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Characterization of a novel thermostable glucose-tolerant GH1 β-glucosidase from the hyperthermophile *Ignisphaera aggregans* and its application in the efficient production of baohuoside I from icariin and total epimedium flavonoids

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ABSTRACT

The minor flavonoid baohuoside I from *Herba epimedii* has better bioactivities than its precursor compounds icariin and other major epimedium flavonoids. In this study, a novel β-glucosidase gene (*Igag_0940*) was cloned and expressed to improve the conversion efficiency in the process of baohuoside I production. For the first time, the recombinant IagBgl1 was purified and then identified uniquely as a trimer in GH 1 family protein from Archaea. The maximum activity of recombinant IagBgl1 was exhibited at 95 °C, pH 6.5, and it retained more than 70% after incubation at 90 °C for 4 h. IagBgl1 had a high catalytic activity towards icariin with a K_{cat}/K_m ratio of 488.19 $mM^{-1}{\cdot}s^{-1}$. Under optimized conditions (65 °C, pH 6.5, 0.8 U/mL enzyme, and 90 min), 10 g/L icariin was transformed into 7.564 g/L baohuoside I with a molar conversion of 99.48%. Meanwhile, 2.434 g/L baohuoside I was obtained from 10 g/L total epimedium flavonoids by a two-step conversion system built with IagBgl1 and two other thermostable enzymes. This is the first report of enzymatic conversion for producing baohuoside I by thermostable enzymes.

1. Introduction

Epimedium flavonoids, a series of flavonoid derivatives consisting of isoprenoid and methoxyl substitutions, have been proven to be the primary bioactive constituents of *Herba Epimedii*. Among the total flavones of Epimedii (TFE) from plants, epimedium flavonoids usually exist in the form of glycosides that are substituted with glucosyl, rhamnosyl or xylosyl residues in various numbers and positions [1]. Among them, four multiple glycosides with high contents, namely, epimedin A, epimedin B, epimedin C, and icariin (Fig. 1), were classified as major epimedium flavonoids (contents of 1.0–2.6% in raw material of Epimedii species). Interestingly, a monoglycoside baohuoside I with quite low contents (less than 0.15% in raw material of Epimedii species) was confirmed to exhibit various attractive pharmacological activities, such as antiosteoporosis [2], antioxidant [3], and anti-osteoblast [4] activities. Moreover, baohuoside I displayed better and broader bioactivities than the major epimedium flavonoids, such as icariin, in many aspects. It was not only reported to enhance the differentiation of osteoblasts but also found to inhibit the differentiation of osteoclasts [5]. Meanwhile, it was found that baohuoside I had a significant inhibition effect on various types of cancers, including osteosarcoma [5], epidermoid carcinoma [6], breast cancer [7], acute myeloid leukemia[8], and hepatoblastoma [9], while icariin did not. Moreover, baohuoside I exhibited greater bioavailability than icariin [10]. The lower polarity of baohuoside I made it more efficiently absorbed into capillaries in the small intestine. Due to the attractive benefits of baohuoside I and the high structural similarity between icariin and baohuoside I, many studies were focused on the preparation of baohuoside I from icariin by releasing its glucose residue. Acid hydrolysis, microbial conversion, and enzymatic transformation have been explored and confirmed as possible ways to produce baohuoside I [11–13]. With high selectivity, high efficiency and mild reaction conditions, the enzymatic transformation method exhibited great application potential for the production of baohuoside I from icariin. Several

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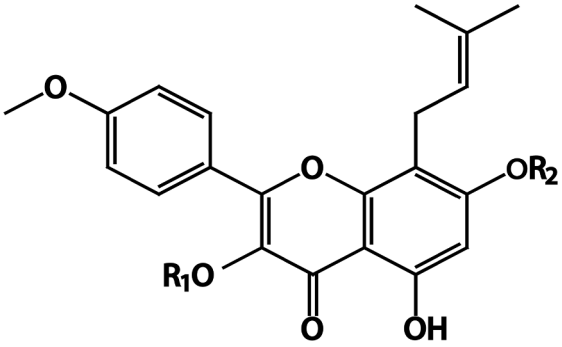


R_1	R_2	Compounds
-Rha-Glc	-Glc	Epmedin A
-Rha-Xyl	-Glc	Epmedin B
-Rha-Rha	-Glc	Epmedin C
-Rha	-Glc	Icariin
-Rha-Glc	-H	Sagittatoside A
-Rha-Xyl	-H	Sagittatoside B
-Rha-Rha	-H	Sagittatoside C
-H	-Glc	Icariside I
-Rha	-H	Baohuoside I
-H	-H	Icaritin

Fig. 1. The chemical structures of epimedium flavonoids.

commercial enzymes [14], including dextranase, cellulase, and β-glucosidase, were reported to be capable of hydrolyzing icariin into baohuoside I; however, commercial β-glucosidase exhibited a better transformation capability.

