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BIOCATALYSIS

Overexpression and characterization of a Ca^{2+} activated thermostable β-glucosidase with high ginsenoside Rb1 to ginsenoside 20(S)-Rg3 bioconversion productivity

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Abstract The thermostable β-glucosidase gene from *Thermotoga petrophila* DSM 13995 was cloned and overexpressed in *Escherichia coli*. The activity of the recombinant β-glucosidase was 21 U/mL in the LB medium. Recombinant β-glucosidase was purified, and its molecular weight was approximately 81 kDa. The optimal activity was at pH 5.0 and 90 °C, and the thermostability of the enzyme was improved by Ca^{2+}. The β-glucosidase had high selectivity for cleaving the outer and inner glucopyranosyl moieties at the C-20 carbon of ginsenoside Rb1, which produced the pharmacologically active minor ginsenoside 20(*S*)-Rg3. In a reaction at 90 °C and pH 5.0, 10 g/L of ginsenoside Rb1 was transformed into 6.93 g/L of Rg3 within 90 min, with a corresponding molar conversion of 97.9 %, and Rg3 productivity of 4620 mg/L/h. This study is the first report of a GH3-family enzyme that used Ca^{2+} to improve its thermostability, and it is the first report on the high substrate concentration bioconversion of ginsenoside Rb1 to ginsenoside 20(*S*)-Rg3 by using thermostable β-glucosidase under high temperature.

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Keywords *Thermotoga petrophila* · Recombinant enzyme · Enzymatic characterization · Biotransformation · Ginsenoside 20(*S*)-Rg3 preparation

Introduction

Panax ginseng C. A. Meyer has been used as traditional medicine for its remarkable beneficial effects on human health including strengthening immunity, providing nutrition and reducing fatigue [2]. The saponin triterpenes (or ginsenosides) have been proven to be the major active components of ginseng [9, 39]. So far, more than 40 different ginsenosides have been isolated and characterized; they show different chemical properties and pharmacological effects because of the complex structure combinations with different triterpenoid skeletons and sugar moieties [2, 41].

Among these ginsenosides, Rg3 has many pharmacological effects, including antitumor [12], anti-metastatic [17], neuroprotective [37], hepatoprotective [16], anti-diabetic [21], and vasodilating effects [13]. The ginsenoside Rg3 exists as two optical isomers, ginsenoside 20(*S*)-Rg3 and 20(*R*)-Rg3. The 20(*S*)-Rg3 isomer is superior to the 20(*R*)-Rg3 isomer in terms of its water solubility and bioavailability because of the spatial arrangement of the hydroxyl group on carbon 20 [18]. However, it is quite difficult to isolate ginsenoside 20(*S*)-Rg3 because it is extremely rare among the wild types of ginseng including red ginseng and Korean white ginseng. However, six ginsenosides, Rb1, Rb2, Rc, Rd, Re and Rg1, account for nearly 90 % of the ginsenosides in ginseng, and they share identical dammarane skeletons with the minor ginsenoside 20(*S*)-Rg3 [34]. Thus, many studies have focused on the conversion from some of the major ginsenosides to the more active minor

ginsenoside 20(*S*)-Rg3 by removing the outer and inner sugar moieties at the C-20 carbon.

Acid treatment and steam treatment can convert the major ginsenosides Rb1, Rb2, Rc, and Rd into ginsenoside 20(*S*)-Rg3 [11, 34], but it is time-consuming and complicated to isolate the 20(*R*)-Rg3 isomer that is inevitably formed as a byproduct [4]. Recently, microbial conversion has been applied to prepare ginsenoside 20(*S*)-Rg3 from ginsenoside Rb1. However, the compositionally complicated crude enzyme expressed by the microorganisms, such as *Aspergillus niger* and *Microbacterium* sp. GS514, was limited because it had poor selectivity in its hydrolysis capability to convert ginsenoside Rb1 [5, 6]. Furthermore, ginsenoside F2 and protopanaxadiol were mixed into the final product, which decreased the productivity [6].

Currently, recombinant enzymes can be easily expressed at high levels, recombinant β-glucosidase form *Microbacterium esteraromaticum* [25] and *Gordonia terrae* [31] could convert Rb1 into Rg3 at normal temperature, but they were limited by substrate concentration and Rg3 productivity. On the other hand, thermostable β-glucosidases have excellent properties and great potentials to be used as bio-catalysts for industrial bioconversion. Some thermostable recombinant enzymes have been obtained with one-step purification methods such as heat treatment or HisTrap affinity chromatography. The high reaction temperatures associated with thermophilic enzymes reduce the risk of microbial contamination and decrease the substrate viscosity, leading to high reaction velocities and improved rates of hydrolysis [10], and these enzymes are suitable for more extensive applications than the enzymes from mesophilic sources. Recombinant β-D-glucosidases from thermophilic organisms have been used to transform ginsenosides into Rare Gypenosides XVII and LXXV,compound K and compound Y [19, 20]. Until now, it has been difficult to find a thermophilic recombinant enzyme that has the capability to convert major ginsenosides to ginsenoside 20(*S*)-Rg3.

In this study, we report the cloning, overexpression, and characterization of Tpebgl3, a ginsenoside-hydrolyzing β-glucosidase from *Thermotoga petrophila* RKU-1 (DSM 13995). This enzyme has high selectivity for transforming ginsenosides Rb1 or Rd to the more active minor ginsenoside 20(*S*)-Rg3. These extraordinary properties make Tpebgl3 a good candidate for producing ginsenoside 20(*S*)-Rg3.

Materials and methods

Bacterial strains, plasmids, growth media, materials

Ginsenosides Rb1, Rd, (S)-Rg3, (R)-Rg3, Rb2, Rc, (S)-Rh2, F2, Re, Rg1 and (S)-Rh1 were purchased from Chendu Must Bio-Technology (Chendu, Sichuan, China). *p*-nitrophenyl (*p*NP)-β-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-α-L-arabinopyranoside, *p*NP-α-L-rhamnopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-β-D-xylopyranoside, and *o*-nitrophenyl (*o*NP)-β-D-glucopyranoside were purchased from Sigma–Aldrich (St Louis, MO, USA).

Thermotoga petrophila DSM 13995 was purchased from DSMZ (www.dsmz.de). It was grown anaerobically at 80 °C as described previously [35]. *Escherichia coli* BL21(DE3) was grown at 37 °C in Luria–Bertani (LB) medium and supplemented with ampicillin when it was required. The expression vector pET-20b (Novagen) was employed as a cloning and expression vector.

