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Enzymatic Bioconversion of Cycloastragenol-6-O-β-D-glucoside into Cycloastragenol by a Novel Recombinant β-Glucosidase from *Phycicoccus sp. Soil748*

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ABSTRACT

Cycloastragenol (CA), the genuine sapogenin of astragaloside from *Astragalus membranaceus*, exhibits diverse pharmaceutical activities. Recently, the efficient production of CA has received considerable attention due to rapidly increasing market demands. In this study, enzyme mining was conducted, based on skeleton and glycosyl similarity, to explore an efficient β-glucosidase for CA preparation. A novel β-glucosidase from *Phycicoccus sp. Soil748* (Bgps) was discovered, possessing the efficient conversion rate for cycloastragenol-6-*O*-β-D-glucoside (CMG) into CA. The optimum temperature and pH value of Bgps were determined as 45 °C and 7.0. The results of kinetic analysis suggested that Bgps catalyzed deglycosylation of CMG more efficiently than other substrates. Furthermore, the optimal substrate concentration of Bgps was up to 80 mg/mL with the conversion rate as 99.2%, suggesting its potential application in CA industrial production by biotransformation.

1. Introduction

Cycloastragenol (CA), the aglycone of astragaloside from *Astragalus membranaceus*, is regarded as a novel functional component [1–3], and has been marketed as a functional health product (TA-65®) in the USA [4,5]. During the past years, there is a rapid increase in the demand for CA in the field of functional foods and medicine due to its potential applications on anti-aging and other beneficial physiological activities, such as antiviral activity, anti-depression and callus repair activity [6–10]. However, it's hardly to efficiently extract CA directly from the plant tissues due to the scarcity of free CA in *Astragalu*. Considering the abundance of major astragaloside—astragaloside IV (ASI) in natural plants (0.2 wt %), transforming ASI into CA has been regarded as an effective strategy for CA preparation [11–13]. Currently, acid hydrolysis and Smith degradation have been used to produce CA from ASI by hydrolyzing the xylose moiety at the C_3 position and glucose moiety at the C_6 position. Unfortunately, the acid hydrolysis results in acid pollution (10%-20% acids) and the formation of byproduct (astragenol), and the Smith degradation needs multiple reaction steps, long reaction time (nearly 2 days) and complex operational steps (redox) [13].

Biotransformation has been characterized as a mild, high-specificity and environment-friendly technology, which has been widely used in the preparation of rare active ingredients, the structural modification of natural products, and the discovery of new drugs [14–18]. So far, the biotransformation using microbial cells and enzymes has been explored for CA preparation [19–22]. *Bacillus* sp. LG-502 has been reported to convert ASI into CA at 35 °C for a week with the conversion rate of 84%, coupling with the production of CA-2H [23], in which the longer time coure limited its industrial applicaiton. Furthermore, two enzymes from *Dictyoglomus thermophilum* were discovered to convert ASI into CA at

Abbreviations: ASI, : Astragaloside IV; Bgam, : β-D-glucosidase from Actinosynnema mirum; Bgmh, : β-D-glucosidase from *Microbacterium hominis*; Bgps, : β-D-glucosidase from *Phycicoccus sp. Soil748*; Bglsk, : β-D-glucosidase from *Sanguibacter keddieii*; CA, : Cycloastragenol; CMG, : Cycloastragenol-6-*O*-β-D-glucoside; CMX, : Cycloastragenol-3-*O*-β-D-xyloside; NTA, : Nitrilotriacetic acid; oNP, : *o*-nitrophenol; pNPG, : *p*NP-β-D-glucopyranoside; pNP, : *p*-Nitrophenol

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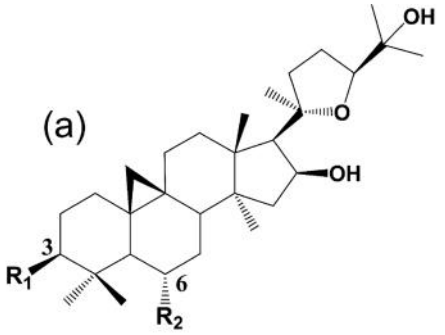
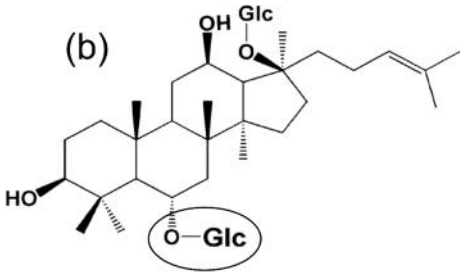


Astragalosides	R_1 (C-3)	R_2 (C-6)
ASI	Xyl-O	Glc-O
CMG	H-O	Glc-O
CMX	Xyl-O	H-O
CA	H-O	H-O



Ginsenoside Rg1

Fig. 1. The chemical structures of astragalosides **(a)** and ginsenoside Rg1 **(b)**.

75 °C with the conversion rate of 94.5% [24], in which the reaction temperature needed to be decreased further considering cost-consumption. In our previous studies, commercial cellulase (Model: Cellic CTec3) from Novozymes Biotechnology Co., Ltd was developed to efficiently convert ASI into cycloastragenol-6-*O*-*β*-D-glucoside (CMG) with the removal of xylose moiety from the C_3 position at 45 °C with the conversion rate of 95% [25] (Fig. 1a). Thus, if an enzyme could specifically and efficiently hydrolyze the glucose moiety at the C_6 position of CMG at medium temperature, CA could be produced effectively from ASI using enzymatic catalysis at the large scale for industry. Unfortunately, to date no enzymes have been discovered to convert CMG into CA or remove the glucose moiety efficiently at the C_6 position of CMG.