β-Glucosidase is well known as one of the main hydrolases in cellulose saccharification; thus, it has been widely used in lignocelluloses biorefineries. However, the functions of β-glucosidase include but are not limited to the hydrolysis of cellulose. Based on its catalysis specificity, β-glucosidase can be classified into three clades [15]: i) aryl-β-glucosidase displays a strong affinity to aryl-β-glucoside, ii) cellobiase hydrolyzes oligosaccharides only, and iii) the third clade of β-glucosidases exhibits broad specificity for a wide range of substrates, including oligosaccharides, aryl-glycosides and alkyl-glycosides with different glycosidic bonds [16,17]. Recently, the third clade of β-glucosidases has attracted increasing attention because of its application potential as a biocatalyst for obtaining glycoside secondary metabolites from natural resources, including polyphenols [18], flavonoids [19], and saponins [20]. Some thermostable β-glucosidases exhibited significant advances in two aspects: i) they exhibited higher specific activity and a stronger catalysis capability for natural glycosides [21], and ii) thermostable β-glucosidase with a high reaction temperature exhibited natural advantages for improving substrate solubility, enhancing the mass transfer rate and reducing the risk of contamination [22]. Thus, thermostable β-glucosidase has been used in industrial applications as an ideal catalyst for the conversion of natural glycosides [23,24]. The use of thermostable β-glucosidase with high specifies to icariin might be a feasible option for further improving the transformation efficiency of baohuoside I production.

In this paper, a novel GH1 thermostable putative β-glucosidase, IagBgl1, from *Ignisphaera aggregans* was cloned and characterized as a multifunctional glycoside hydrolase with β-glucosidase/β-xylosidase/β-galacosidase/α-arabinosidase activities. It displayed high selectivity for producing baohuoside I from icariin with high productivity. Moreover, it exhibited excellent thermostability, organic solvent resistance and sugar tolerance. These extraordinary properties make IagBgl1 more suitable for production of baohuoside I under high concentrations of icariin. Meanwhile, the total epimedium flavonoids (TEF) was used as a substrate to produce baohuoside by a two-step conversion system consisting of IagBgl1 and two other thermostable enzymes from Thermotoga petrophila.

2. Materials and methods

2.1. Materials

The artificial substrates, including *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), *p*NP-β-D-galactopyranoside (*p*NPGal), *p*NP-α-L-arabinopyranoside (*p*NPArp), *p*NP-β-D-xylopyranoside (*p*NPXyl), *p*-nitrophenyl-α-L-rhamnopyranoside (*p*NPR*)*, and *p*NP-α-L-arabinofuranoside (*p*NPArf), were purchased from Sigma-Aldrich (St Louis, MO, USA). The standards, including epimedin A, epimedin B, epimedin C, icariin, sagittatoside A, sagittatoside B, sagittatoside C, baohuoside I, and icaritin were purchased from Chendu Must Bio-Technology (Chendu, Sichuan, China). The TFE, containing epimedin A (2.65%), epimedin B (5.73%), epimedin C (12.23%), icariin (18.53%), and baohuoside I (1.95%), was obtained from Sobeo biotech Co. Ltd. (Xian, Shanxi Province, China). The recombinant rhamnosidase TpeRha and recombinant xylosidase TpeXyl3 were kindly provided by Professor Linguo Zhao from the Department of Biopharmaceuticals, Institute of Chemical Engineering, Nanjing Forestry University, Nanjing, China.

2.2. Bioinformatic analysis of IagBgl1

BLAST from the NCBI was employed to search databases and obtain sequence identity. Clustal X version 2.0 was used for multiple alignments of protein sequences [25,26]. ESPript 3.0 was applied to generate protein alignment figures. The phylogenic trees were created by Mega 6.0.