DNA manipulation

DNA was manipulated by standard procedures [3, 27]. A QIAGEN Plasmid Kit and a QIAGEN MinElute Gel Extraction Kit (Qiagen, USA) were employed for purifying the plasmids and PCR products. DNA restriction enzymes were purchased form TaKaRa (Dalian, China). DNA transformation was manipulated by using GenePulser (Bio-Rad, USA) for the electroporation.

Plasmid constructions

The β-glucosidase encoded gene Tpebgl3 was amplified from *T. petrophila* DSM 13995 genomic DNA by a polymerase chain reaction with Pfu DNA polymerase. The gene was amplified using the following primers: Tpebgl3-f (CGC *CAT ATG* ATG GGA AAG ATC GAT GAA A) and Tpebgl3-r (CCG *CTC GAG* TGG TTT GAA TCT CTT CTC T). The underlined sequences represent the restriction enzyme sites. The PCR products were digested with *Nde*I and *Xho*I and inserted into pET-20b at the *Nde*I and *Xho*I sites, then the expression plasmid pET-Tpebgl3 was obtained from the positive transformants.

Sequence analysis of Tpebgl3

The potential ORF of Tpebgl3 was searched for using the ORF search tool provided by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Databases were searched by using BLAST from NCBI and against CAZy (www.cazy.org). The multiple sequence alignment tool Clustal X1.9 was used for multiple protein sequence alignment [15].

Expression and purification of Tpebgl3

Escherichia coli BL21(DE3), transformed with the recombinant plasmid pET-Tpebgl3, was grown in Luria–Bertani

(LB) ampicillin medium at 37 °C to an optical density at 600 nm (OD_{600}) of 0.6. Protein expression was induced to express recombinant Tpebgl3 by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, and the bacteria were further incubated at 30 °C for approximately 8 h. Bacteria (200 mL) were harvested by centrifugation at 5000×g for 20 min at 4 °C, and they were washed twice with distilled water; resuspended in 50 mL of 5 mM imidazole, 0.5 mM NaCl, and 20 mM Tris–HCl buffer (pH 7.9); and French-pressed three times. The cell extracts were heat treated (75 °C, 20 min) and then cooled in an ice bath and centrifuged (20,000×g, 4 °C, 30 min). An immobilized metal affinity column (Novagen, USA) was used to purify the resulting supernatants, and the pure enzyme protein was eluted with 1 M imidazole, 0.5 M NaCl, and 20 mM Tris–HCl buffer (pH 7.9). The protein was examined by SDS-PAGE, and the protein bands were analyzed by density scanning with an image analysis system (Bio-Rad, USA). The protein concentration was determined by the Bradford method using BSA as a standard. The Bradford protein Assay Kit (Sangon Biotech, Shanghai, China) was employed for the determination.

Enzyme characterization

The specific activity of the purified Tpebgl3 was determined using *p*NP-β-D-glucopyranoside as a substrate in 50 mM sodium phosphate buffer and 1 mM *p*-nitrophenyl-β-D-glucopyranoside as substrates, the volume of the assay system was 200 μL, which was kept at pH 5.0 and 90 °C. The reaction was stopped after 5 min by adding 600 μL of 1 M Na_2CO_3 [24, 29]. The released *p*NP was immediately measured at 405 nm. For ginsenosides Rb1, Rd, (*S*)-Rg3, (*R*)-Rg3, Rb2, Rc, (*S*)-Rh2, F2, Re, Rg1 and (*S*)-Rh1 as substrates, the reaction mixture contained 50 mM sodium phosphate buffer (pH 5.0) and 1 mM ginsenoside substrates, and it was incubated for 5 min at 90 °C. The reaction was stopped by adding 250 μL of methanol, and the samples were assayed by HPLC. For 10 mM sucrose, cellobiose, laminaribiose and gentiobiose as substrates, the reaction was terminated in an ice bath. The released glucoses were measured by a glucose assay kit (Dingguo, China). One unit of enzyme activity was defined as the amount of enzyme necessary to liberate 1 μmol of *p*NP, ginsenosides Rd and Rg3, and glucose per min under the assay conditions.

The optimum pH for Tpebgl3 activity was determined by incubation at 90 °C for 5 min in a 50 mM sodium phosphate buffer from pH 3.5 to 7.5. The optimum temperature for the enzyme activity was determined by standard assays ranging from 65 to 100 °C in the 50 mM sodium phosphate buffer at pH 5.0. The results were expressed as percentages of the activity obtained at either the optimum pH or the optimum temperature. The pH stability of the enzyme was determined by measuring the remaining activity after incubating the enzyme at 75 °C for 1 h in the 50 mM sodium phosphate buffer from pH 3.5 to 7.5. After incubation, each enzyme sample was diluted by using 50 mM sodium phosphate buffer (pH 5.0) at 1:50 of dilution ratio. Then, the residual activity of the enzyme incubated at variant pH was determined, immediately. To determine the effect of temperature on the stability of Tpebgl3, the enzyme was pre-incubated in the 50 mM sodium phosphate buffer (pH 7.0) for various times at 70, 80 and 90 °C in the absence of the substrate. The activity of the enzyme without pre-incubation was defined as 100 %.

The effects of metals and chemical agents on the pure Tpebgl3 enzyme were determined. Ni^{2+}, Fe^{3+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, K^{+}, Al^{3+}, Li^{2+}, Cu^{2+}, Co^{2+} and Hg^{2+} were assayed at the final concentrations of 1 and 5 mM in the reaction mixture. The chemical agents DTT and EDTA were assayed at concentrations of 1 and 5 mM in the reaction mixture, respectively. The effects of organic solvents on the enzyme were determined by adding 5, 10, 15, and 30 % organic solvents (ethanol, methanol and DMSO) to the reaction mixture. The enzyme was incubated with each reagent for 10 min at 90 °C before adding *p*-nitrophenyl-β-D-glucopyranoside to initiate the enzyme reaction. The activity was determined as described above and was expressed as a percentage of the activity obtained in the absence of the chemical agents and metal cations.

The substrate specificity of the enzyme was tested by using *p*NP-β-D-glucopyranoside, ginsenosides Rb1 and Rd, *p*NP-β-D-galactopyranoside, *p*NP-α-L-rhamnopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-α-L-arabinofuranoside, sucrose, gentiobiose, laminaribiose and cellobiose. The kinetic constant of Tpebgl3 was determined by measuring the initial rates at various *p*NP-β-D-glucopyranoside concentrations (0.2, 0.4, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, and 2.4 mM) under standard reaction conditions.