In this study, by comparing the backbone (tetracyclic triterpenoids) and the position of glucose residue (C_6) of CMG with ginsenoside, it was discovered that ginsenoside Rg1 and CMG had a high degree of structural similarity (Fig. 1b) and both possessed a glucose residue at C_6 positions [26]. Subsequently, we screened CMG hydrolase in the NCBI based on a strategy of skeleton similarity and glycosyl similarity. Using DNA mining, we cloned and overexpressed several *β*-glucosidases with high structural homology and then tested their ability to convert CMG. A novel *β*-glucosidase from *Phycicoccus sp. Soil748* showed the highest efficiency of CMG conversion, and then this enzyme was characterized and applied to CA preparation.

2. Materials and Methods

2.1. Materials

The crude extracts of astragaloside IV (ASI) (80 % purity), the standards of ASI and cycloastragenol (CA) were purchased from Sichuan Weikeqi Biotechnology Co., Ltd (China). The commercial cellulase (Model: Cellic CTec3) was purchased from Novozymes. The substrate cycloastragenol-6-*O*-*β*-D-glucoside (CMG) was prepared from ASI by cellulase (pH: 5.0, T = 45 °C). The *p*NP-*β*-D-glucopyranoside (*p*NPG) was purchased from Sigma (USA).

2.2. Gene mining

The DNA mining was carried out in NCBI database by amino acid sequence alignment with ginsenoside Rg1 hydrolase (GenBank accession no. ACZ20402.1). Given the existence of hundreds of DNA sequence, the blast results were selected according to two principles: (1) the sequence similarity is higher than 65%; (2) in the same genus only one gene was selected for further experiments.

2.3. Molecular cloning, expression and purification of enzymes

The gene sequences corresponding to *β*-D-glucosidases were from *Sanguibacter keddieii* (Bglsk, GenBank accession no. ACZ20402.1), *Actinosynnema mirum* (Bgam, GenBank accession no. **WP_015801787.1**), *Microbacterium hominis* (Bgmh, GenBank accession no. **WP_060960396.1**) and *Phycicoccus sp. Soil748* (Bgps, GenBank accession no. **WP_056884270.1**). The gene sequences were optimized and synthesized by Sangon Biotech (China), followed by amplified with BamHI and KpnI restriction sites by Pro-Flex PCR System (Thermo Scientific, USA). After being digested with restriction enzyme (Thermo Scientific, USA) at 37 °C for 1 h, the DNA fragments and the pETduet-1 vector were ligated using T_4 DNA ligase (Thermo Scientific, USA) at 22 °C for 2 h to generate the recombinant plasmids. Chemical transformation was used to transform the recombinant plasmids into *E. coli* Trans 5*α* (Trans Gene Biotech, China). Then, the positive recombinant plasmids were transformed into *E. coli* BL21 (DE3) (Trans Gene Biotech, China), growing in Luria-Bertani medium with 100 μg/mL ampicillin at 37 °C, and the recombinant proteins were induced for the expression analysis. The *E. coli* cells were collected by centrifuging at 8000 rpm, 4 °C, and then lysed by using Ultrasonic Cell Disruptor (Ningbo Scientz Biotechnology Co., Ltd. China). The crude enzymes with 6×His-tag were purified using His-trap Ni-NTA (nitrilotriacetic acid) Fast Flow column (GE Healthcare, USA), and analyzed by 12% SDS-PAGE [27].The concentration of purified enzymes was determined by bicinchoninic acid protein assay kit (Pierce Chemicals, USA) with bovine serum albumin (Sigma, Germany) as the standard.

2.4. The enzyme screening

Screening experiments were conducted using 5 mM CMG as substrate in 50 mM sodium phosphate buffer. In order to optimize conversion rate of each purified enzyme towards CMG, the enzymatic reaction were conducted with different pH values (5.5,6.0, 6.5, 7.0, 7.5 and 8.0) at 35 °C, or different temperatures (25 °C, 30 °C, 35 °C, 40 °C, 45 °C and 50 °C) at pH 7.5. After the enzymatic biotransformation, the samples were analyzed with HPLC.

2.5. CA production by Bgps biotransformation

The purified Bgps (0.265 mg/mL) was used to further test the biotransformation capacity from ASI (5 mM) or CMG (5 mM) into CA at 45 °C. The sample solutions were taken and analyzed by TLC and HPLC, and the molecular weight of products were analyzed by Mass Spectrometry.

2.6. Characterization of Bgps

The enzyme activities of the purified Bgps for ASI and CMG were determined by incubating with 5 mM ASI or 5 mM CMG in pH 7.0 at 45 °C for 1 h or 10 min. One unit (U) of Bgps activity for *p*NPG, ASI and CMG were defined as the amount of Bgps to generate 1 μmol *p*NP per min, or 1 μmol ASI/1 μmol CMG per min. The special activity for *p*NPG, ASI and CMG were described as the enzyme activities per mg of Bgps.