2.3. Expression and purification of IagBgl1

The target gene *Igag_0940* from *Ignisphaera aggregans* DSM17230 (CP002098.1) was synthesized by Generay (Generay Biotechnology, Co., Ltd., Shanghai, China). The codons of coding gene IagBgl1 were optimized following the codon bias of *Escherichia coli*. The synthesized *Igag_0940* was inserted into pET20b vector (Novagen Company, USA) by using *Nco*I and *Xho*I restriction sites at the 5′-end and 3′-end, respectively. The positive transformant was obtained by screening with LB plates, adding 100 μg/mL ampicillin (Amp). The optimal inducer concentration was determined by adding different concentrations (0 mM, 0.005 mM, 0.01 mM, 0.05 mM, 0.1 mM, and 0.25 mM) of isopropyl-β-D-thiogalactoside (IPTG); then, the bacterium was incubated at 32 °C for approximately 7 h. The recombinant IagBgl1 was expressed after adding IPTG to the culture with a final concentration of 0 mM and 0.005 mM and incubation at different temperatures (27 °C, 32 °C, 37 °C, and 42 °C). All the pellets incubated under different induction conditions were collected through centrifugation (8,000*g*, 4 °C) for 20 min after ice-baths.

The positive transformants were grown in 400 mL of Amp-resistant LB medium at 37 °C for 7 h. All pellets from 1.2 L mediums (3 × 400 mL) were harvested and resuspended by using binding buffer (containing 25 mM Tris-HCl, 500 mM sodium chloride, and 20 mM imidazole); then, the pellets were fully treated by ultrasonication in an ice-bath. The soluble enzyme was obtained by centrifugation of crude enzyme under 12,000*g* at 4 °C for 25 min. Then, the enzyme was loaded on a Ni^{2+} affinity chromatography column. The purified recombinant IagBgl1 was obtained by using elution buffer (containing 25 mM Tris-

HCl, 400 mM sodium chloride, and 20 mM imidazole). To remove imidazole, the purified protein IagBgl1 was dialyzed with phosphate buffer (50 mM citrate phosphate, 200 mM sodium chloride, pH 6.5). As a standard of the Bradford method, bovine serum albumin (BSA) was used for the determination of protein concentration, and the protein homogeneity was estimated by SDS-PAGE (12%).

2.4. Size exclusion chromatography ofrecombinant IagBgl1

The molecular weight of purified IagBgl1 was analyzed by size exclusion chromatography (*SEC*). Approximately 250 μg of the sample was loaded on a Superdex 200 10/300 GL column (General Electric Healthcare Company, USA), which was equilibrated with 50 mM citrate phosphate buffer (pH 6.5, 0.2 M sodium chloride) at 2 column volumes (CVs). A constant rate of 0.75 mL/min was applied for elution with a 1.5 CV. The standard curve of this column was calibrated with protein molecular weight standards, includingovalbumin (44 kDa), albumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa), thyroglobulin (669 kDa) and blue dextran (2000 kDa).

2.5. Assay of enzyme activity and characterization of therecombinant IagBgl1

The enzyme activity assays were performed as previously reported [27]. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol *p*-nitrophenyl (*p*NP) or glucose under assay conditions.

The substrate specificity of IagBgl1 was detected by the following substrates: *p*NPG, *p*NPGal, *p*NPArp, *p*NPXyl, *p*NPR, *p*NPArf, cellobiose,sucrose and lactose. The reaction mixture (200 μL) contained 1 mM artificial substrate or 10 mM oligosaccharides and 25 mM citrate phosphate buffer (pH 6.5). The reaction was stopped by adding 600 μL of 1 M Na_2CO_3 after incubation for 10 min. The quantity of released *p*NP was determined by measuring the ultraviolet absorbance at 405 nm. A glucose assay kit (Jiancheng Biotech Co., Ltd., Nanjing, China) was used to detect the amount of released glucose when cellobiose, sucrose and lactose were used as substrates.