Analysis of ginsenoside Rb1 degradation

The ginsenoside Rb1 was treated with purified Tpebgl3, and the degradation was analyzed by HPLC. The reaction mixture (50 μL) contained 50 mM sodium phosphate buffer (pH 5.0), 10 g/L of ginsenoside Rb1, and 0.75 U/mL of Tpebgl3. The reaction was performed for various durations at 90 °C and stopped by an ice bath. The reaction was measured by HPLC at various times.

HPLC analysis

The ginsenosides were analyzed using an HPLC 1200 system (Agilent, USA) and a C18 column (4.6 × 250 mm;i.d., 5 μm; S.No. USNH017518, USA)

with acetonitrile (A) and distilled water (B) at A/B ratios 30:70–60:40 and run times of 0–20 min, A/B ratios of 60:40 and run times of 20–25 min, A/B ratios of 60:40–30:70 and run times of 25–28 min, and A/B ratios of 30:70 and an ending run time of 32 min. The flow rate was 1.2 mL/min, and detection was performed by monitoring the absorbance at 203 nm.

Results

Analyzing the sequence of Tpebgl3

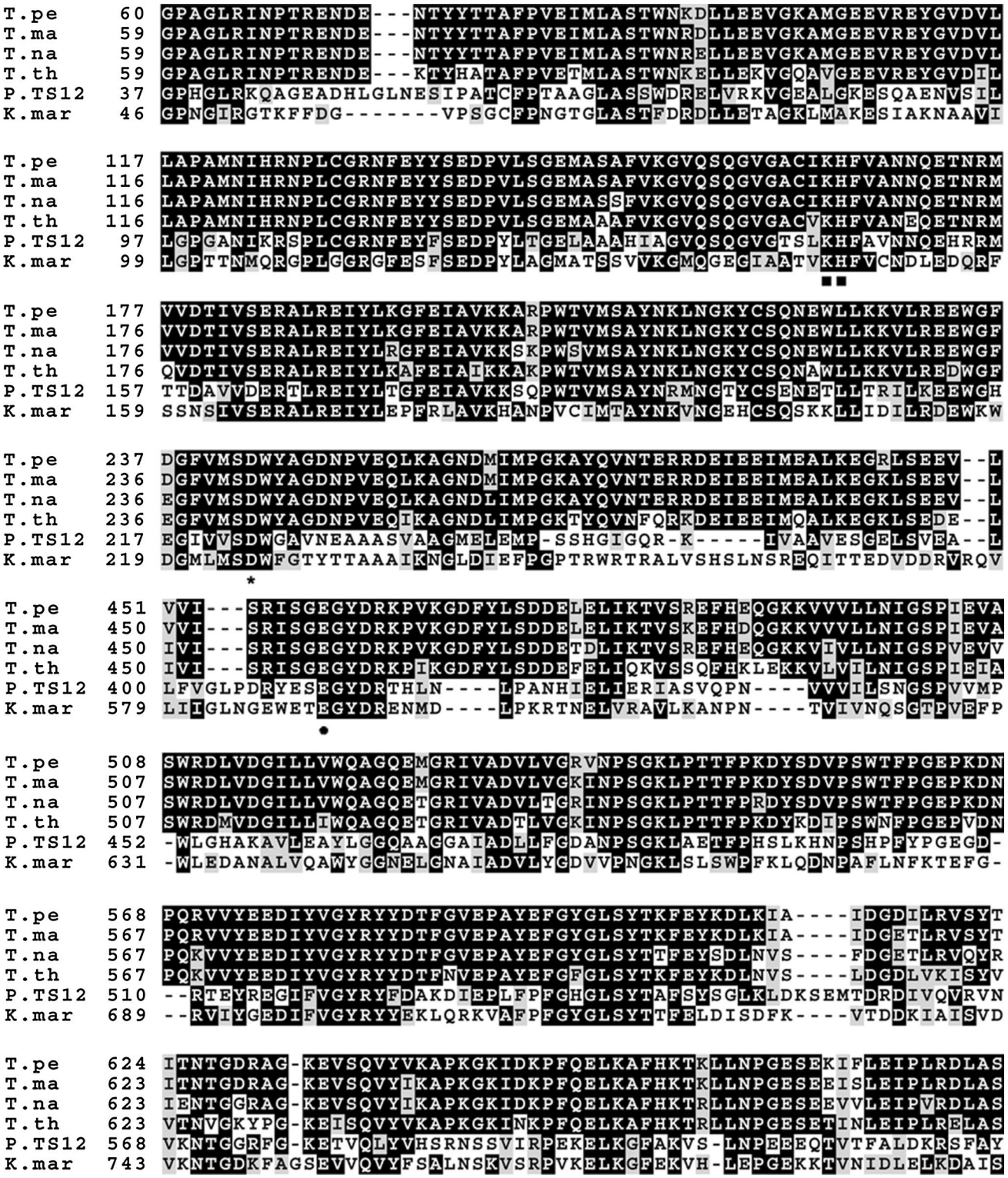
The full-length β-glucosidase gene Tpebgl3 (GenBank accession No. CP000702.1, Tpet_0898) was 2169 bp in length and encoded a protein of 722 amino acids with a predicted molecular weight of 81.243 kDa and a pI of 5.28. It shares a high similarity with thermophilic bacterial GH3 β-glucosidases, exhibits a 97 % identity with the β-glucosidase from *Thermotoga* sp. RQ2 (GenBank accession No. WP_012310822.1) and *T. maritima* (GenBank accession No. WP_004082478.1), shows 97 % homology to the β-glucosidase from *T. naphthophila* (GenBank accession No. WP_0128896088.1), and shares 88 % identity with the β-glucosidase from *T. neapolitana* (GenBank accession No. WP_015919165.1). Alignment of the Tpebgl3 cluster with several GH3 β-glucosidases indicated that they share some conserved motifs (Fig. 1): CIHKFV (residues 162–167), GFVMSDWYAGDN (residues 238–249) and IVISRISGEGYDRK (residues 452–463). Based on the crystalline structure analysis, the catalytic residues of *T. na*, *P. TS 12* and *K. mar* was clearly invistigeted, and there were also on the conserved motifs [33, 38, 40]. We predicted that the conserved residues D242 and E457 of Tpebgl3 (Fig. 1) are the typical catalytic nucleophile and catalytic acid of the GH3 enzyme, respectively. The conserved residue sequence HK is a putative carbohydrate-binding site [36]. Moreover, the protein encoded by Tpebgl3 is a member of the clusters of orthologous groups, the glycosyl hydrolase family 3 *N*-terminal superfamily and the glycosyl hydrolase family 3 C-terminal superfamily. Therefore, the results confirmed that Tpebgl3 belonged to a bacterial member of the GH3 family.