2.6.1. The effect of pH on Bgps activity

Enzymatic reactions were conducted at different pH values (2.0 to 11.0) at 45 °C to analyze the optimum pH for Bgps activity. The effect of pH on Bgps stability was studied by measuring the residual enzyme activities of Bgps, in which the Bgps was pre-treated with different pH buffer (pH 2.0 to 11.0) for 4 h at 4 °C. The pH values of reaction systems were obtained using different buffers, including disodium hydrogen phosphate-citric acid (pH 2.0 to 5.0), disodium hydrogen phosphate sodium dihydrogen phosphate (pH 6.0 to 8.0), Tris–HCl (pH 9.0), glycine-sodium hydroxide (10.0), and borax-sodium carbonate (pH 11.0).

2.6.2. The effect of temperature on Bgps activity

The optimum temperature for Bgps activity was studied at various temperatures ranging from 5 °C to 70 °C in pH 7.0. The thermostability of Bgps was studied by pretreating Bgps, at temperatures ranging from 0 to 80 °C in pH 7.0 for 1 h, and then measuring Bgps activity.

2.6.3. The effect of metal ions and chemicals on Bgps activity

NaCl, KCl, $CaCl_2$, $FeCl_3$, $CuCl_2$, $MgCl_2$, $MnCl_2$, $FeCl_2$, $ZnSO_4$, $CoCl_2$, DTT, SDS, *β*-mercaptoethanol and EDTA were selected as the effectors to analyze the effect of metal ions and chemicals on Bgps activity. The activity of Bgps was determined after reacting for 5 min at pH 7.0 and 45 °C with the concentration of effectors at 1 mM or 10 mM, and the activity of Bgps without effectors was used as the control.

2.6.4. The substrate specificity of Bgps

The *p*NPG, *p*NP-*α*-D-glucopyranoside, *p*NP-*α*-D-xylopyranoside, *p*NP-*α*-L- rhamnopyranoside, *o*NP-*β*-D-glucopyranoside, *p*NP-*β*-D-xylopyranoside, *p*NP-*β*-D- glactopyranoside and *o*NP-*β*-D-galactopyranoside were selected as the substrates with the concentration of 5 mM to study the substrate specificity of Bgps. The definition of Bgps activity was the amount of Bgps required to generate 1 μmol *p*NP or *o*-nitrophenol (*o*NP) per min. The relative activity of Bgps against *p*NP-*β*-D-glucopyranoside was assumed to be 100%.

2.6.5. The kinetic parameters of Bgps

The kinetic parameters of Bgps for *p*NPG, CMG and ASI were determined in 50 mM sodium phosphate buffer (pH 7.0) at 45 °C. The concentrations of *p*NPG, CMG and ASI were varied from 0.05 mM to 5 mM, from 0.1 mM to 10 mM or from 0.1 mM to 10 mM, respectively. The K_m, V_{max} and K_{cat} were obtained by the enzyme kinetic program [28]. All assays were performed in triplicate.

2.7. Reaction temperature optimization of CA production

Experiments were carried out at 38 °C, 40 °C, 42 °C, 45 °C, 48 °C and 50 °C to determine the optimum temperature for CA production. The substrate CMG (80 mg/mL) was incubated with 1.65 mg/mL crude Bgps in 1 mL solution system at pH 7.0. Since the content of Bgps in crude enzyme solution could be determined directly, the enzyme concentration in Bgps crude solution was described with the enzyme activities against *p*NPG. The amount of CA at 2 h, 4 h, 6 h, 8 h, and 10 h was measured by HPLC to determine conversion rate of CMG at the different time points.

2.8. CMG concentration optimization of CA production

High substrate concentration of enzymes is critical for their industrial application. Biotransformation was conducted with substrate concentration of 40 mg/mL, 60 mg/mL, 80 mg/mL, 100 mg/mL, 120 mg/mL and 140 mg/mL in 1 mL system (1.65 mg/mL crude Bgps enzyme, pH 7.0 and 42 °C) to determine the optimal CMG concentration for CA production. The amounts of CA at 2 h, 4 h, 6 h, 8 h, and 10 h were measured by HPLC to determine conversion rate of CMG at different time points.

2.9. TLC analysis

The thin layer chromatography (TLC) analysis was performed on silica gel plate (T225100 F, Synthware, China). The developing solvent was $CHCl_3$:CH_3OH:H_2O (13:5:2, v/v) and visualization of product spot was achieved by spraying 20% (v/v) H_2SO_4 followed by heating at 80 °C for 10 min.

2.10. HPLC analysis

The quantitative analysis of astragaloside was performed by high-performance liquid chromatography (HPLC) system (Shimadzu LC-20AT, Japan). The separation was carried out using a C_{18} column (5 μm, 250 × 4.6 mm, Shimadzu, Japan) with 25% solvent A (water) and 75% solvent B (methanol). The flow rate was 1 mL/min, and the injection volume was 20 μL. The astragalosides were detected by the evaporative light scattering detectors (ELSD) with the gas pressure at 350 MPa.

2.11. Mass spectrometry analysis

The molecular weight and a molecular formula of astragalosides were identified by mass spectrometry (MS) analysis (Waters Xevo G2 Q-TOF, USA).

3. Results

3.1. Cloning, expression, screening and purification of recombinant enzymes

After gene mining in database, seven genes were finally selected for the further cloning and expression in *E. coli* strain. However, the expression of the enzyme from *Clavibacter michiganensis* (GenBank accession no. **WP_015489650.1**) failed, and the proteins expressed from *Cryocola sp. 340MFSha3.1* (GenBank accession no. **WP_020076619.1**) and *Leifsonia* (GenBank accession no. **WP_089878197.1**.) did not exhibit any enzymatic activity towards CMG. The rest of the recombinant *β*-D-glucosidases Bglsk, Bgam, Bgmh and Bgps (Table S2), were purified and analyzed by 12% SDS-PAGE. The position of the bands on SDS-PAGE (Fig. S2) demonstrated that the molecular masses of glucosidases were consistent with the theoretical values (Bgam, 66.8 kDa; Bgmh, 69.2 kDa; Bgps, 68.3 kDa; Bglsk, 68.1 kDa).

