacterium pennivorans* DSM9078 can also hydrolyze the outer glucose at the C-3 position in PPD-type ginsenosides leading to the transformation of ginsenoside Rb1, Rb2, Rc and Rd into ginsenoside GypXVII, C-O, C-Mc1 and F2. However, BcelFp belongs to the GH5 family while *AnGlu04478* shares only 9.92% amino acid sequence similarity with BcelFp and lacks similar domains (Zheng et al. 2022).

AnGlu04478 and the glucosidase from *Aspergillus tubingensis* had 99.79% similarity in amino acid sequence, but the sequence was submitted without protein expression and functional verification. *AnGlu04478* and the glucosidase from *Aspergillus niger* strain have the highest similarity of 98.55% in amino acid sequence. Only the preliminary studies on cellulose degradation have been conducted without any protein purification or ginsenoside transformation being carried out. Therefore, the β -glucosidase derived from *Aspergillus niger*, which can catalyze the hydrolysis of glucose at C3 position of Rb1, Rb2, Rb3 and Rc at the same time has not been previously reported. In comparison to glycosidase with strong substrate recognition specificity, β -glucosidase *AnGlu04478* can recognize different ginsenoside substrates and could be better used in the biotransformation of ginsenosides in industry. In the future, homologous modeling, molecular docking and directed evolution can be used to improve the enzyme activity and the efficiency of biotransformation of ginsenosides, which will help to improve the application value of β -glucosidase *AnGlu04478* in industrial production.

Conclusion

In this study, a ginsenoside β -glucosidase *AnGlu04478* was successfully cloned and overexpressed in *Transetta* (DE3). The optimum pH was 4.5, optimum temperature was 40°C, Cu^{2+} had a certain inhibitory effect on *AnGlu04478*, most of the other metal ions had little effect on *AnGlu04478*. It had a certain tolerance to ethanol, feedback inhibition of conversion products (glucose) had little effect. The Michaelis constant (K_m) of *AnGlu04478* was 1.55mmol/L, the maximum reaction rate ($V_{\max}$) was 0.014 mmol/min when using *p*NPG as substrate. Enzyme activity tests showed that *AnGlu04478* could transform ginsenoside Rb1 into GypXVII, Rb2 into C-O, Rb3 into C-Mx1 and C-Mx, Rc into C-Mc1, which would provide a choice of more multi-functional enzymes for the biotransformation of ginsenosides.

Materials and methods

Materials

4-nitrophenyl β -D-glucopyranoside (*p*NPG) were obtained from Sigma-Aldrich Chimie S.a.r.l. (Lyon, France). Ginsenoside Rb1, Rb2, Rb3 and Rc were obtained from the 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 purchased from Transgen Biotech Co., Ltd. (Beijing, China). Protein Marker were purchased from Cofitt Life Sciences Co., Ltd. (Kunming, China). IPTG and Kanamycin were purchased from BioFroxx Biotech Co., Ltd. (Kunming, China).

Strains, Plasmid, and Culture Conditions

Aspergillus niger NG1306 was screened by our laboratory in the previous work. DH5 α for routine cloning, and *Transetta* (DE3) (Transgen Biotech, Beijing, China) for protein expression. pEASYT1 (Transgen Biotech, Beijing, China) was used as the T-cloning vector, and pET29a (Novagen, Madison) was used to express the His-tag fused recombinant *Anglu04478*. *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) medium or on LB agar plates supplemented with appropriate antibiotics(kanamycin, 50 $\mu\text{g/mL}$) when necessary. *Aspergillus niger* NG1306 were grown at 28°C on Potato Dextrose Agar (PDA) medium.

Transcriptome sequencing and Unigenes differential expression analysis

Transcriptome sequencing was completed by Wuhan Huada Medical Research Institute Co., Ltd. The transcriptome data of *Aspergillus niger* NG1306 was referenced by its own genome and annotated by Rfam, Pfam, Reactome, COG, EggNOG and InterPro databases. The quantity of differential expression Unigenes were analyzed by Dr.Tom interactive multi-omics data mining system of BGI.

GO Classification and KEGG Pathway Classification

Gene Ontology was divided into three functional categories: molecular function, cellular component and biological process. According to the results of differential gene detection, functional classification analysis were carried out.

The KEGG metabolic pathway involved in transcriptional genes was divided 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 classified and statistically analyzed.

Identification of candidate genes involved in Ginsenosidehydrolyzing process

According to GO annotations and classification results, differential expression Unigenes were classified functionally, focusing on catalytic activity under molecular function. The phyper function in R software was used for enrichment analysis, P value was calculated, and then FDR correction is carried out on P value. Usually, functions with Q value ≤ 0.05 are regarded as significant enrichment. Further, KEGG enrichment analysis was carried out on the genes in catalytic activity, focusing on Carbohydrate metabolism under Metabolism, and looking for the key genes related to hydrolase activity in the secondary metabolic process.

Ginsenosides from *Panax notoginseng* leaves induced *Aspergillus niger* Fermentation and RNA extraction

0.2% ginsenosides from *Panax notoginseng* leaves was added to fermentation medium (NaNO_3 0.3 g, K_2HPO_4 0.1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 g, KCl 0.05 g, FeSO_4 0.001 g, sucrose 2 g, ddH₂O 1000 mL), and *Aspergillus niger* was induced to ferment until the fifth day. The cells were collected and total RNA was extracted using a Geneode Fungus RNA Kit, following exactly the protocol recommended by the manufacturer (Geneode bio-tek, Wuhan, China). The RNA was quantified and its purity determined using a UV-Vis spectrophotometer (BioDropuLITE, Holliston, USA) and its integrity was assessed on a 1.2% agarose gel.

Cloning, expression and purification of β -glucosidase Anglu04478

cDNA was obtained by reverse transcription PCR through PrimeScript RT kit with gDNA Eraser (Takara, DaLian). Gene *Anglu04478* was PCR amplified from cDNA with primers *Anglu04478*-F: 5

ATGGGTTCAGCAACAACCTCAAC 3 and *Anglu04478*-R: 5 TAATGTCTTCTCGATATACTGGCTGAA 3 . The PCR program was used as follows: 94°C for 2 min, followed by 31 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min, and a 10 min final extension at 72°C. The amplicons were cloned into pEASY-T1 and sequenced by Sangon Biotech (shanghai, China). The *Kpn* I and *Hind* III restriction enzyme sites were designed into the forward and reverse primers (underlined) *Anglu04478* (*Kpn* I)-F: 5

GGGGTACCATGGGTTCAGCAACAACCTCAAC 3 and *Anglu04478* (*Hind* III)-R: 5

CCCAAGCTTTAATGTCTTCTCGATATACTGGCTGAA 3 . The amplicons were digested with *Kpn* I and *Hind*

III, purified with a TIANGel Purification Kit (TIANGEN Bio-Tek, Beijing, China), and ligated into pET29a expression vector, which was treated in the same protocol.

The expression constructs were transformed via heat shock into *Transetta* (DE3) expression cells (Transgen Biotech, Beijing, China). The transformants were selected on solid Lauria–Bertani (LB) medium containing 50 µg/ml of kanamycin. Through sequencing verified recombinant strain was incubated overnight in 10 mL LB medium supplemented with 50 µg/mL kanamycin, at 37°C shaking at 180 rpm, and then was used for the 1% (v/v) inoculation of 50 mL LB medium for expression. When the OD₆₀₀ reached around 0.6, isopropyl-b-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, and the culturing was continued under the same conditions for additional 7 hours. Cells were recovered by centrifugation at 5,000 rpm for 10 min at 4°C and resuspended in 10 mL PBS buffer (pH 7.4). Ultrasonication was used to disrupt cells, debris was removed by centrifugation at 12,000 rpm for 20 min at 4°C, the supernatants were filtered with a 0.22 µm Millipore column. The expression quantity and molecular weight were assessed by 15% SDS–PAGE with Coomassie Blue staining.