Cloning and overexpression of Tpebgl3

The β-glucosidase gene was amplified from the genomic DNA of *T. petrophila* DSM 13995. Then, the Tpebgl3 gene was successfully subcloned into the expression vector pET-20b (−) at *Nde*I and *Xho*I to generate the plasmid pET-Tpebgl3. The recombinant vector was transformed into *E. coli* BL21(DE3) and expressed by adding 0.5 mM IPTG at 30 °C for approximately 7 h. The target protein was overexpressed with a high β-glucosidase activity of approximately 21 U/mL.

Fig. 1 Comparison of the conserved sequences TpeBgl3 with family 3 Glycoside hydrolases by using Multi-alignment. Multiple sequence alignment of TpeBgl3 with selected glycoside hydrolase family 3 enzymes was performed by using Clustal X1.8. Full species names and Genbank IDs of the selected glycoside hydrolases in family 3 are as follows: *Thermotoga petrophila* RUK-1β-glucosidase (*T.pe*), WP_011943475.1; *Thermotoga maritime* β-glucosidase (*T. ma*), WP_004082478.1; *Thermotoga neapolitana* β-glucosidase (*T.ne*), ABI29899; *Thermotoga thermarum* β-glucosidase (*T.th*), WP_013932959.1; *Paenibacillus* sp.TS12 β-glucosidase (*P.TS12*), BAC16750; *Kluyveromyces marxianus* β-glucosidase (*K.mar*), ACY95404.1. The conserved residues and the three-dimensional protein construction of P. TS12, T. ne and K.mar have been analyzed. The filled diamond below the conserved sequence was indicated as a putative carbohydrate-binding site. The asterisk below the conserved sequence was indicated as the catalytic nucleophile site. The filled circle below the conserved sequence was indicated as the catalytic acid

The total cell protein, insoluble protein and soluble protein of the target protein, which were analyzed by SDS-PAGE (Fig. 2, lane 13–15), indicated that the concentration of the soluble target protein was higher than in the inclusion bodies. Theoretically, the concentration of the soluble target protein still has the possibility to increase by reducing the formation of inclusion bodies. The formation of inclusion bodies was affected by various factors: the insolubility of the product at the concentrations being produced, the inability to fold correctly in the bacterial environment, or the lack of appropriate bacterial chaperone proteins [28]. Therefore, we tried to design a different induction strategy, includes controlling the induction temperature and changing the IPTG concentration. The recombinant *E. coli* was induced at 30 °C with IPTG final concentrations from 0.01 to 0.5 mM, but the formation of inclusion bodies of Tpebgl3 did not significantly decrease (Fig. 2, lane 9, 12, 15). And the concentration of the soluble target protein almost kept the same level (Fig. 2, lane 8, 11, 14). Then, we expressed the gene at 26 °C with a 0.5 mM IPTG final concentration; the results showed that the inclusion bodies were reduced, but the production of total protein decreased at the same time. This decrease may have been caused by the slow growth rate result at the low induction temperature. Though we did not successfully increased the concentration of the soluble target protein furthermore, the soluble protein was high expressed at final induction condition (0.5 mM IPTG, 30 °C), approximately 21U/mL β-gulcosdiase activity was been detected. In addition, these phenomena also indicated that the induction strategy (IPTG concentration from 0,01 to 0.05 mM) could not effectively influence the expression level of the target protein Tpebgl3. Therefore, we tried to express the Tpebgl3 gene at 37 and 30 °C with no IPTG. Interestingly, the results showed that the inclusion bodies of Tpebgl3 clearly decreased (Fig. 2,



lane 1–6); approximately 13 U/mL of β-glucosidase activity was detected in the soluble fraction, which indicated that some thermostable β-glucosidase could be expressed without the expensive and toxic IPTG. This phenomenon was similar to the expression of Tt-BGL from *T. thermarum* [41], the unexpected expression with no IPTG may have been caused by a low concentration of lactose from the LB medium [32], which could activate the T7 promoter. Though the expression level of Tpebgl3 without IPTG was lower than which expressed at final induction condition,

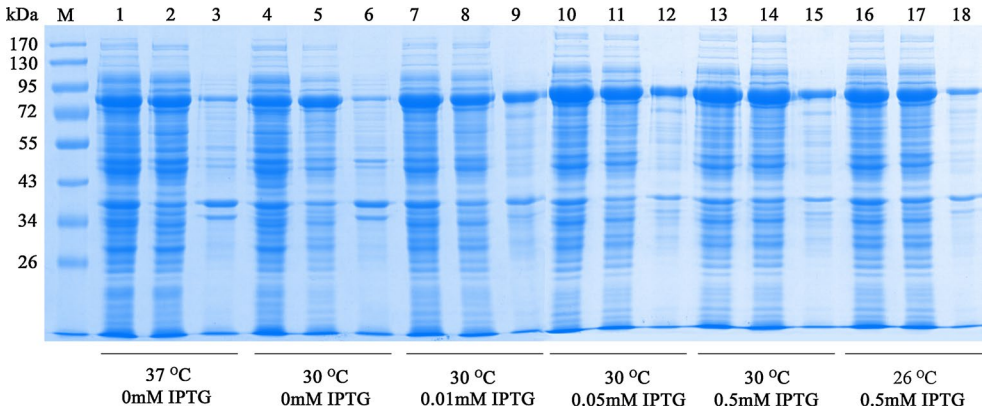


Fig. 2 SDS-PAGE analysis of the total protein, soluble protein and insoluble protein of the recombinant TpeBgl3 expressed in different induction condition. Lane M: protein molecular mass marker, lane 1, 4, 7, 10, 13, 16: the total protein of *E. coli* BL21 (DE3) harboring PET-TpeBgl3; lane 2, 5, 8, 11, 14, 17: the soluble protein of *E. coli* BL21 (DE3) harboring PET-TpeBgl3; lane 3, 6, 9, 12, 15, 18: the insoluble protein of *E. coli* BL21 (DE3) harboring PET-TpeBgl3

Table 1 Purification scheme for the recombinant protein TpeBgl3

Purification step	Total protein (mg)	specific activity (U/mg)	Total activity (U)[a]	Yield (%)	Purification (fold)
Crude extract	125.3	15.6	1960.4	100.0	1.0
Heat treatment	20.7	66.0	1362.3	69.5	4.2
Ni–NTA resin affinity chromatography	10.4	95.7	992.6	50.6	6.1

Substrates for TpeBgl3 were *p*NP-β-D-glucopyranoside

[a] One unit of TpeBgl3 was defined as the amount of enzyme liberating 1 μmol/min of *p*-nitrophenol

these results still have possibly provided a safe, convenient and cheap expression method for the potential application of thermostable enzymes in the food and healthcare product industries.