The CMG transforming capabilities of four purified enzymes were next tested. The results showed that the conversion rates of Bgam, Bgmh and Bglsk were less than 40%, and Bgps exhibited the highest conversion of CMG, up to 90% (Fig. 2). The enzyme Bglsk, which had a high activity for hydrolyzing ginsenoside Rg1 to F1, did not showed a higher conversion rate towards CMG, possibly due to the compatibility differences between substrate and active center of enzyme. This also indicated that it's critical to select various homologous genes for further

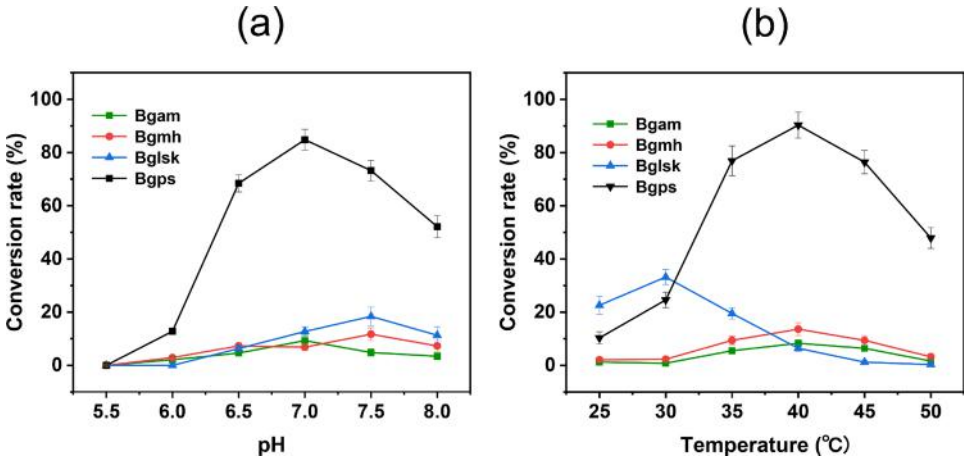


Fig. 2. The conversion rate of cycloastragenol-6-*O*-*β*-D-glucoside (CMG) by enzymes in different pH **(a)**, and temperature **(b).**

experimental test during gene mining for the purpose of obtaining target gene. Thus, the Bgps with the highest conversion of CMG was selected for the further study.

Different induction conditions were studied to maximize the yield of the Bgps, and the induction with 1 mM IPTG at 16 ˚C for 24 h showed the best results (data not shown). The absence of recombinant enzyme in the precipitation pellet indicated the higher soluble expression of the enzyme (Fig. 3(a)). The purified protein showed a single band at 68.3 KDa that agreed with the theoretical calculation, and the concentration of purified Bgps protein was 0.53 mg/mL.

3.2. Biotransformation of astragalosides by Bgps

Fig. 3 indicated that most of the CMG was converted into product A by Bgps based on the R_f values (Fig. 3b) and the HPLC profiles (Fig.3c). Furthermore, product A was analyzed by MS, and the result showed an ion peak at *m/z* 513.356 ($[M + Na]^+$) (Fig. 4a), indicating that the molecular formula of product A was $C_{30}H_{50}O_5$ (M_w, 490.71), corresponding to CA. The data from MS, TLC and HPLC analysis finally identified product A as CA, suggesting that Bgps could hydrolyze the glucose moiety of CMG to produce the CA. Both ASI and CMG have a glucose at C_6 position (Fig. 1), and next the glucosidase activity of Bgps towards ASI was studied. Bgps also showed a hydrolyzing capacity toward the glucose at C_6 position of ASI (Fig. 3(b) and Fig. 3(d)), and the product B had an ion peak at *m/z* 645.396 ($[M + Na]^+$) (Fig. 4b), indicating the molecular formula was $C_{35}H_{58}O_9$ (M_w, 622.84), corresponding to CMX (Fig. 1). On the basis of HPLC, TLC and MS analysis, the product B was identified as cycloastragenol-3-*O*-*β*-D-xyloside (CMX), resulting from the removal of glucose moiety from the C_6 position of ASI. These results indicate that the Bgps hydrolyzed the glucose moiety at C_6 position of ASI or CMG with higher steroselectivity. The biotransformation pathways are shown in Fig. 4(c) and Fig. 4(d).

CMX has been reported only as a trace by-product during chemical synthesis or microbial transformation. And the highly-purified CMX is difficult to obtain due to its complex chemical synthesis pathway and uncontrolled biotransformation processes [23,29]. Thus, the physiological activity of CMX has not been extensively studied. The present study demonstrated that the CMX can be produced by Bgps from ASI, providing a potential way to produce CMX at large scale.

3.3. Characterization of Bgps

As shown in Fig. 5(a), Bgps exhibited the activity with pH ranging from 4.0 to 9.0 and its optimum pH was pH 7.0. The enzyme could be stable with pH from 6.0 to 8.0, indicating the stability of Bgps through a wide pH range. The optimum temperature for Bgps activity was 45 ˚C (Fig. 5b), and this enzyme could be quite stable with the temperature lower than 40 ˚C, possessing at least 80 % of the maximum activity. Moreover, more than 70% of the maximum activity will be lost at temperatures beyond 50 ˚C.