For purification, the *AnGlu04478* protein was purified by using a Ni²⁺-affinity column (Transgen Biotech, Beijing, China) according to the manufacturer's instruction. The purified *AnGlu04478* protein was loaded into a PD-10 desalting column (GE Healthcare) for desalting, and was then concentrated by using a centrifugal filter device. Concentrated *AnGlu04478* was dissolved in PBS buffer with 10% glycerol (pH 7.4) for further analysis. Protein concentration was determined by using a BCA protein concentration assay kit (Takara, Dalian, China)

Characterization of the Recombinant *AnGlu04478*

The *AnGlu04478* activity as determined with *p*NPG as the substrate. One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per minute under standard assay conditions. Enzyme activity reaction system: 1 µL of recombinant purified enzyme solution, 50 µL of *p*NPG, PBS buffer was added to make the final volume of 100 µL (final concentration of *p*NPG was 5 mmol/L). At 30 °C for 10 min, the reaction was terminated by adding 100 µL 0.5 mmol/L Na₂CO₃ solution. The absorbance was measured at 405 nm.

The effect of temperature on enzyme activity was investigated in PBS buffer with a temperature range of 25–80 °C and a gradient of 5 °C. In order to investigate the effect of pH on enzyme activity, pH 2.0–10.0 was chose, incubate at 30 °C for 10 min.

The effect of different metal ions (Na⁺, K⁺, Ca²⁺, Co²⁺, Ni²⁺, Mg²⁺ and Cu²⁺) and chemical reagents (SDS and EDTA-Na₂) on the activity of *AnGlu04478* was determined. The effect of glucose and ethanol concentration on the activity of *AnGlu04478* was investigated.

Enzyme Kinetics

By measuring the initial rates of *p*NPG at different 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 *AnGlu04478* for *p*NPG were calculated by double reciprocal mapping (Raza et al. 2020).

Enzyme assays and product analysis

0.2% ginsenosides Rb1, Rb2, Rb3 and Rc, 0.5 U *AnGlu04478* and PBS buffer (50 mmol/L, pH 4.5) were added into 100 μ L reaction system. After 60 min reaction at 40 °C, 100 μ L n-butanol was added to extract the conversion product, vortex for 2 min, 9000 r/min, centrifuged for 10 min, and the n-butanol phase was taken and detected by TLC. The developing solvent was CHCl_3 – CH_3OH (85:15, v/v), the spots on the TLC plates were detected by spraying with CH_3OH – $\text{C}_4\text{H}_8\text{O}_2$ – H_2SO_4 (85:10:5, v/v/v, low phase) followed by blower heating.

The remaining n-butanol phase was evaporated in water bath, redissolved with chromatographic pure methanol, filtered with 0.45 μ m organic membrane, and further analyzed by HPLC. Chromatographic conditions: Column C18, 4.6 $\times$ 250 mm; flow rate: 1 mL/min; column temperature: 30 °C; Wavelength: 203 nm; the mobile phase and elution condition were as following: acetonitrile(A) and distilled water (B) at A/B of ratios 31:69 to 65:35 and run times of 0–53 min, A/B ratios of 65:35 to 100:0 and run times of 54–69 min, and A/B ratios of 31:69 and an ending run time of 10 min.

Declarations

Acknowledgement

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Author Contributions

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

Conflicts of interest The authors declare that they have no competing interests. All authors declare that they have no conflict of interest.

References

1. An C, Ma S, Shi X, Liu C, Ding H, Xue W (2022) Diversity and Ginsenoside Biotransformation Potential of Cultivable Endophytic Fungi Associated With *Panax bipinnatifidus* var. *bipinnatifidus* in Qinling Mountains, China. *Frontiers in pharmacology* 13:762862. <http://doi.org/10.3389/fphar.2022.762862>

2. An DS, Cui CH, Lee HG, Wang L, Kim SC, Lee ST, Jin F, Yu H, Chin YW, Lee HK, Im WT, Kim SG (2010) Identification and characterization of a novel *Terrabacter ginsenosidimutans* sp. nov. beta-glucosidase that transforms ginsenoside Rb1 into the rare gypenosides XVII and LXXV. *Applied and environmental microbiology* 76(17):5827-5836. <http://doi.org/10.1128/AEM.00106-10>
3. Attele AS, Wu JA, Yuan CS (1999) Ginseng pharmacology: multiple constituents and multiple actions. *Biochemical pharmacology* 58(11):1685-1693. [http://doi.org/10.1016/s0006-2952\(99\)00212-9](http://doi.org/10.1016/s0006-2952(99)00212-9)
4. Bauer S, Vasu P, Persson S, Mort AJ, Somerville CR (2006) Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. *Proceedings of the National Academy of Sciences of the United States of America* 103(30):11417-11422. <http://doi.org/10.1073/pnas.0604632103>
5. Cao L, Wu H, Zhang H, Zhao Q, Yin X, Zheng D, Li C, Kim MJ, Kim P, Xue Z, Wang Y, Li Y (2020) Highly efficient production of diverse rare ginsenosides using combinatorial biotechnology. *Biotechnology and bioengineering* 117(6):1615-1627. <http://doi.org/10.1002/bit.27325>
6. Chang KH, Jo MN, Kim KT, Paik HD (2012) Purification and characterization of a ginsenoside Rb(1)-hydrolyzing beta-glucosidase from *Aspergillus niger* KCCM 11239. *International journal of molecular sciences* 13(9):12140-12152. <http://doi.org/10.3390/ijms130912140>
7. Choi CJ, Ju HJ, Park BH, Qin R, Jahng KY, Han DM, Chae KS (2005) Isolation and characterization of the *Aspergillus nidulans* egIC gene encoding a putative beta-1,3-endoglucanase. *Fungal genetics and biology : FG & B* 42(7):590-600. <http://doi.org/10.1016/j.fgb.2005.02.002>
8. Choi S, Kim T (2023) Compound K - An immunomodulator of macrophages in inflammation. *Life sciences* 323:121700. <http://doi.org/10.1016/j.lfs.2023.121700>
9. Chu LL, Hanh NTY, Quyen ML, Nguyen QH, Lien TTP, Do KV (2023) Compound K Production: Achievements and Perspectives. *Life* 13(7):1565. <http://doi.org/10.3390/life13071565>
10. Fu Y, Yin ZH, Yin CY (2017) Biotransformation of ginsenoside Rb1 to ginsenoside Rg3 by endophytic bacterium *Burkholderia* sp. GE 17-7 isolated from *Panax ginseng*. *Journal of applied microbiology* 122(6):1579-1585. <http://doi.org/10.1111/jam.13435>
11. Fuzzati N (2004) Analysis methods of ginsenosides. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences* 812(1-2):119-133. <http://doi.org/10.1016/j.jchromb.2004.07.039>
12. Hong H, Cui CH, Kim JK, Jin FX, Kim SC, Im WT (2012) Enzymatic Biotransformation of Ginsenoside Rb1 and Gypenoside XVII into Ginsenosides Rd and F2 by Recombinant beta-glucosidase from *Flavobacterium johnsoniae*. *Journal of ginseng research* 36(4):418-424. <http://doi.org/10.5142/jgr.2012.36.4.418>
13. Huang Q, Li J, Chen J, Zhang Z, Xu P, Qi H, Chen Z, Liu J, Lu J, Shi M, Zhang Y, Ma Y, Zhao D, Li X (2023) Ginsenoside compound K protects against cerebral ischemia/reperfusion injury via Mfn1/Mfn2-mediated mitochondrial dynamics and bioenergy. *Journal of ginseng research* 47(3):408-419. <http://doi.org/10.1016/j.jgr.2022.10.004>