Purification and characterization of recombinant Tpebgl3

The Tpeblg3 gene was fused with a His-tag originating from the expression vector pET-20b (−), which was successfully overexpressed and purified simply by heat treatments and Ni^{2+}-affinity column chromatography. The specific activity of purified Tpebgl3 was 6.1-fold higher than that of the crude soluble fraction, and the purification yield was approximately 50.6 % (Table 1). All of these purification preparations gave a single band on an SDS-PAGE gel, and the molecular mass of the enzyme was estimated to be 81 kDa (Fig. 3, lane 3).

The enzymatic properties of Tpebgl3 were characterized by using the purified recombinant Tpebgl3. The optimal pH of Tpebgl3 was determined to be 5.0 (Fig. 4a). The enzyme retained over 50 percent of its optimum pH activity between 4.0 and 5.5, while the residual β-glucosidase activity was more than 50 percent of the original purified enzyme activity after 1 h of incubation at 75 °C in the pH range from

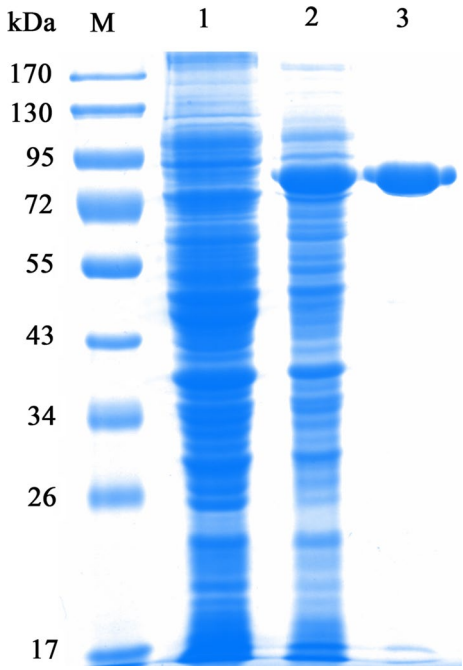


Fig. 3 SDS-PAGE analysis of recombinant TpeBgl3 expressed in *E. coli* BL21 (DE3). Lane M: protein molecular mass marker, lane 1: the crude extract of *E. coli* BL21 (DE3) harboring pET-20b, lane 2: the crude extracts of *E. coli* BL21 (DE3) harboring PET-TpeBgl3, lane 3: TpeBgl3 purified by Ni–NTA resin affinity chromatography

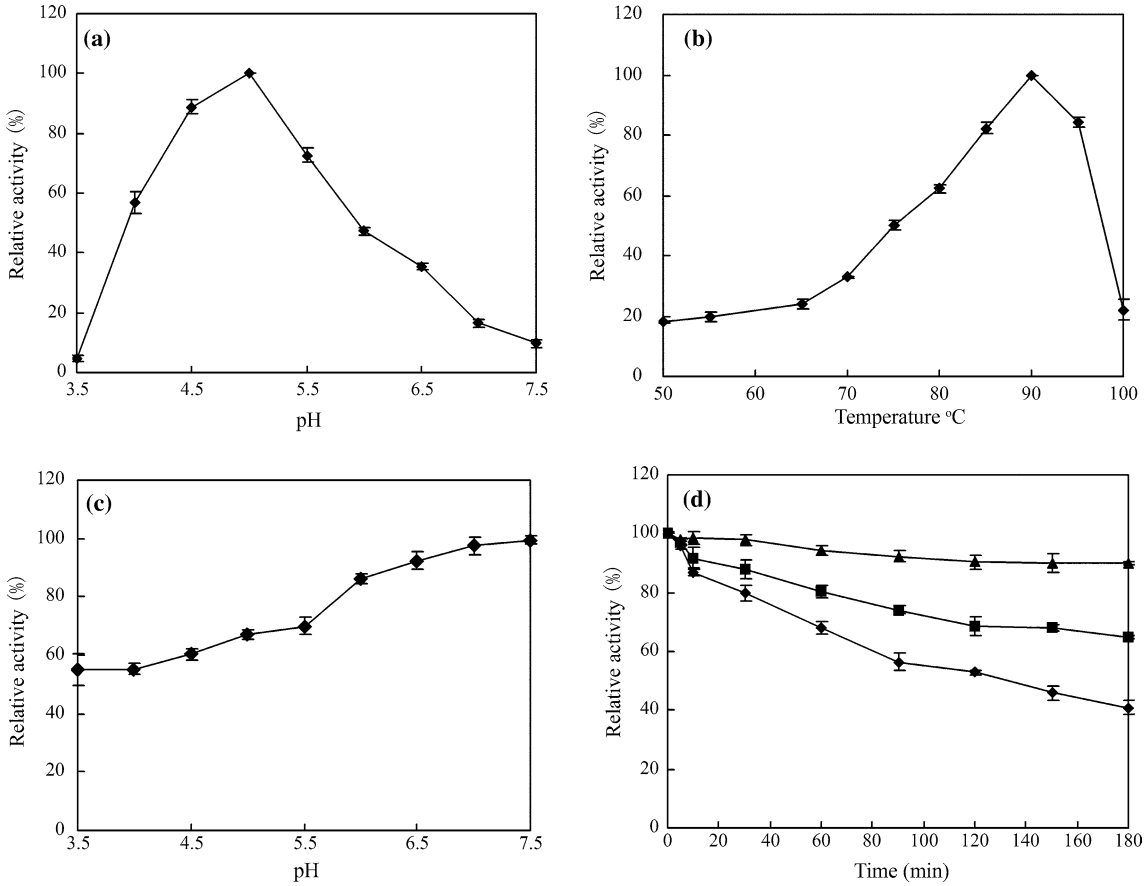


Fig. 4 The effects of pH and temperature on the activity and stability of the recombinant TpeBgl3. **a** Effect of pH on TpeBgl3 activity. **b** Effect of temperature on TpeBgl3 activity. **c** The pH stability of the enzyme TpeBgl3. **d** The thermostability of the enzyme TpeBgl3; the residual activity was monitored, while the enzyme was incubated at 90 °C (*filled diamonds*), 80 °C (*filled squares*), and 70 °C (*filled triangles*). The initial activity was defined as 100 %. These activities were expressed as relative values. Data represent the means of three experiments, and *error bars* represent the standard deviation