The optimum pH of IagBgl1 was determined at 90 °C with 25 mM citrate phosphate buffer (pH 3.0–7.5) and Tris-HCl (pH 7.5–9.0). pH stability was examined by pre-incubation of IagBgl1 at 90 °C for 2 h in a pH range from 3.0 to 9.0, and then the residue activity of IagBgl1 was determined under 95 °C at pH 6.5. The optimum temperature of IagBgl1 was determined by performing the β-glucosidase assay in a temperature range from 50 °C to 100 °C under pH 6.5. The thermostability of IagBgl1 was measured by incubating enzyme at a temperature range from 70 °C to 100 °C for 4 h under pH 6.5. Then, the residual activities of IagBgl1 incubated for different durations were obtained by measuring β-glucosidase activity.

The effects of chemicals on IagBgl1 were determined by measuring the β-glucosidase activity by adding different reagents to reaction mixtures: 1. metal ions (Mn^{2+}, Mg^{2+}, Cu^{2+}, Ca^{2+}, Co^{2+}, Li^{+}, Zn^{2+}, Ni^{2+} and Ba^{2+}) and the chelating reagent EDTA were added at final concentrations of 1 mM and 5 mM, respectively; 2. three organic reagents (methanol, ethanol and DMSO) were added at final concentrations of 1%, 2%, 5% and 10% to the reaction mixtures; and 3. four kinds of monosaccharides, including glucose, arabinose, galactose and xylose, were added to the reaction mixture with final concentrations ranging from 0 mM to 3000 mM. All residual activities were measured under standard conditions (pH 6.5, 90 °C).

2.6. The kinetic parameters of recombinant IagBgl1

To obtain the kinetic parameters of recombinant IagBgl1 via artificial substrates, three artificial substrates, pNPG (0.2–1.2 mM), pNPGal (1–10 mM) and pNPX (1–10 mM), were added to the reaction system with different final concentrations. All mixtures were reacted under standard assay conditions.

To obtain the kinetic parameters of IagBgl1 via the major epimedium flavonoids, icariin, epimedin A, epimedin B, and epimedin C were added to the reaction mixture with final concentrations from 0.02 mM to 0.4 mM. The reactions were stopped by adding 400 mL of methanol after incubating at 95 °C for 10 min, and then all samples were passed through a 0.22 μm filter before HPLC analysis.

2.7. Enzymatic transformation of icariin into baohuoside I

The reaction mixture (100 uL) contained 20 mM citrate phosphate buffer (pH 6.5) and 10 g/L icariin. To obtain the optimal conversion conditions for producing baohuoside I from icariin, the effects of reaction factors, including reaction temperature, reaction pH, enzyme dosage, icariin concentration and transformation duration, were investigated.

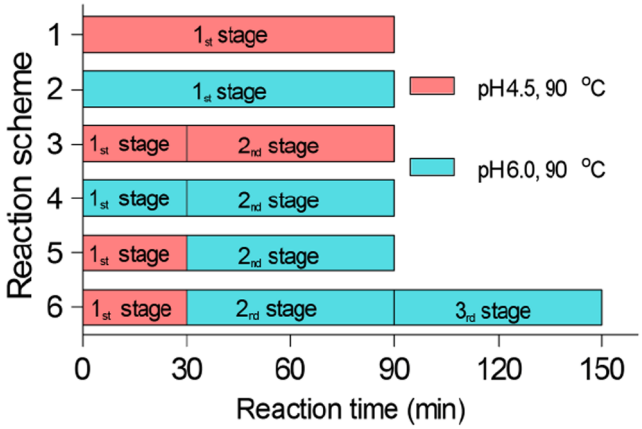
To determine the suitable reaction temperature and pH condition of for converting icariin into baohuoside I by IagBgl1, icariin and IagBgl1 were added to the reaction mixtures with final concentrations of 1 g/L and 50 U/L, respectively. The optimal conversion temperature for IagBgl1 was obtained by detecting the product concentration of conversion mixtures that were incubated in the range from 70 °C to 100 °C at pH 6.5. The optimal conversion pH for IagBgl1 was obtained by detecting the product concentration of conversion mixtures with 20 mM citrate phosphate buffer from a pH range of 4.0 and 7.5 under 90 °C. The suitable icariin concentration was obtained by determining the product concentration of conversion mixtures in the range from 1 g/L to 10 g/L icariin by adding 500 U/mL IagBgl1 at 90 °C and pH 6.5. The optimal enzyme dosage was obtained by determining the product concentration of conversion mixtures by adding IagBgl1 at final concentrations from 550 U/mL to 850 U/mL and reacting under optimal conditions (pH 6.5, 90 °C, 10 g/L of icariin). After 90 min of incubation at 90 °C and pH 6.5, the time effect curve for enzymatic transformation was generated by estimating the amounts of substrate and products with the addition of 800 U/L IagBgl1 and 10 g/L icariin. All the conversion reactions were stopped in an ice bath. Then, all the samples were sufficiently vibrated after adding 400 μL of methanol and passed through a 0.22 μm filter. Finally, the products of conversion mixtures were detected by an HPLC analysis system.