14. Jeong EB, Kim SA, Shin KC, Oh DK (2020) Biotransformation of Protopanaxadiol-Type Ginsenosides in Korean Ginseng Extract into Food-Available Compound K by an Extracellular Enzyme from *Aspergillus niger*. *Journal of microbiology and biotechnology* 30(10):1560-1567. <http://doi.org/10.4014/jmb.2007.07003>
15. Ji YJ, Kim HD, Lee ES, Jang GY, Seong HA (2023) Heat Treatment Enhances the Neuroprotective Effects of Crude Ginseng Saponin by Increasing Minor Ginsenosides. *International journal of molecular sciences* 24(8):7223. <http://doi.org/10.3390/ijms24087223>
16. Kim DH (2012) Chemical Diversity of *Panax ginseng*, *Panax quinquefolium*, and *Panax notoginseng*. *Journal of ginseng research* 36(1):1-15. <http://doi.org/10.5142/jgr.2012.36.1.1>
17. Kim MJ, Upadhyaya J, Yoon MS, Ryu NS, Song YE, Park HW, Kim YH, Kim MK (2018) Highly regioselective biotransformation of ginsenoside Rb₂ into compound Y and compound K by beta-glycosidase purified from *Armillaria mellea* mycelia. *Journal of ginseng research* 42(4):504-511. <http://doi.org/10.1016/j.jgr.2017.07.001>
18. Li N, Pang Q, Zhang Y, Lin J, Li H, Li Z, Liu Y, Fang X, An Y, Bai H, Li D, Cao Z, Liu J, Yang Q, Hu S (2023) Ginsenoside compound K reduces neuronal damage and improves neuronal synaptic dysfunction by targeting Aβ. *Frontiers in pharmacology* 14:1103012. <http://doi.org/10.3389/fphar.2023.1103012>
19. Li Z, Ji GE (2017) Ginseng fermented by mycotoxin non-producing *Aspergillus niger*: ginsenoside analysis and anti-proliferative effects. *Food science and biotechnology* 26(4):987-991. <http://doi.org/10.1007/s10068-017-0117-z>
20. Lin F, Guo X, Lu W (2015) Efficient biotransformation of ginsenoside Rb₁ to Rd by isolated *Aspergillus versicolor*, excreting beta-glucosidase in the spore production phase of solid culture. *Antonie van Leeuwenhoek* 108(5):1117-1127. <http://doi.org/10.1007/s10482-015-0565-5>
21. Liu J, Wang Y, Yu Z, Lv G, Huang X, Lin H, Ma C, Lin Z, Qu P (2022) Functional Mechanism of Ginsenoside Compound K on Tumor Growth and Metastasis. *Integrative cancer therapies* 21:1-13. <http://doi.org/10.1177/15347354221101203>
22. Murugesan M, Mathiyalagan R, Boopathi V, Kong BM, Choi SK, Lee CS, Yang DC, Kang SC, Thambi T (2022) Production of Minor Ginsenoside CK from Major Ginsenosides by Biotransformation and Its Advances in Targeted Delivery to Tumor Tissues Using Nanoformulations. *Nanomaterials* 12(19):3427. <http://doi.org/10.3390/nano12193427>
23. Opassiri R, Pomthong B, Onkoksoong T, Akiyama T, Esen A, Ketudat Cairns JR (2006) Analysis of rice glycosyl hydrolase family 1 and expression of Os4bglu12 beta-glucosidase. *BMC plant biology* 12(29):6-33. <http://doi.org/10.1186/1471-2229-6-33>
24. Park MK, Cui CH, Park SC, Park SK, Kim JK, Jung MS, Jung SC, Kim SC, Im WT (2014) Characterization of recombinant beta-glucosidase from *Arthrobacter chlorophenolicus* and biotransformation of ginsenosides Rb₁, Rb₂, Rc, and Rd. *Journal of microbiology* 52(5):399-406. <http://doi.org/10.1007/s12275-014-3601-7>

25. Quan LH, Min JW, Yang DU, Kim YJ, Yang DC (2012) Enzymatic biotransformation of ginsenoside Rb1 to 20(S)-Rg3 by recombinant beta-glucosidase from *Microbacterium esteraromaticum*. *Applied microbiology and biotechnology* 94(2):377-384. <http://doi.org/10.1007/s00253-011-3861-7>
26. Raza A, Pothula R, Abdelgaffar H, Bashir S, Jurat-Fuentes JL (2020) Identification and functional characterization of a beta-glucosidase from *Bacillus tequelensis* BD69 expressed in bacterial and yeast heterologous systems. *PeerJ* 8:e8792. <http://doi.org/10.7717/peerj.8792>
27. Ru W, Wang D, Xu Y, He X, Sun YE, Qian L, Zhou X, Qin Y (2015) Chemical constituents and bioactivities of *Panax ginseng* (C. A. Mey.). *Drug discoveries & therapeutics* 9(1):23-32. <http://doi.org/10.5582/ddt.2015.01004>
28. Shi ZY, Zeng JZ, Wong AST (2019) Chemical Structures and Pharmacological Profiles of Ginseng Saponins. *Molecules* 24(13):2443. <http://doi.org/10.3390/molecules24132443>
29. Shim H, Waminal NE, Kim HH, Yang TJ (2021) Dynamic evolution of *Panax* species. *Genes & genomics* 43(3):209-215. <http://doi.org/10.1007/s13258-021-01047-6>
30. Shin KC, Oh DK (2014) Characterization of a novel recombinant beta-glucosidase from *Sphingopyxis alaskensis* that specifically hydrolyzes the outer glucose at the C-3 position in protopanaxadiol-type ginsenosides. *Journal of biotechnology* 172:30-37. <http://doi.org/10.1016/j.jbiotec.2013.11.026>
31. Shin KC, Oh DK (2016) Classification of glycosidases that hydrolyze the specific positions and types of sugar moieties in ginsenosides. *Critical reviews in biotechnology* 36(6):1036-1049. <http://doi.org/10.3109/07388551.2015.1083942>
32. Shin KC, Oh HJ, Kim BJ, Oh DK (2013) Complete conversion of major protopanaxadiol ginsenosides to compound K by the combined use of alpha-L-arabinofuranosidase and beta-galactosidase from *Caldicellulosiruptor saccharolyticus* and beta-glucosidase from *Sulfolobus acidocaldarius*. *Journal of biotechnology* 167(1):33-40. <http://doi.org/10.1016/j.jbiotec.2013.06.003>
33. Sun BS, Gu LJ, Fang ZM, Wang CY, Wang Z, Lee MR, Li Z, Li JJ, Sung CK (2009) Simultaneous quantification of 19 ginsenosides in black ginseng developed from *Panax ginseng* by HPLC-ELSD. *Journal of pharmaceutical and biomedical analysis* 50(1):15-22. <http://doi.org/10.1016/j.jpba.2009.03.025>
34. Upadhyaya J, Yoon MS, Kim MJ, Ryu NS, Song YE, Kim YH, Kim MK (2016) Purification and characterization of a novel ginsenoside Rc-hydrolyzing beta-glucosidase from *Armillaria mellea* mycelia. *AMB Express* 6(1):112. <http://doi.org/10.1186/s13568-016-0277-x>
35. Wang P, Tang C, Liu Y, Yang J, Fan D (2023) Biotransformation of High Concentrations of Ginsenoside Substrate into Compound K by beta-glycosidase from *Sulfolobus solfataricus*. *Genes* 14(4):897. <http://doi.org/10.3390/genes14040897>
36. Xiao Y, Liu C, Im WT, Chen S, Zuo K, Yu H, Song J, Xu L, Yi TH, Jin F (2019) Dynamic changes of multi-notoginseng stem-leaf ginsenosides in reaction with ginsenosidase type-I. *Journal of ginseng research* 43(2):186-195. <http://doi.org/10.1016/j.jgr.2017.10.001>
37. Yang M, Luoh SM, Goddard A, Reilly D, Henzel W, Bass S (1996) The bglX gene located at 47.8 min on the *Escherichia coli* chromosome encodes a periplasmic beta-glucosidase. *Microbiology* 142