3.0 to 7.5 in the absence of the substrate. Furthermore, the recombinant Tpebgl3 was more stable at the neutral pH condition (Fig. 4c). The optimal temperature for Tpebgl3 was 90 °C, and the β-glucosidase activity was more than 50 percent of the maximum activity in the temperature range from 75 to 95 °C (Fig. 4b), which was similar to the thermostable GH3 β-glucosidases from *Thermotoga maritima* [8], *Dictyoglomus turgidum* [14] and *Dictyoglomus thermophilum* [42]. Few thermostable β-glucosidases isolated or cloned from thermophilic bacteria have been reported [7, 23]. The β-glucosidase from *Thermus thermophilus* HB27 and *Thermoascus aurantiacus* [23] were previously defined as thermostable β-glucosidases with optimal temperatures of 88 and 80 °C, respectively. The optimal temperature for Tpebgl3 activity (90 °C at pH 5) is one of the highest optimal temperatures compared with the published values for thermostable β-glucosidases. Thermostability assays of the recombinant Tpebgl3 showed that its residual activity was more than 50 % after being incubated at 90 °C for 3 h, and it retained more than 90 % of its activity after being incubated at 70 °C for 3 h (Fig. 4d).

The effects of various metal cations on the activities of Tpebgl3 were also investigated with final concentrations of 1 and 5 mM (Table 2). The activity of Tpebgl3 was completely inhibited by Hg^{2+}, and it was strongly inhibited by Al^{3+} and NH^{4+}. However, most of the other divalent metal cations had different effects on the activity of Tpebgl3. In particular, the activity of Tpebgl3 was significantly increased by Mn^{2+}, Ca^{2+} and Co^{2+}. The catalytic efficiency of the reaction system with Co^{2+} and Mn^{2+} added at 5 mM final concentrations was nearly 1.5 times higher than that of the metal ion-free controls. This result was similar to what was observed for the rBglA from *Thermotoga neapolitana* [22], indicating that Co^{2+} and Mn^{2+} could be stabilizing factors for the protein structure or the components of the catalytic active center. However, the effect of EDTA (5 mM) on the enzyme activity was not significant, which indicates that the chelating agent EDTA did not influence the β-glucosidase activity.

Table 2 Effects of metal cations and reagents on the recombinant protein TpeBgl3 activity

Cation of reagent[a]	Reletive activity	
	1 mM	5 mM
Contrl	100.0	100.0
Zn^{2+}	118.1	129.2
Ca^{2+}	98.5	158.2
K^{+}	118.1	122.5
Al^{3+}	63.3	32.6
Co^{2+}	120.4	196.6
Mg^{2+}	128.1	129.3
Mn^{2+}	164.0	181.9
NH_4^{+}	60.8	55.1
Ba^{2+}	126.1	145.7
Na^{+}	118.5	118.8
Hg^{2+}	0.0	0.0
Ni^{2+}	110.3	122.2
Cu^{2+}	105.4	59.4
Li^{+}	104.9	95.2
EDTA	103.0	89.5
DTT	98.3	103.2

Values shown are the mean of duplicate experiments, and the variation about the mean was below 5 %

[a] Final concentration, 1 and 5 mM as indicated

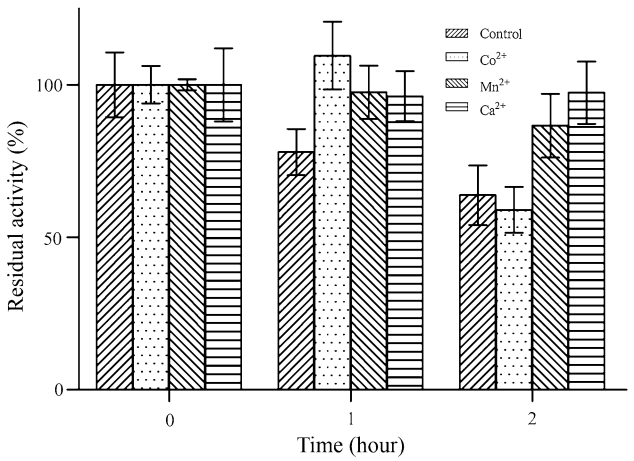


Fig. 5 The effect of Co^{2+}, $Mn^{2+}$and Ca^{2+} on the thermostability of the recombinant TpeBgl3. The 50μL incubate system contained 2 μg purified TpeBgl3, 50 mM pH 5.5 buffer and 5 mM metal iron, including Co^{2+}, $Mn^{2+}$and Ca^{2+}. The double distilled water replaced the metal iron as the Control. Samples were incubated at 90 °C for 0, 1 and 2 h, respectively. The average activity of Control from three experiments was defined as 100 %. Others were expressed as residual activity values. Data represent the means of three experiments, and error bars represent the standard deviation

These results indicated that divalent cations, such as Mn^{2+}, Ca^{2+} and Co^{2+}, were not necessary for enzymatic activity and that Tpebgl3 is not a metalloprotein. In addition, the

Table 3 Effects of organic solvent for the recombinant protein TpeBgl3 activity

Final concentration of organic solvent[a]	Relative activity (%)		
	Methanol	Ethanol	DMSO
0	100.0	100.0	100.0
5	114.0	102.0	85.9
10	104.4	99.3	84.6
15	94.0	80.6	74.2
30	80.3	54.5	57.8

Values shown are the mean of duplicate experiments, and the variation about the mean was below 5 %

[a] Final concentration of Substrate was 1.0 mM

Table 4 Substrate specificity of the recombinant protein TpeBgl3

Substrate[a]	Reletive activity (mean % ± SD)
p-Nitrophenyl-β-D-glucopyranoside	100 ± 0[b]
p-Nitrophenyl-β-D-galactopyranoside	ND[c]
p-Nitrophenyl-α-L-arabinofuranoside	ND
p-Nitrophenyl-α-L-arabinopyranoside	ND
p-Nitrophenyl-α-L-rhamnopyranoside	ND
p-Nitrophenyl-β-D-xylopyranoside	ND
o-Nitrophenyl-β-D-glucopyranoside	9.10 ± 0.16
Gentiobiose (10 mM)	9.08 ± 0.11
Cellobiose (10 mM)	1.89 ± 0.01
Laminaribiose(10 mM)	ND
Sucrose (10 mM)	ND

Data represents the means of three experiments, and the SD represents the standard deviation

[a] Final concentration of Substrate was 1.0 mM

[b] The activity against *p*NP-β-D-glucopyranoside was assumed to be 100 %, and corresponded to a specific activity of 81.4U/mg

[c] Not detected, specific activity is not detected by the analytical methods uesd in this stduy

β-glucosidase activity of Tpebgl3 was not affected by DTT, which is a well-known thiol group inhibitor, suggesting that sulfhydryl groups may not be involved in the catalytic center of the protein structure, also similar to the rBglA from *Thermotoga neapolitana* [22].