The activity of the Bgps was inhibited by Cu^{2+} and Zn^{2+} (Table 1). Mn^{2+} increased the enzyme activity of Bgps by 1.2 times in comparison with that of control, suggesting the possibility of Mn^{2+} as an activator for Bgps activity. The other metal ions did not show significant effect on Bgps. At the range of concentration from 1 mM to 10 mM, *β*-mercaptoethanol, DTT and EDTA did not show a significant inhibition on the Bgps, as previously reported for *β*-glucosidase from *Terrabacter ginsenosidimutans* [30], suggesting that the sulfhydryl groups may not be involved in the catalytic center, and the divalent cations may not be essential for the activity of Bgps. SDS, a well-known protein denaturant, caused more than 95% loss of enzyme activity. The substrate specificity of Bgps indicated that only *p*NPG and *o*NP-*β*-D-glucopyranoside could be hydrolyzed by Bgps (Table 2). The specific hydrolysis of Bgps on the glucoside bond was in agreement with above studies (Fig. 4(c) and Fig. 4(d)).

The kinetic parameters of Bgps were studied by using *p*NPG, CMG and ASI as substrates (Table 3). The specific activities of Bgps to CMG and ASI were determined as 0.24 U/mg and 0.01 U/mg, respectively. The catalytic efficiency constants (K_{cat} / K_m) of Bgps for *p*NPG, ASI and CMG decreased in the following order: CMG > *p*NPG > ASI, indicating the better affinity of Bgps towards CMG.

Given the unique difference between ASI and CMG in the structure, we speculate that the presence of attached xylose may create some spatial resistance to the binding of Bgps and ASI, just as the attached rhamnose moiety of ginsenoside Re did towards BglQM [17]. However, the detailed mechanism on the reduce of the affinity of Bgps towards ASI needs to be studied in future.

3.4. Optimization of CA production by Bgps biotransformation

Different reaction temperatures were tested to determine an optimum temperature for the preparation of CA. The optimum reaction temperature for CA production was 42 ˚C, and CMG was completely converted into CA at 42 ˚C within 6 h (Fig. 6(a)). CMG concentrations were further tested (Fig. 6(b)) for the optimum substrate concentrations towards CA production. When substrate concentration was lower than 80 mg/mL, CMG was almost completely converted into CA by Bgps within 6 h (99.2%). However, when the substrate concentration was higher than 100 mg/mL, the conversion decreased significantly (78%). Thus, the optimal substrate concentration was determined to be 80 mg/mL.