(7):1659-1665. <http://doi.org/10.1099/13500872-142-7-1659>

38. Yang W, Zhou J, Gu Q, Harindintwali JD, Yu X, Liu X (2023) Combinatorial Enzymatic Catalysis for Bioproduction of Ginsenoside Compound K. *Journal of agricultural and food chemistry* 71(7):3385-3397. <http://doi.org/10.1021/acs.jafc.2c08773>
39. Yang WZ, Hu Y, Wu WY, Ye M, Guo DA (2014) Saponins in the genus *Panax* L. (Araliaceae): a systematic review of their chemical diversity. *Phytochemistry* 106:7-24. <http://doi.org/10.1016/j.phytochem.2014.07.012>
40. Zheng F, Zhao H, Wang N, Zhong P, Zhou K, Yu S (2022) Cloning and characterization of thermophilic endoglucanase and its application in the transformation of ginsenosides. *AMB Express* 12(1):136-149. <http://doi.org/10.1186/s13568-022-01473-z>

Figures

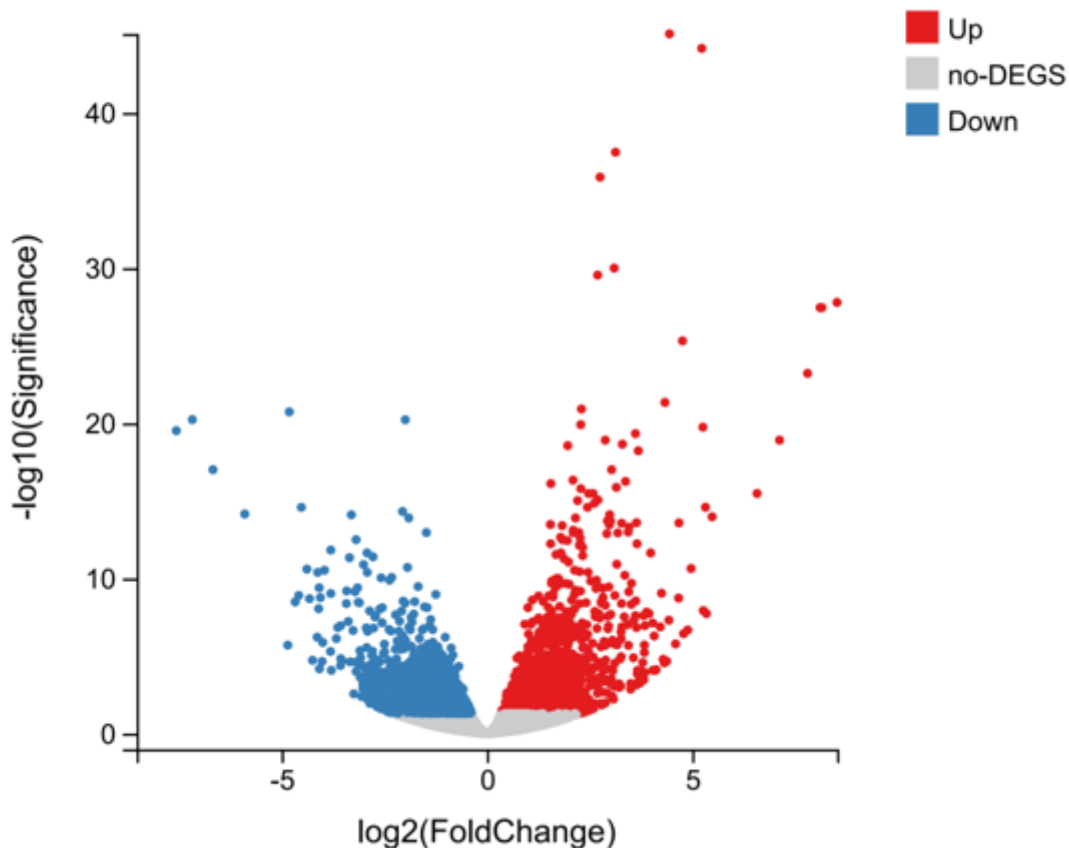


Figure 1

Volcanic map of differentially expressed genes

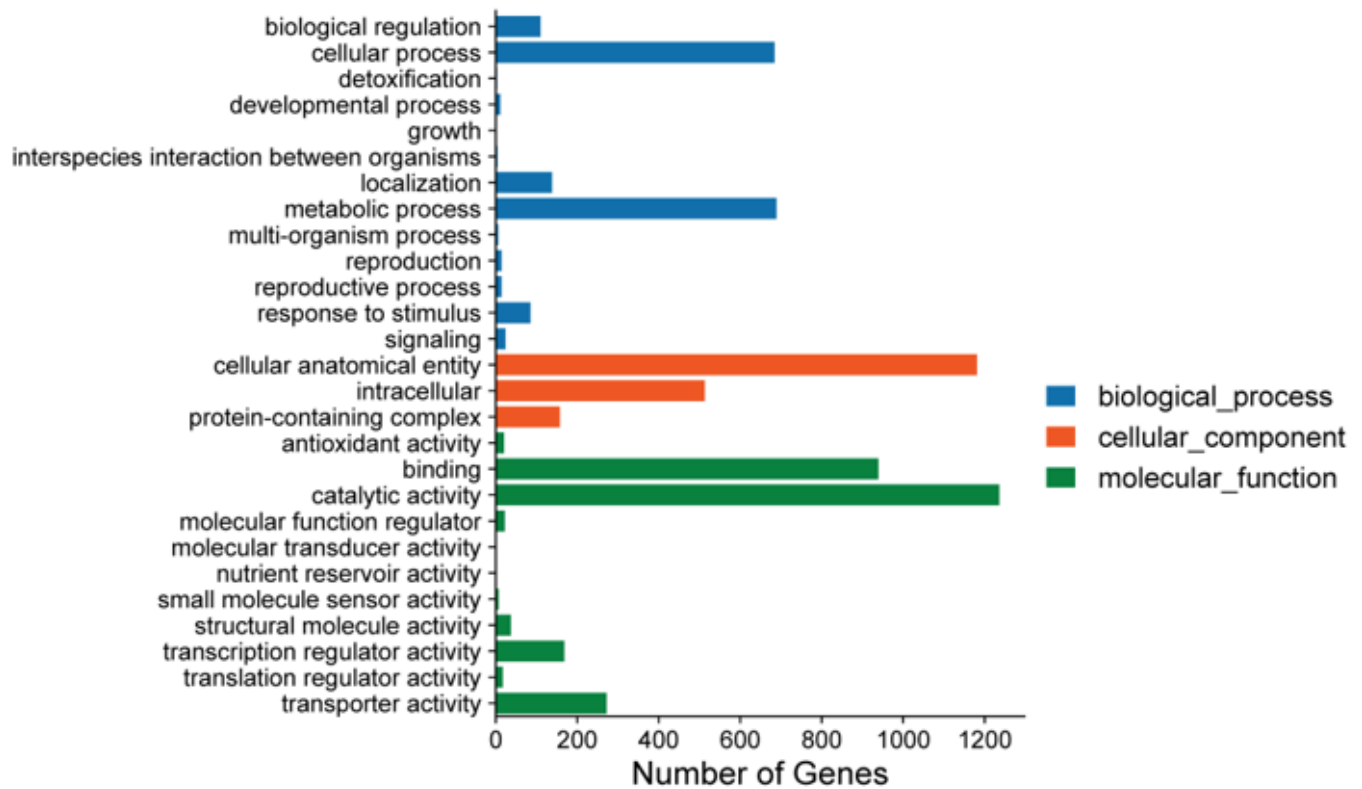


Figure 2

GO analysis of differentially expressed genes

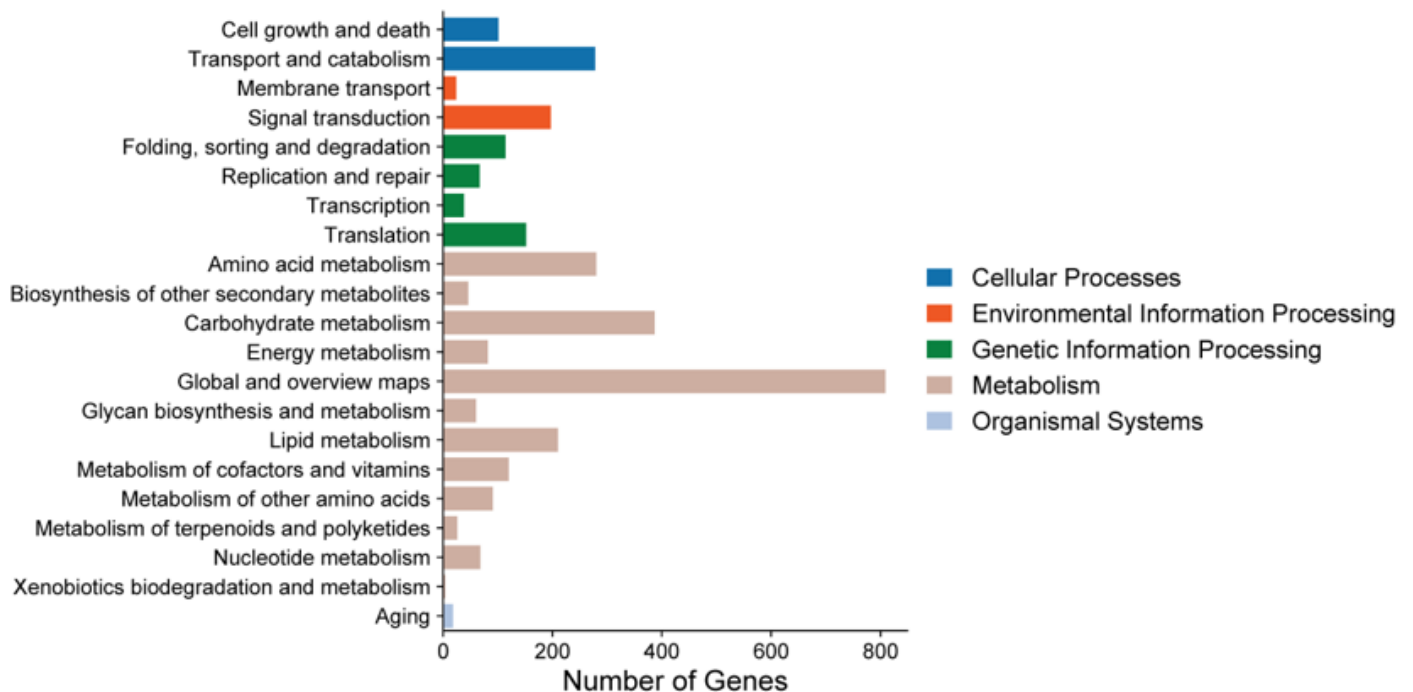


Figure 3

KEGG Pathway classification of differentially expressed genes

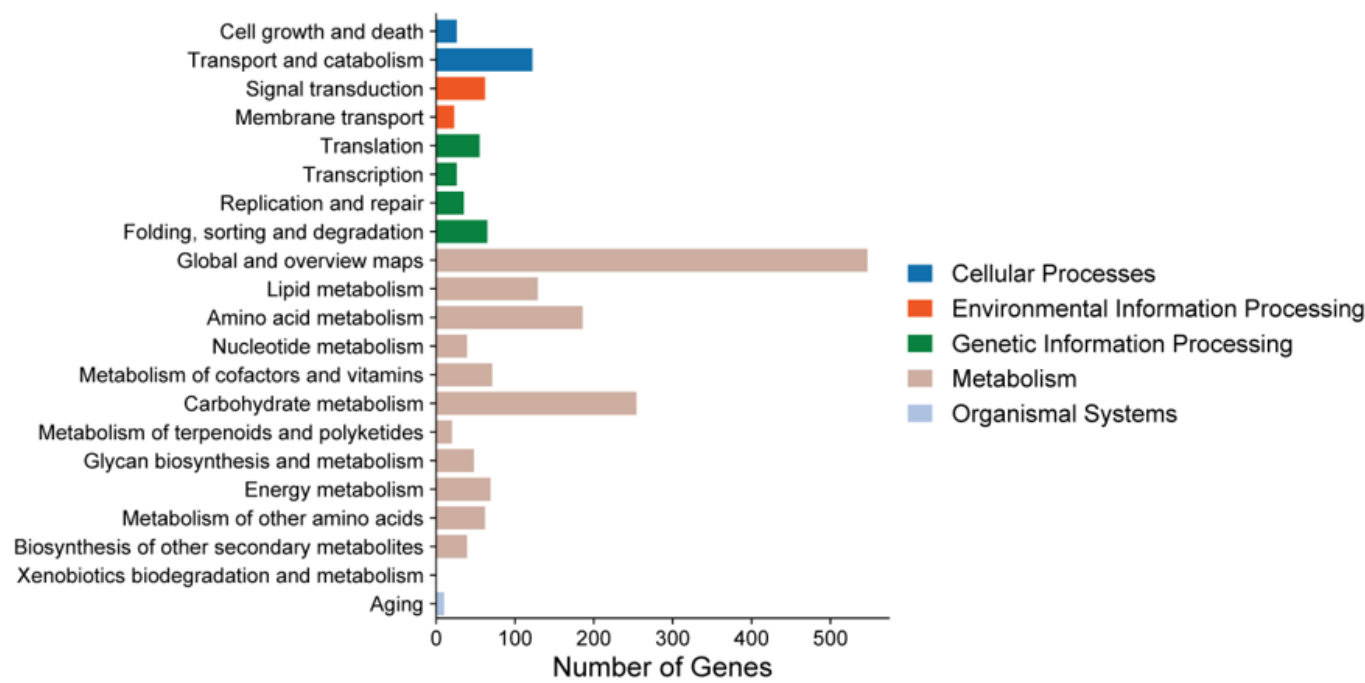


Figure 4

KEGG Pathway classification

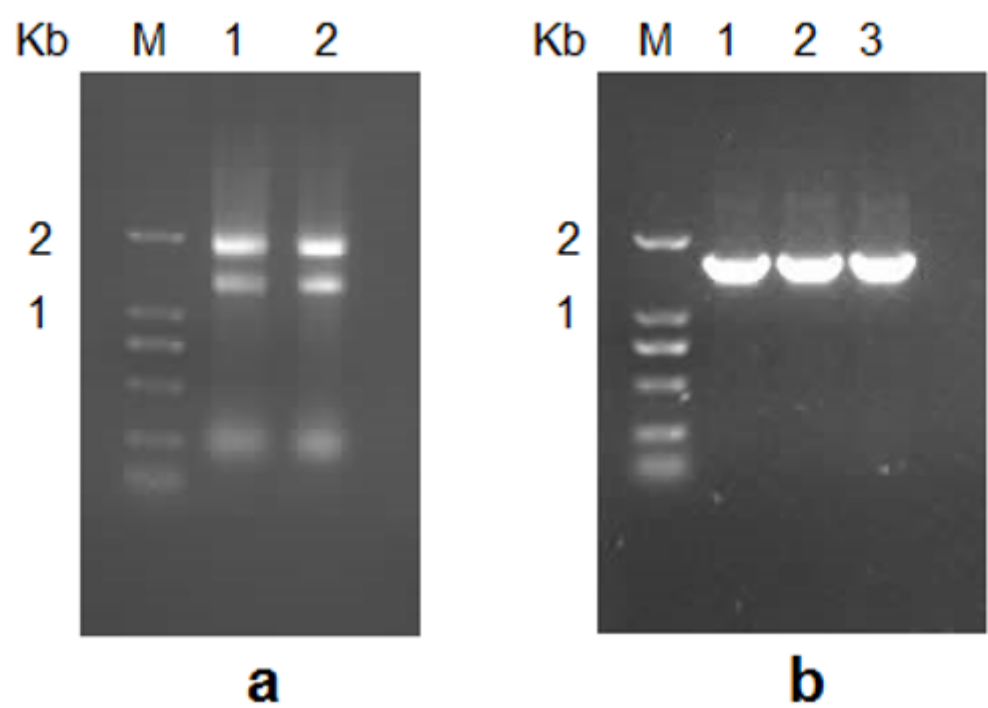


Figure 5

Cloning of full length β -glucosidase gene *Anglu04478*. a. Total RNA of *Aspergillus niger* NG1306 (M Marker, lanes 1-2: Total RNA samples). b. *Anglu04478* RT-PCR amplification products (Lanes 1-3 with size 1452 bp were *Anglu04478* PCR products)