2.8. The enzymatic transformation of baohuoside I from total epimedin flavonoids by three thermostable enzymes

The enzymatic properties of α-L-rhamnosidase TpeRha, β-D-xylosidase TpeXyl3 and β-D-glucosidase IagBgl1 were determined by using their optimal artificial substrates. To improve the productivity of baohuoside I, six different enzymatic conversion schemes were designed to select a suitable addition protocol of these three enzymes. The details are summarized in Fig. 2.

The optimal dosage of TpeRha was measured by the product content of the reaction mixture with the addition of TpeRha at final concentrations of 25, 30, 45, 50, 55, and 60 U/mL for 30 min of incubation in the first transformation stage. The optimal incubation time for the first transformation stage was detected by estimating the product content of the reaction mixture after incubation for 10, 15, 20, 25, 30, and 35 min.

To detect the reaction factors of the second transformation stage, all reaction mixtures were incubated at 90 °C for 30 min after adding 55 U/mL TpeRha, and then these reaction mixtures were mixed with IagBgl1 at final concentrations of 0.2, 0.4, 0.6, 0.8, 1, and 2 U/mL. The optimal dosage of IagBgl1 was obtained by measuring the product content of reaction mixtures incubated at 90 °C for 60 min after adding IagBgl1. Meanwhile, the reaction mixtures previously treated by TpeRha were mixed with 2 U/mL Iagabel1 and TpeXyl3 added at final concentrations of 0.2, 0.4, 0.6, 0.8, 1, and 2 U/mL. Then, the optimal dosage of TpeXyl3 was obtained by measuring the sagittatoside B content of the



Scheme	1st stage	2nd stage	3rd stage
	enzyme, dosage (U/mL)		
1 / 2	TpeRha, 70 IagBgl1, 1 TpeXyl3, 150	–	–
3 / 4 / 5	TpeRha, 70	IagBgl1, 1 TpeXyl3, 150	–
6	TpeRha, 70	IagBgl1, 1	TpeXyl3,150

Fig. 2. The schematic diagram for six different transformation schemes built with three enzymes.

reaction mixtures after incubation at 90 °C for 60 min.

The variation curve for two-step enzymatic transformation was generated by estimating the amounts of substrate and products upon the addition of TpeRha at 55 U/mL for 30 min in the 1st stage and 1 U/mL IagBgl1 and 200 U/mL TpeXyl3 for 60 min in the 2nd stage. All the samples were analyzed by HPLC after adding 400 μL of methanol, sufficiently vibrated, and passed through a 0.22 μm filter.

2.9. HPLC analysis for the identification of transformation products

All the samples were analyzed by using an HPLC 2695 system (Waters, USA) with a Poroshell 120 EC-C18 column (3 × 150 mm, 2.7 μm; S.No. USCFW12101) in the stationary phase, with 0.1% acetic acid solution (A) and acetonitrile (B) as the mobile phases. The injection volume was 20 μL for each sample, the flow rate was 0.3 mL/min, the column temperature was 40 °C, and the absorbance at 269 nm was monitored.

A 46 min binary gradient elution was employed for the analysis of icariin and baohuoside I. An isocratic elution of 25% solvent B lasted for the initial 5 min; a linear gradient elution of 25–30% solvent B was performed from 5 to 25 min, followed by a linear gradient elution of 30–63% solvent B from 25 to 32 min; this was followed by a linear gradient elution of 63–95% solvent B from 32 to 40 min; and finally, the column was equilibrated with the starting conditions for 6 min.