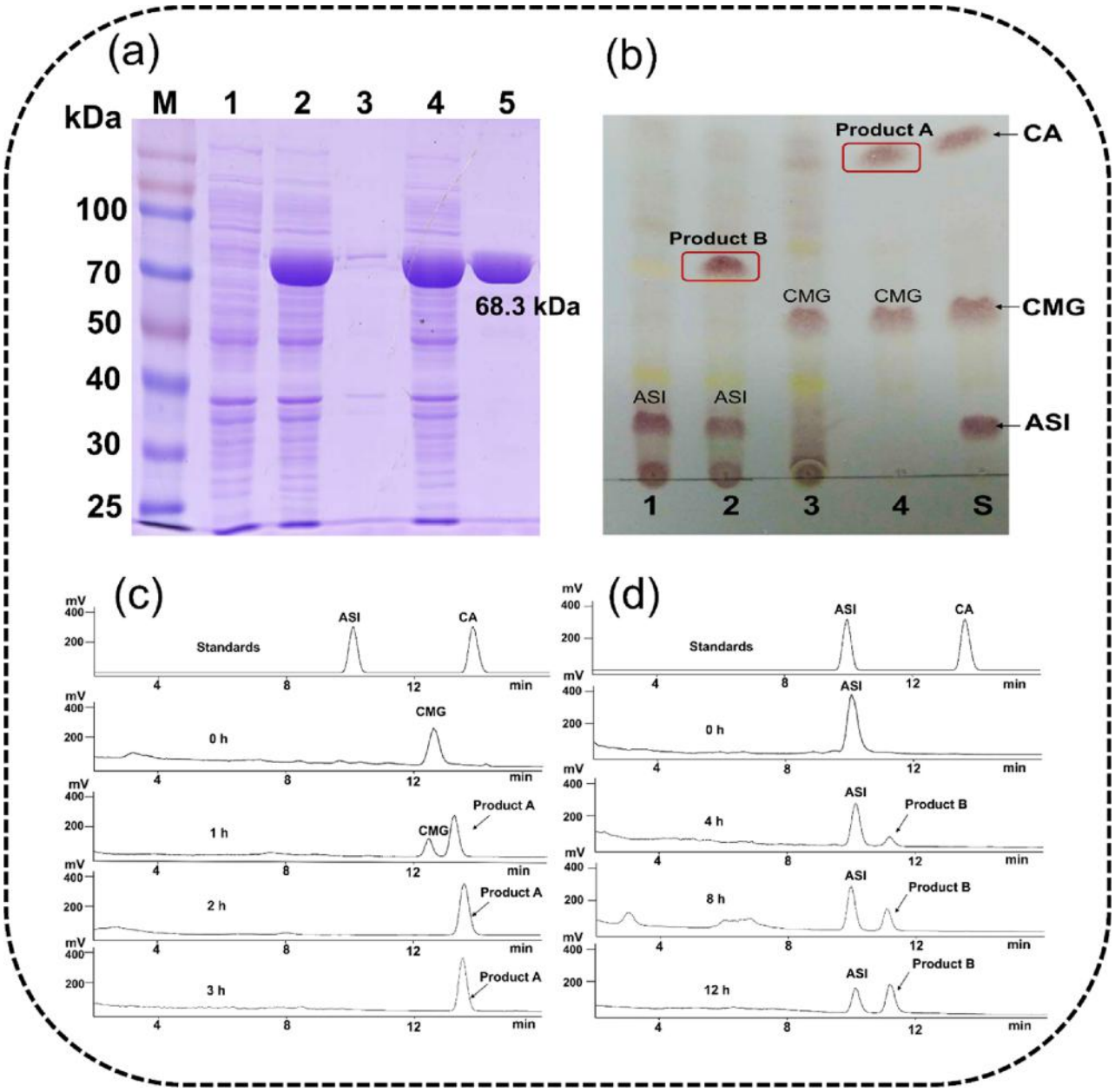


Fig. 3. (a), The SDS-PAGE analysis of Bgps, lane M: molecular mass standard; lane 1: the recombinant BL21(DE3) cells without IPTG induction; lane 2: the crude extract of recombinant BL21(DE3) with IPTG induction; lane 3: the pellet of the crude extract; lane 4: the supernatant of the crude extract; lane 5: the purified Bgps protein by using His-trap Ni-NTA (nitrilotriacetic acid) Fast Flow column. **(b),** TLC analysis of astragalosides hydrolyzed by Bgps, lane S: the mixture of astragaloside IV (ASI), CMG and cycloastragenol (CA) standard; lane 1: ASI; lane 2: the hydrolysate of ASI by Bgps; lane 3: CMG; lane 4: the hydrolysate of CMG by Bgps; **(c),** HPLC analysis results of the biotransformation of cycloastragenol-6-*O*-*β*-D-glucoside (CMG) into product A by Bgps at different time; **(d),** HPLC analysis results of the biotransformation of astragaloside IV (ASI) into product B by Bgps at different time.

4. Discussion

In this study, the gene mining was applied for the discovery of enzyme that could highly convert CMG into CA, during which a novel *β*-glucosidase (Bgps) from *Phycicoccus sp. Soil748* was selected, further cloned, overexpressed and characterized.

During the past years, various microorganisms have been investigated for the bioconversion of ASI, and we also explored diverse filamentous fungi, rich in glycosidase, for the possibility of converting ASI into CA (data not shown); however, most reported microorganisms showed the hydrolyzing capacity of xylose group at C_3 position of ASI or exhibit other types of biotransformation; although *Bacillus* sp. LG-502 has been reported to convert ASI into CA, the low transforming efficiency and undesirable byproducts limited the application of this technology [19–23]. After the failure of microbial conversion, numerous commercial glycosidases were investigated for the cleavage of glucosidic bond at C_6 position, and the results were also not positive (data not shown). In the present study, we adopted an alternative strategy through gene mining based on the substrate similarity. Astragalosides and ginsenosides both belong to the tetracyclic triterpenoid saponins. More than 180 ginsenosides have been identified in Ginseng and these are characterized with the attachment of different amount and types of glycosyl residues substituted at diverse C positions [31,32]. Among those reported ginsenosides, ginsenoside Rg1 had the highest structural similarity with CMG and both had a glucose moiety at C_6 position (Fig.1(b)). Furthermore, a glycosidase (Bglsk) from