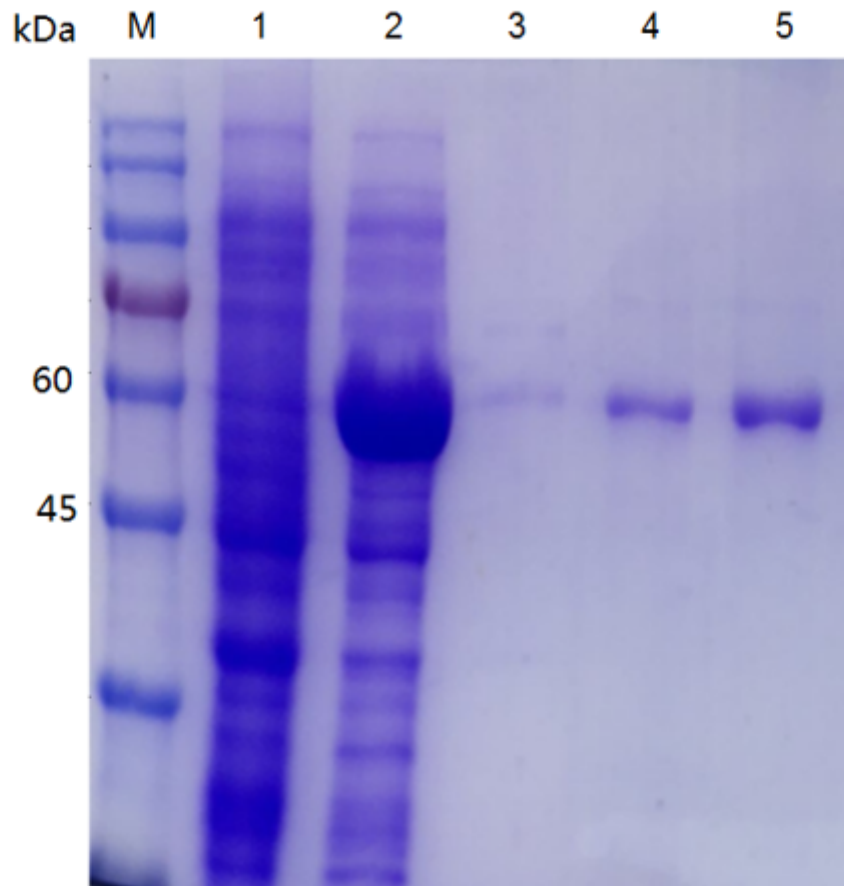


Figure 6

Purification of *AnGlu04478* protein. (1: control; 2: crude enzyme solution; 3-5: Purified *AnGlu04478*)

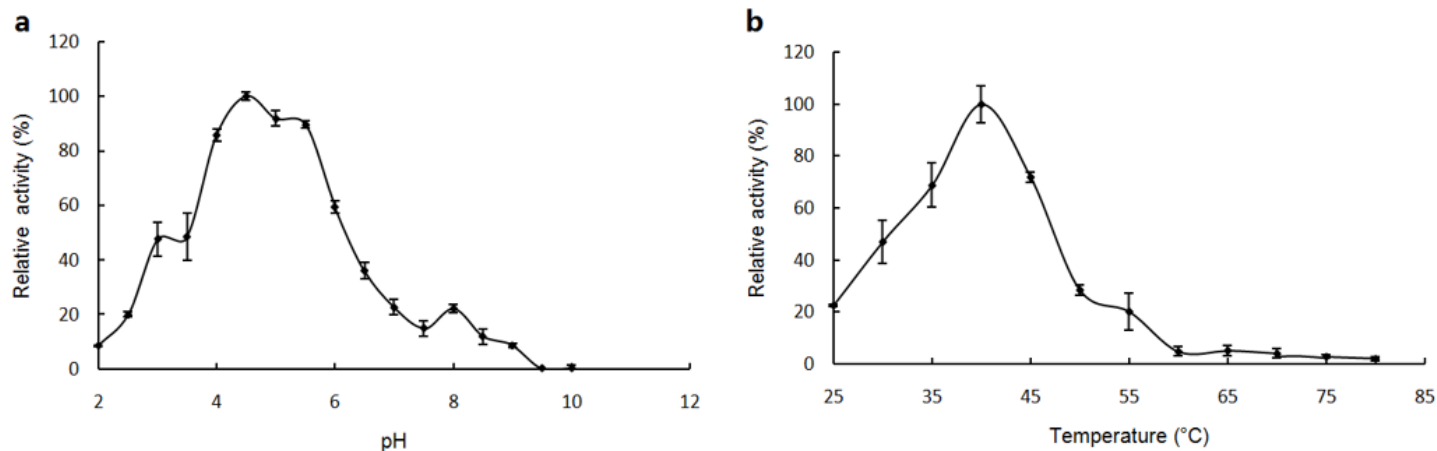


Figure 7

Effects of pH(a) and temperature(b) on the activity of recombinant *AnGlu04478* determined using *pNPG* as a substrate

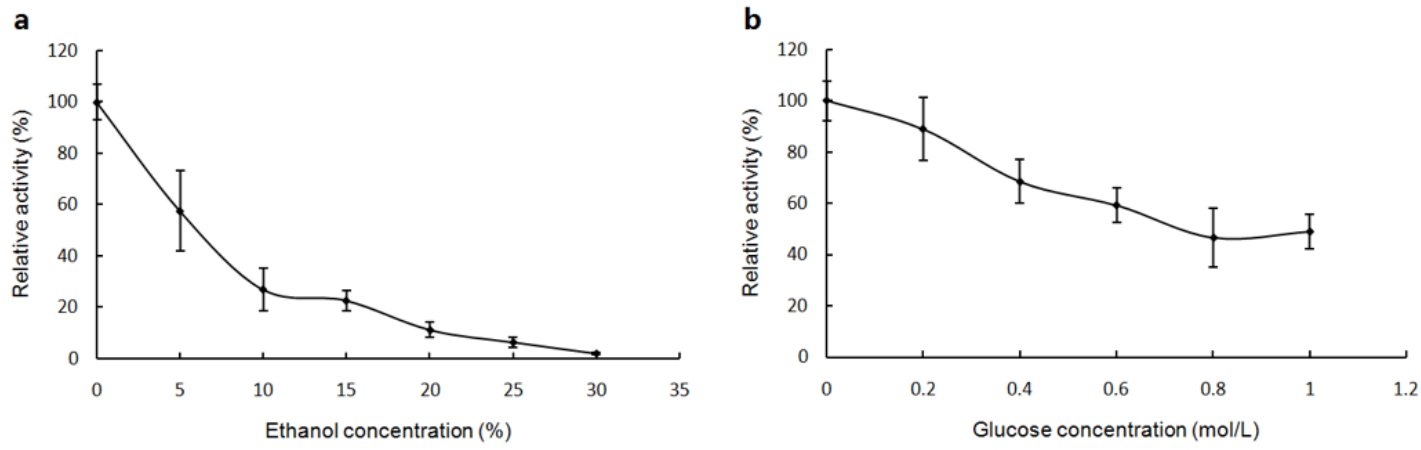


Figure 8

AnGlu04478 tolerance to ethanol(a) and glucose(b)