Next, we incubated Tpebgl3 with Co^{2+}, Mn^{2+} and Ca^{2+} at 90 °C for 0, 1 and 2 h. The results shown in Fig. 5 indicated that Ca^{2+} improved the thermostability of TpeBlg3 at high temperatures. The β-glucosidase activity of Tpebgl3 with a 5 mM Ca^{2+} final concentration remained at almost 100 % after 2 h of incubation at 90 °C, while the Tpebgl3 without metal ions only retained 50 % activity. These phenomena were similar to those observed in Xyn10A [30], CjXyn10A and CfCjXyn10A [1], so it is likely that the protein may have

Table 5 Substrate specificity of recombinant protein TpeBgl3 with different Ginsenosides

Type	Substrate[a]	Procuct	Reletive activity (mean % ± SD)[b]
PPD	Ginsenoside Rb1	Ginsenoside Rd	100 ± 1.21
	Ginsenoside Rb2		ND[c]
	Ginsenoside Rc		ND
	Ginsenoside Rd		83.60 ± 2.25
	Ginsenoside F2	Ginsenoside (*S*)-Rh2	90.86 ± 1.93
	Ginsenoside (*S*)-Rg3		ND
	Ginsenoside (*S*)-Rh2		ND
	Ginsenoside CK	APPD	35.27 ± 0.82
PPT	Ginsenoside Re	Ginsenoside Rg2	81.04 ± 0.73
	Ginsenoside Rg1	Ginsenoside (*S*)-Rh1	63.56 ± 1.25
	Ginsenoside (*S*)-Rh1		ND
	Ginsenoside Rg2		ND

[a] Final concentration of Substrate was 1.0 mM

[b] The activity against Ginsenoside Rb1 was assumed to be 100 %, and corresponded to a specific activity of 10.8U/mg

[c] Not detected, specific activity is not detected by the analytical methods uesd in this stduy

a metal-ion binding loop that has the capability to retain the thermostability of the protein structure by binding metal ions such as Co^{2+}, Mn^{2+} and Ca^{2+}. In addition, the longer half-life indicates less consumption of the enzyme; superior enzyme thermostability is desired for industrial production.

The residual enzyme activity was more than 80 percent with a concentration of methanol below 30 % and more than 50 percent with a concentration of ethanol and DMSO below 30 % (Table 3). The results showed that the enzyme could be applied for biotransformation in the presence of an organic solvent, showing the organic solubility property of some special ginsenosides.

Substrate specificity and enzyme kinetic assays

The substrate specificity of Tpebgl3 was determined by using 1.0 mM *p*NP- and *o*NP-glycosides. The results summarized in Table 4 show that Tpebgl3 was most active against *p*NPGlc, followed by *o*NPGlc, while no enzyme activity was detected against *p*NPGal, *p*NPXyl, *p*NPArf, *p*NPArp and *p*NPRha. These results indicate that Tpebgl3 has highly selective specific activity on residual glucose and has a strong affinity to aryl-β-glucose. Furthermore, Tpebgl3 also has the catalytic capability to hydrolyze oligosaccharides because it has β-glucosidase activity against cellobiose, gentiobiose, and ginsenoside Rb1. Moreover, it also hydrolyzes the alkyl-β-glucose linked to carbon 20 of ginsenoside Rd (Fig. 5). These results indicated that Tpebgl3 has broadly specific β-glucosidase activity against a wide range of substrates with different glycosidic bonds, including aryl-β-glucosidic bonds and alkyl-β-glucosidic bonds, and oligosaccharides [26]. Additionally, we detected the substrate specificity of Tpebgl3 about different ginsenosides (Table 5), which indicated that Tpebgl3 has the highest specific activity on ginsenoside Rb1, followed by ginsenoside F2, ginsenoside Rd, ginsenoside Re, ginsenoside Rg1 and ginsenoside CK. Tpebgl3 did not show catalytic activity on ginsenoside (*S*)-Rh1 and ginsenoside (*S*)-Rh2, which indicates that Tpebgl3 cannot hydrolyze the aryl-β-glycosidic bond in PPD- and PPT-type ginsenosides.

The dependence of the rate of the enzymatic reaction on the substrate concentration followed Michaelis–Menten kinetics, with K_m and V_{max} values of 1.6 mM and 109 U/mg for *p*NPGlu and 0.35 mM and 11.2 U/mg for ginsenoside Rb1 under optimal conditions.

Analysis of ginsenoside Rb1 degradation

The hydrolysis product ginsenoside Rg3 exists as 20(S) and 20(R) optimal isomers, and there are two pathways for the bioconversion of ginsenoside Rb1 to Rg3: graded hydrolysis of the two glucoses at C-20 (Fig. 6a) or direct hydrolysis of the inner glucoses at C-20 (Fig. 6b) [25]. To verify the ginsenoside Rb1 biotransformation by Tpebgl3, a time-course experiment was performed, and the hydrolyzed products were analyzed by HPLC. As shown in Fig. 7, the reaction mixture (50 μL), containing 50 mM buffer (pH

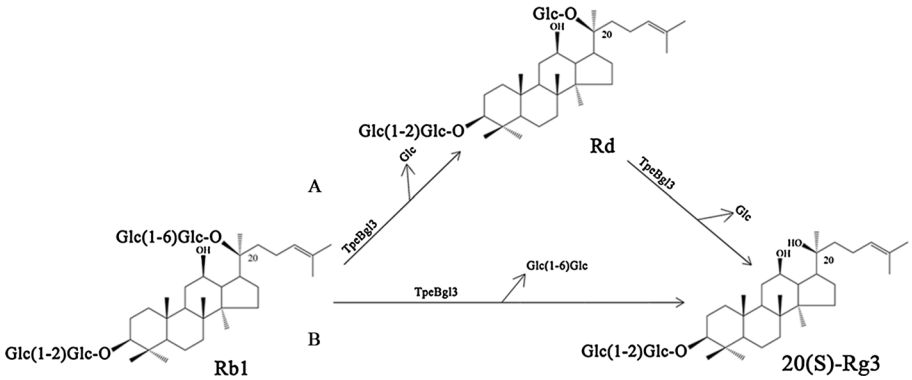


Fig. 6 Biotransformation pathway for production of ginsenoside Rb1: *A* ginsenoside Rb1 was gradually hydrolyzed into ginsenoside (S)-Rg3; *B* ginsenoside Rb1 was directly converted to ginsenoside (*S*)-Rg3