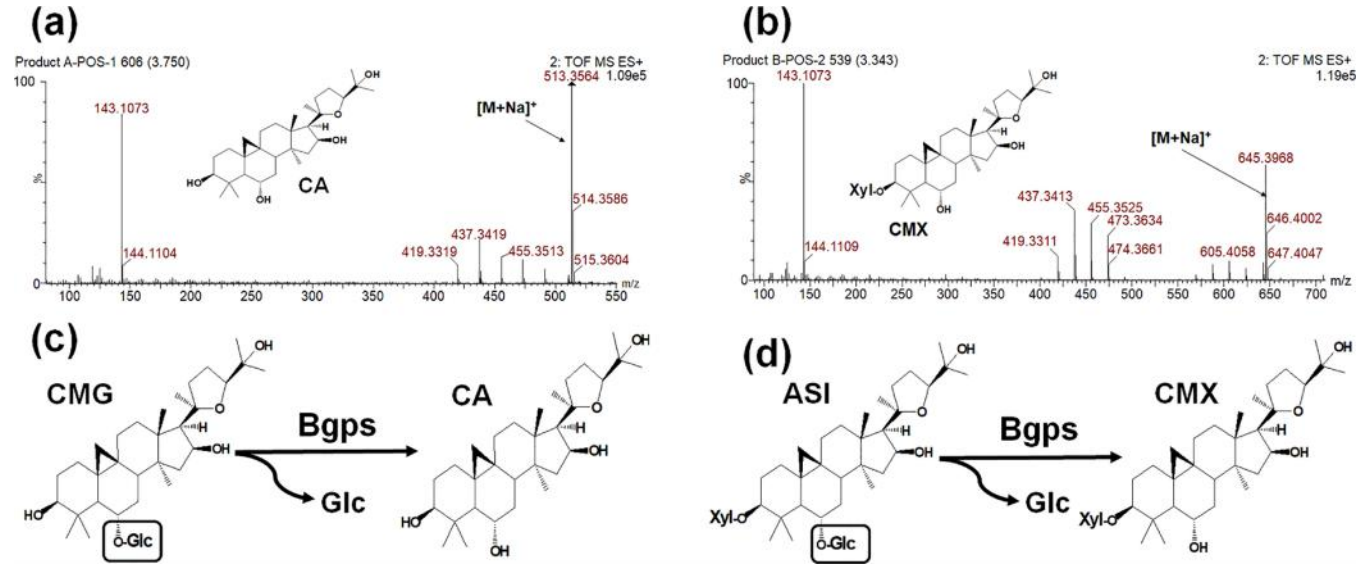


Fig. 4. Mass spectra data of product A **(a)** and product B **(b)**. Product A, *m/z* 513.356 = [M_w + Na], M_w 490.71; Product B, *m/z* 645.396 = [M_w + Na], M_w 622.84; **(c)**, Biotransformation pathway of cycloastragenol-6-*O*-*β*-D-glucoside (CMG) catalyzing by Bgps; **(d)**, Biotransformation pathway of astragaloside IV (ASI) catalyzing by Bgps.

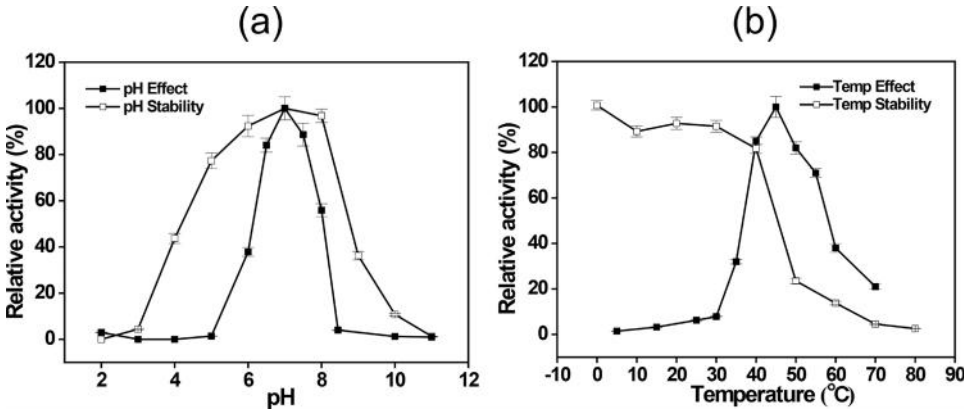


Fig. 5. Effect of pH **(a)** and temperature **(b)** on Bgps activity. The relative activity of the maximum activity for Bgps was defined as 100%.

Table 1
Effect of chemicals on Bgps activity. The relative activities were described as a percentage of the activity of the control.

Metal ions and reagents	Relative activity ± SD (%) at:	
	1 mM	10 mM
Control	100.0 ± 1.85	100.0 ± 2.13
Na^+	101.81 ± 4.56	99.53 ± 3.34
K^+	104.10 ± 2.14	101.02 ± 4.94
Ca^{2+}	99.24 ± 3.02	100.15 ± 2.13
Fe^{2+}	104.54 ± 4.27	92.54 ± 2.15
Fe^{3+}	100.75 ± 5.94	103.42 ± 4.32
Mg^{2+}	105.39 ± 3.67	101.23 ± 4.03
Mn^{2+}	120.52 ± 2.12	122.31 ± 1.97
Zn^{2+}	77.63 ± 2.69	54.23 ± 3.01
Cu^{2+}	45.49 ± 2.13	10.23 ± 1.49
Co^{2+}	95.23 ± 2.16	89.21 ± 4.53
SDS	13.35 ± 2.65	4.24 ± 3.45
DTT	94.02 ± 4.19	95.21 ± 5.18
EDTA	104.8 ± 5.87	101.3 ± 4.05
β-mercaptoethanol	96.28 ± 2.02	97.48 ± 3.67

Table 2
The substrate specificity of Bgps. The relative activity of Bgps against *p*NP-*β*-D-glucopyranoside was defined as 100%.

Substrate	Relative activity ± SD (%)
*p*NP-*α*-D-glucopyranoside	0
*p*NP-*α*-D-xylopyranoside	0
*p*NP-*α*-L-rhamnopyranoside	0
*p*NP-*β*-D-glactopyranoside	0
*p*NP-*β*-D-xylopyranoside	0
*p*NP-*β*-D-glucopyranoside	100.0 ± 1.56
*o*NP-*β*-D-glucopyranoside	96.0 ± 2.13
*o*NP-*β*-D-galactopyranoside	0

Sanguibacter keddieii was reported to remove glucose moiety at the C_6 position of the Rg1, resulting in F_1 [33]. The high similarity of CMG and ginsenoside Rg1 skeletons and a glucose moiety at the same C position inspired us to screen gene with capacity of transforming CMG into CA by Bglsk and its homology. Based on this strategy, a novel *β*-glucosidase (Bgps) from *Phycicoccus sp. Soil748* was discovered that effectively hydrolyzed the glucose at C_6 position of CMG from a limited number of candidates. Compared with general microbial screening or enzyme screening, this effective strategy improved the probability and efficiency of enzyme mining, which could also be applied in other saponins' enzymatic catalysis and biotransformation in future, such as the biotransformation of ginsenoside.