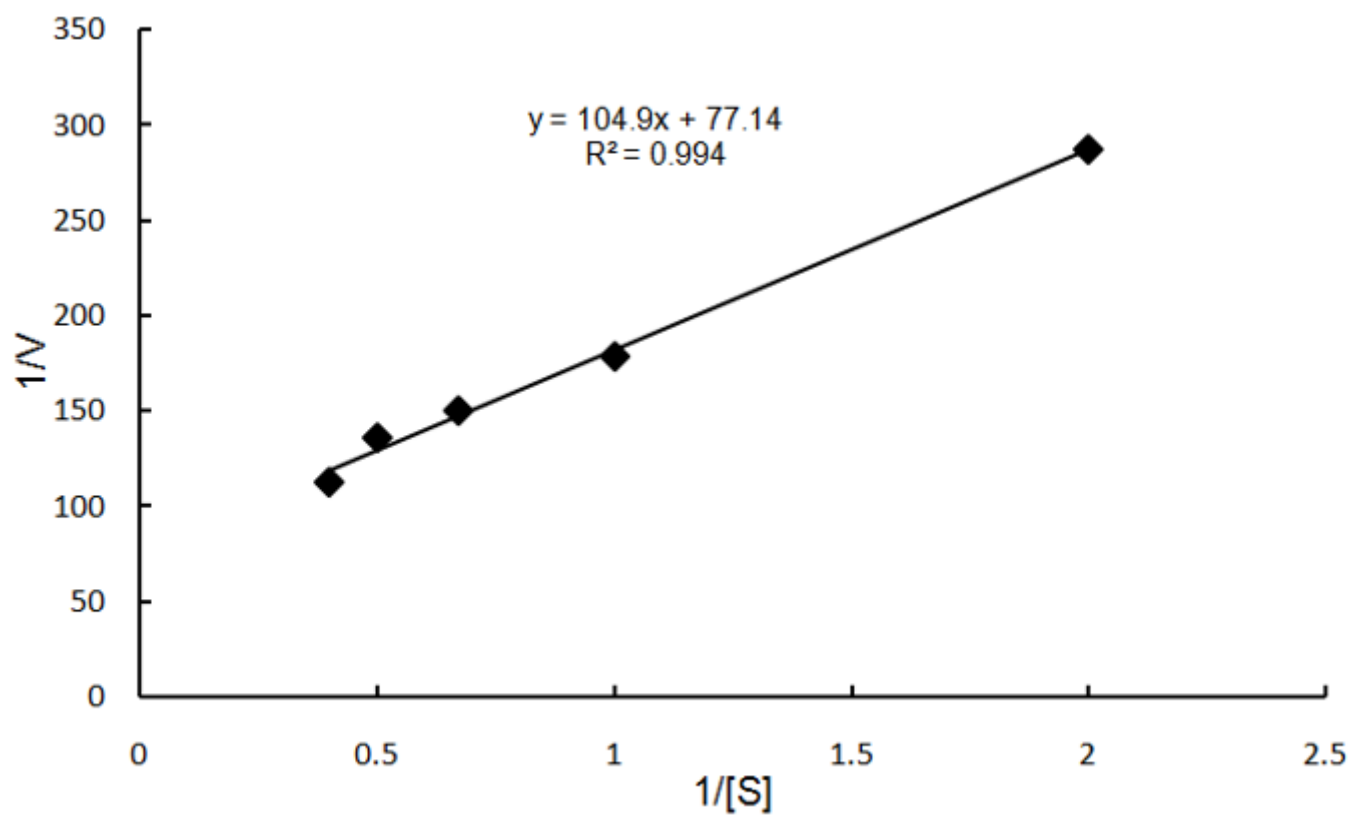


Figure 9

Lineweaver-Burk curve with *p*NPG as substrate

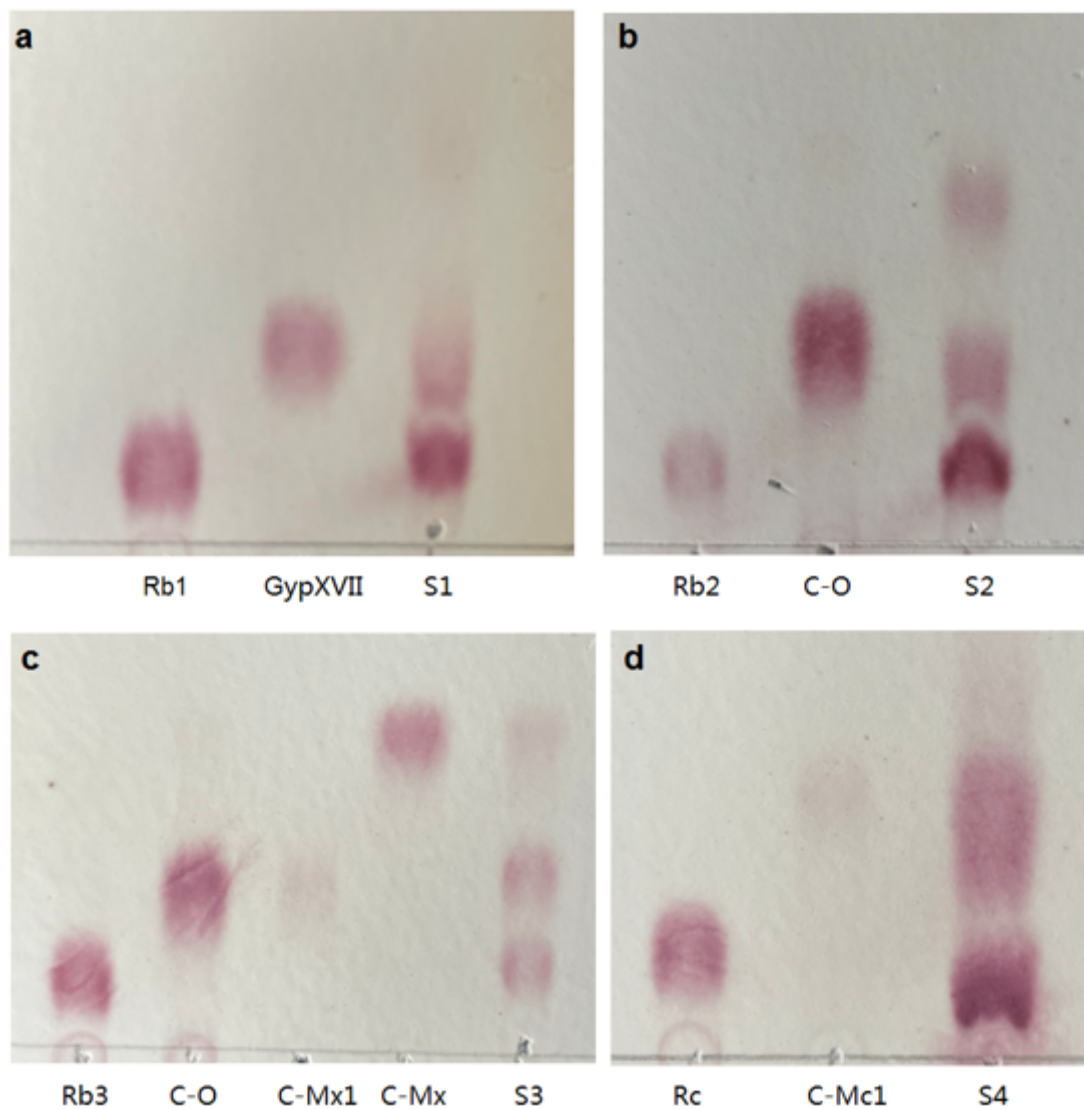


Figure 10

TLC detection of different ginsenoside conversion products. Ginsenoside reference substances: Rb₁, GypXVII, 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