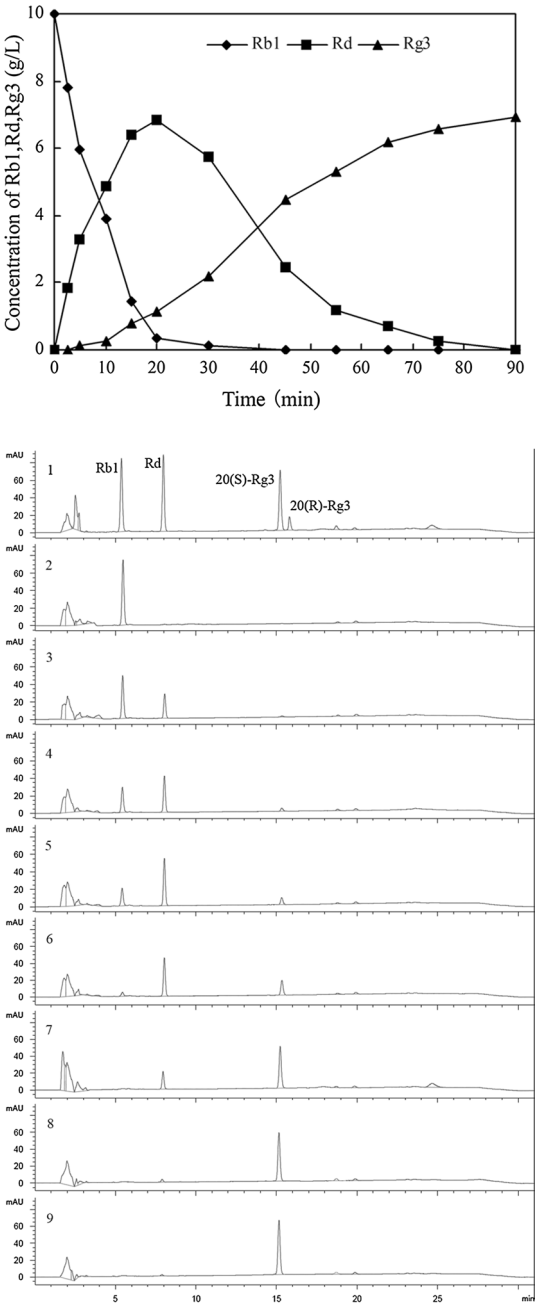


Fig. 7 HPLC analysis of ginsenoside Rb1 hydrolysis by TpeBgl3,1: ginsenoside Rb1, ginsenoside Rd and ginsenoside 20(*S*)-Rg3, lane 2, 3, 4, 5, 6, 7, 8, 9: ginsenoside Rb1 (10 g/L) incubated with TpeBgl3 (12 U/mL) for 0, 5, 10, 15, 30, 45, 75, and 90 min, respectively

5.0), 10 g/L of ginsenoside Rb1, and 10 U/mL of Tpebgl3 in 0.1 mL, was incubated for 90 min at 90 °C. The ginsenoside Rb1 was almost completely converted after 30 min of reaction, and the concentration of ginsenoside Rd was gradually reduced after it increased. Otherwise, the concentration of ginsenoside Rg3 continuously increased after it was detected at a reaction time of 10 min. At the end of the reaction, the final product was identified as ginsenoside 20(*S*)-Rg3 (Fig. 7). As shown in Fig. 7, 10 g/L of ginsenoside Rb1 was transformed into 6.93 g/L of ginsenoside Rg3 after a reaction time of 90 min, which represents a molar conversion yield of 97.9 %.

It reported the ginsenodise Rg3 was also prepared by using crude β-gulcosidases from *Aspergillus niger* [5], *Microbacterium* sp. GS514 [6]. Since the crude β-gulcosidases have complicated properties, which reduced its selective hydrolysis ability and substrate specificity. In addition, the ginsenoside F2 was detected in the final products [6], which affected the productivity of ginsenoside Rg3. Therefore, some recombinant β-gulcosidases from *Microbacterium esteraromaticum* [25] and *Gordonia terrae* [31] was used to produce ginsenoside Rg3, which could selectively convert Rb1 into Rd without other byproducts. While the specific activity of Tpebgl3 against ginsenoside was higher than the β-gulcosidase from *Gordonia terrae* [31]. The Rg3 productivity of Tpebgl3 was 4620 mg/L/h, while the Rg3 productivity of Bgp1 from *Microbacterium esteraromaticum* [25] and β-gulcosidase from *Gordonia terrae* [31] was 71 and 1132 mg/L/h, respectively. In addition, the yield of them were 100 %, which was higher than the yield of Tpeblg3, We believed the loss of product may caused by high temperature. Otherwise, the β-gulcosidase from *Gordonia terrae* had higher specific activity against Rb1 than which against Rd [31]. This was similar to Tpebgl3 (Table 5), which indicated that Rb1 was the optimum substrate for Tpebgl3 on biotransformation.

These results also suggested that Tpebgl3 hydrolyzed the ginsenoside Rb1 along the pathway Rb1→Rd→Rg3 (Fig. 6a) [25], suggesting hydrolysis of the outer glucose molecules at position C-20, followed by hydrolysis of the inner glucose molecules at the same position. Additionally, Tpebgl3 shows specific affinity for the glucose at position C-20, which indicates that the recombinant Tpebgl3 is highly selective for biotransforming the ginsenoside Rb1 into 20(*S*)-ginsenoside Rg3 with high productivity.

Conclusions

A thermostable β-glucosidase, Tpebgl3 from *T. petrophila* DSM13995, was cloned and overexpressed in *E. coli* BL21 with IPTG. The recombinant protein could be expressed without IPTG. The biochemical characterization showed that Tpebgl3 had a high optimal temperature, and the thermostability was improved by Ca^{2+}. Tpebgl3 also had a highly selective capability to hydrolyze the alkyl-β-glycosidic bond and the 1,6 glycosidic bond linked to the C-20 carbon of ginsenoside Rb1, and it had high catalytic efficiency for biotransforming ginsenosides Rb1 or Rd to the minor ginsenoside 20(*S*)-Rg3, which has greater pharmacological activity. This study demonstrates that

recombinant Tpebgl3 has great potential for industrial applications, including bioconversion for producing natural medicine.

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