Bgps should belong to the glycoside hydrolase family 3 on the basis

Table 3
The kinetic parameters of Bgps

Substrate	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_{cat} (s^{-1})	K_{cat} / K_m (mM^{-1} s^{-1})
*p*NPG	0.73 ± 0.03	0.24 ± 0.01	0.27 ± 0.021	0.32 ± 0.015
ASI	2.72 ± 0.24	0.01 ± 0.0015	0.011 ± 0.001	0.004 ± 0.00001
CMG	0.36 ± 0.01	0.27 ± 0.02	0.31 ± 0.025	0.86 ± 0.12

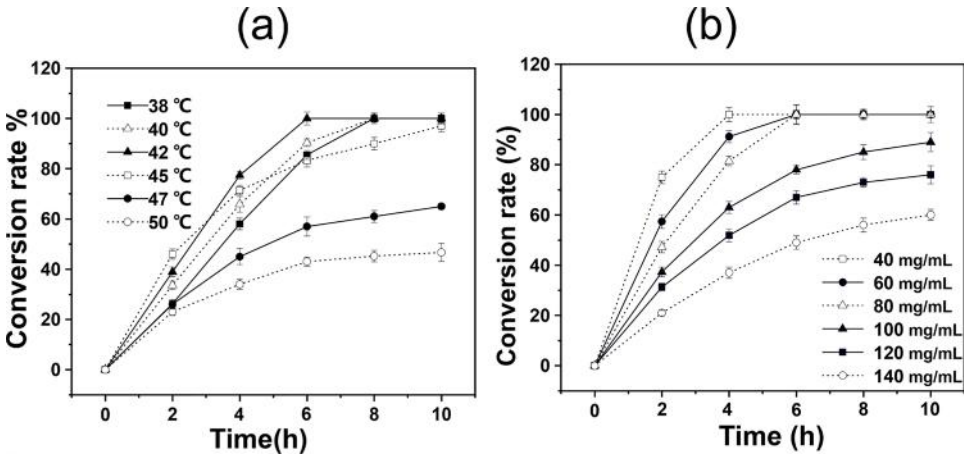


Fig. 6. The time course curves of cycloastragenol (CA) production from cycloastragenol-6-*O*-*β*-D-glucoside (CMG) catalyzing by Bgps at the different temperature **(a)** and different substrate concentration **(b).**

of amino acid sequence similarities and it is similar to the *β*-glucosidase from *Phycicoccus sp. Root 563* (86.56%, GenBank accession no. **WP_082580769.1**), *Terrabacter ginsenosidimutans* (83.99%, GenBank accession no. ACZ66247.3), and *Intrasporangiaceae bacterium URHB0013* (82.19%, GenBank accession no. **WP_026862722.1**). To the best of our knowledge, the Bgps was first characterized in the present study. The substrate specificity of Bgps indicated that only *p*NPG and *o*NP-*β*-D-glucopyranoside could be hydrolyzed similar to that of Bglsk [33]. Compared to other ginsenoside glycosidases (BglAm, bgpA, BglBX10, Bglsk) of the glycosidase family 3, Bgps exhibited better pH stability; however, all these enzymes lost most of its activity when the temperature was higher than 50 °C, suggesting the poor thermal stability [16,18,30,33]. Interesting, the Mn^{2+} could increase the enzyme activity of Bgps to some extent, which was different from bgpA, BglAm, bgp3 and BglSk [34].

Recently, the microbial production of CA was first realized by *Bacillus sp. LG-502*. However, the long fermentation period (6 days), low transforming efficiency, and undesirable byproducts (CA-2 H) limited the application of this technology at industrial scales [23]. In addition, enzymatic catalysis was also explored to transform ASI into CA by the pathway of ASI→CMX→CA, whose operating conditions were 75 °C, pH 5.5 and 15 mg/mL (substrate concentration) [24]. In the present study, Bgps exhibited a lower reaction temperature (42 °C) and higher conversions (99.2%), and the optimum concentration of substrate was determined as 80 mg/mL. These beneficial characterizations suggest Bgps as a highly competitive biocatalyst, suitable for the production of CA. The Bgps also showed better affinity to CMG than ASI, indicating a more reasonable transformation path ASI→CMG→CA, rather than ASI→CMX→CA for CA production by the combination of cellulase (Cellic CTec3) and Bgps. The preliminary bioconversion of ASI to CA indicated that the efficiency of the CA production by the path of ASI→CMG→CA was much higher than that by the path of ASI→CMX→CA, which confirmed our hypothesis (Fig. S3). In the future, the operating parameters of the rational combination of commercial glucosidase step and Bgps step remain to be optimized. Additionally, it will be meaningful to uncover the mechanism of different affinity of Bgps towards ASI and CMG.

5. Conclusion

In conclusion, a rational gene-mining strategy was applied to screen the target enzymes on the basis of the skeleton similarity and glycosyl similarity between ginsenoside Rg1 and CMG. A novel *β*-glucosidase (Bgps) from *Phycicoccus sp. Soil748* was discovered and further characterized. This enzyme showed selective hydrolysis of the glucose moiety at C_6 position of CMG with higher substrate concentration, up to 80 mg/mL. Under the optimal reaction conditions, CMG was almost completely transformed into CA (99.2%). The present study provides not only an effective strategy of enzyme mining, but also a mild, high optimal substrate concentration and high-efficiency technology for the biotransformation of CMG into CA. In addition, the rational combination of commercial cellulase and Bgps provides an enzymatic catalysis way for the industrial production of CA from ASI.

Declarations of interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.procbio.2019.11.006.

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