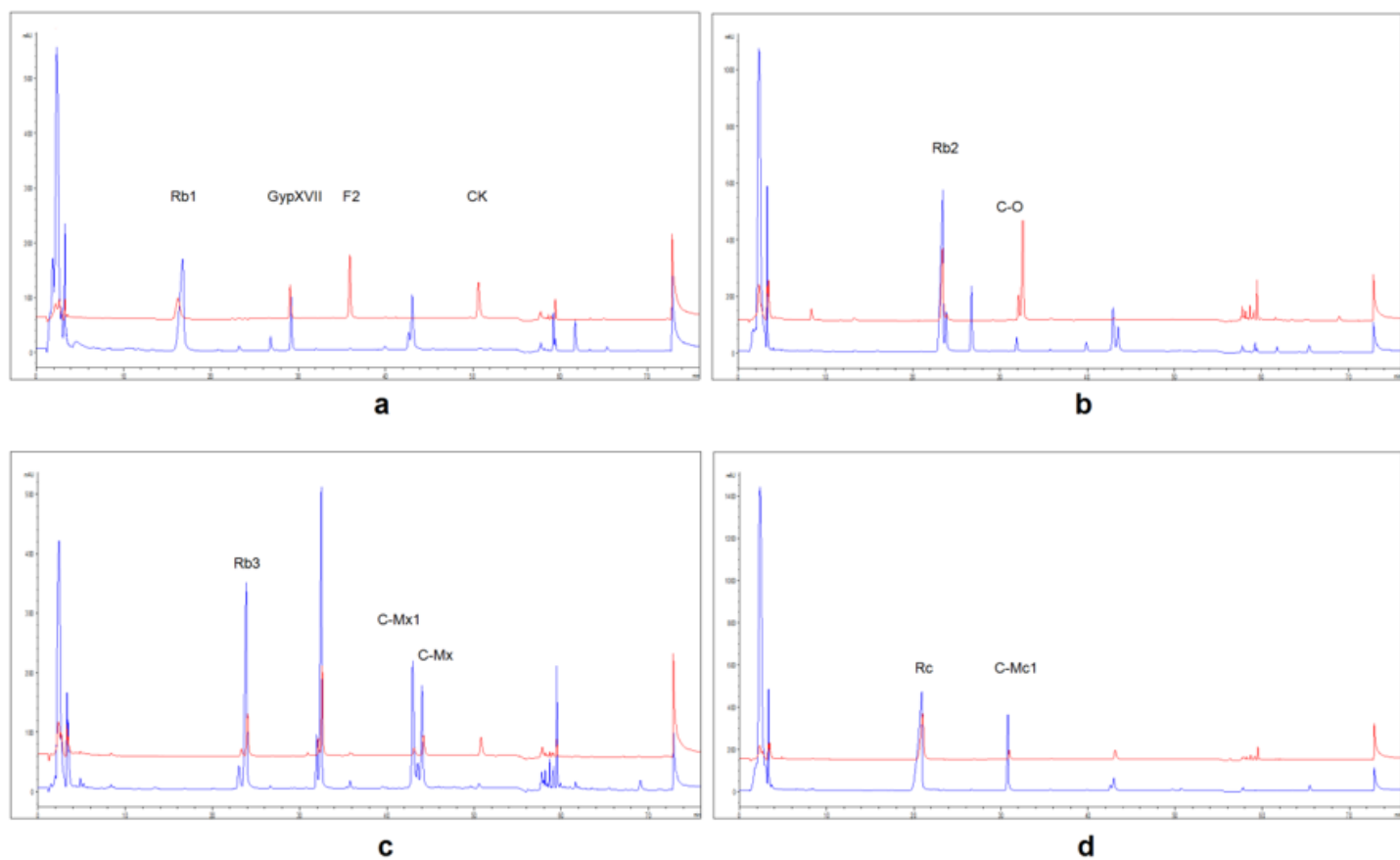


Figure 11

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

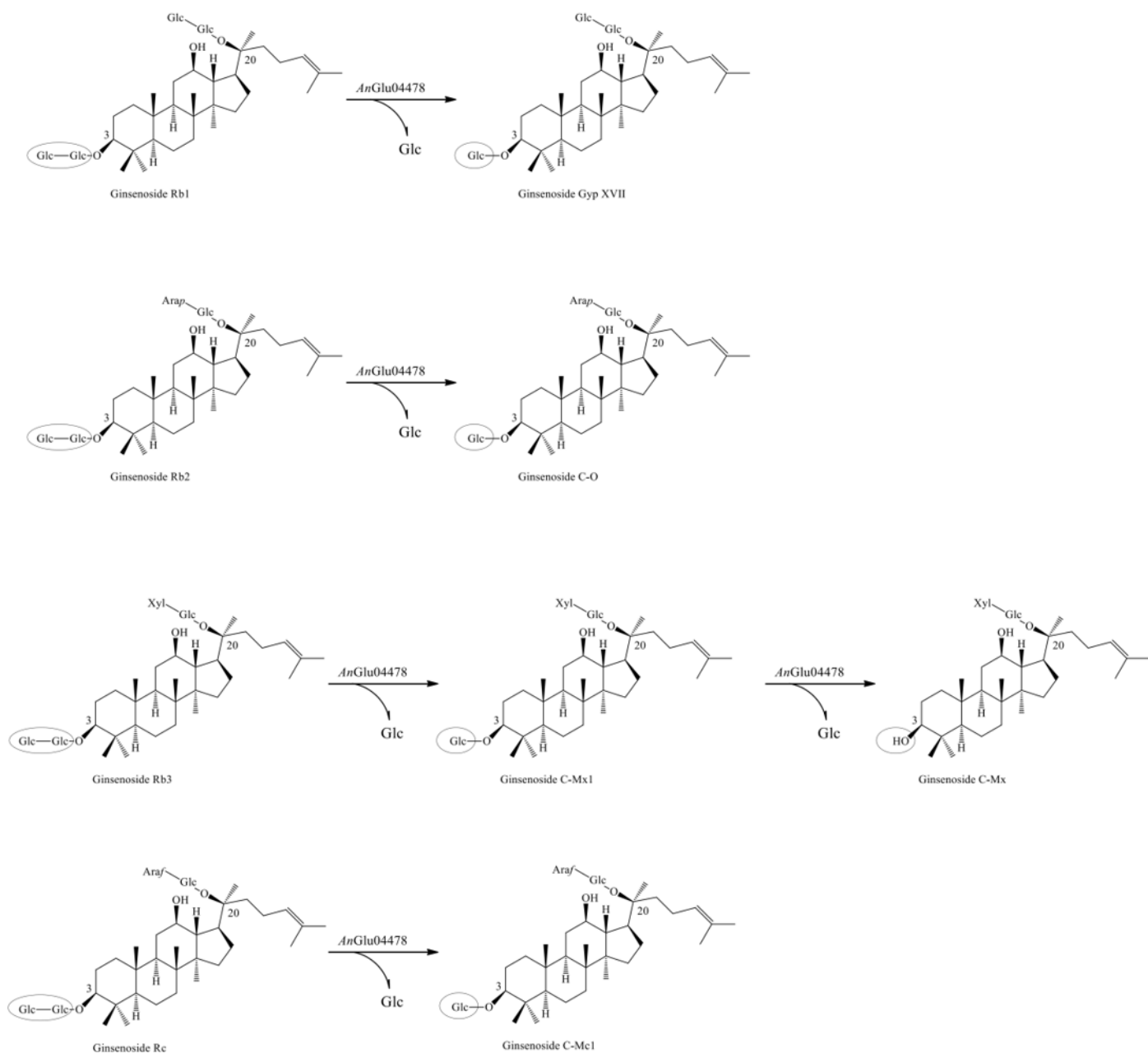


Figure 12

Diagrammatic representation of the *AnGlu04478* to ginsenoside conversion pathway (Rb1 → GypXVII, Rb2 → C-O, Rb3 → C-Mx1 → C-Mx, Rc → C-Mc1)