A 65 min binary gradient elution was used for the analysis of TEF and its products after enzymatic hydrolysis. An isocratic elution of 25% solvent B lasted for the initial 5 min; a linear gradient elution of 25–30% solvent B was performed from 5 to 25 min; a linear gradient elution of 30–42% solvent B was performed from 25 to 48 min; a linear gradient elution of 42–95% solvent B was performed from 48 to 55 min; an isocratic elution of 95% solvent B was performed from 55 to 58 min; and finally, the column was equilibrated with the starting conditions for 7 min.

3. Results and discussion

3.1. Bioinformatic analysis of IagBgl1

Five putative β-glucosidase-encoding genes were revealed after investigating the genome information of *Ignisphaera aggregans* DSM 17,230 from the CAZy database [25]. BlastP analysis with different databases revealed a novel coding gene, *Igag_0940*, consisting of 486 amino acids, which was chosen for further investigation because of its significant distant relationship with other characterized β-glucosidase genes. This putative β-glucosidase shares the highest identities of 69%, 63% and 61% with unidentified GH 1family proteins (RLG75229.1, RLG79985.1) from *Thermoprotei archaeon* and (ADG91379.1) from *Thermosphaera aggregans*, respectively. Moreover, this putative β-glucosidase only exhibits 60% to 51% identities with β-glycosidase (PDB ID: 1QVB) from *Thermosphaera aggregans* [28], β-glucosidase(PDB ID: 3APG) from *Pyrococcus furiosus* [29], β-galactosidase (PDB ID: 4HA3) from *Acidilobus saccharovorans* 345-15 [30] and β-glycosidase (PDB ID: 1UWI) from *Saccharolobus solfataricus* [31]. On the other hand, two well-supported evolutionary trees were generated according to the evolutionary relationships detected through the neighbor-joining method and the maximum likelihood method. Significant evolutionary distances between IagBgl1 and the other GH family 1 enzymes from archaebacteria were clearly displayed by both of the evolutionary trees (Fig. 3b, 3c).

Alignment of the putative β-glucosidase with thermophilic GH family 1 proteins whose structures and enzymatic properties were integrally analyzed previously revealed two special strictly conserved motifs (Fig. 3a): TMN*E*PNVV (residues 207–214) and T*E*NG (residues 387–389). Based on the structural information of these identified GH 1family enzymes, the two glutamate residues (E^{210} and E^{388}) annotated in conserved motifs (Fig. 2) were identified as a potential catalytic core acting as the acid/base and the nucleophile catalyst, respectively. They also share a few other conserved residues related to substrate binding [28], suggesting that IagBgl1 has the same catalytic mechanism as other β-glucosidases.

3.2. Expression and purification of IagBgl1

The induction conditions were optimized for improving expression of target gen in *E. coli* BL21 (DE3) after incubation for 7 h at 37 °C without IPTG (Supplementary Fig. 1). The recombinant protein IagBgl1 with His-Tag fused at its C-terminus was purified by affinity chromatography and gel chromatography. The results are summarized in Supplementary Table 1 and Fig. 4. Finally, approximately 1.5 mg of purified protein was obtained with a yield of 54%.

To determine the molecular mass of monomer recombinant IagBgl1, the purified IagBgl1 was analyzed by SDS-PAGE, and it showed a clear band with a molecular mass of approximately 55 kDa, which corresponded to its theoretical molecular weight of 56356.44 Da (Fig. 4, lane 2). Meanwhile, a slender band with a molecular mass between 180 kDa and 130 kDa was displayed clearly. It might be a part of the incompletely denatured IagBgl1 after SDS-heat treatment, a phenomenon also occasionally reported for some other multimeric proteins from thermophilic bacteria [32]. This suggested that the native IagBgl1 existed in a multimeric form with high possibility.

The multimerization of proteins with noncovalent bonds is a common phenomenon, especially for enzymes from thermophilic microorganisms [33–35]. As shown in Clade III of Fig. 3b and 3c, the quaternary structural features of the characterized archaea GH1 enzymes were classified and annotated. Almost all of them were previously identified as dimers and tetramers. Meanwhile, thermostable enzymes in two evolutionary clusters of Clade I were previously identified as multimers. The gel chromatography analysis showed that IagBgl1 had a molecular weight of approximately 169 kDa (Fig. 5f), which adequately proved that native IagBgl1 existed in a trimeric form

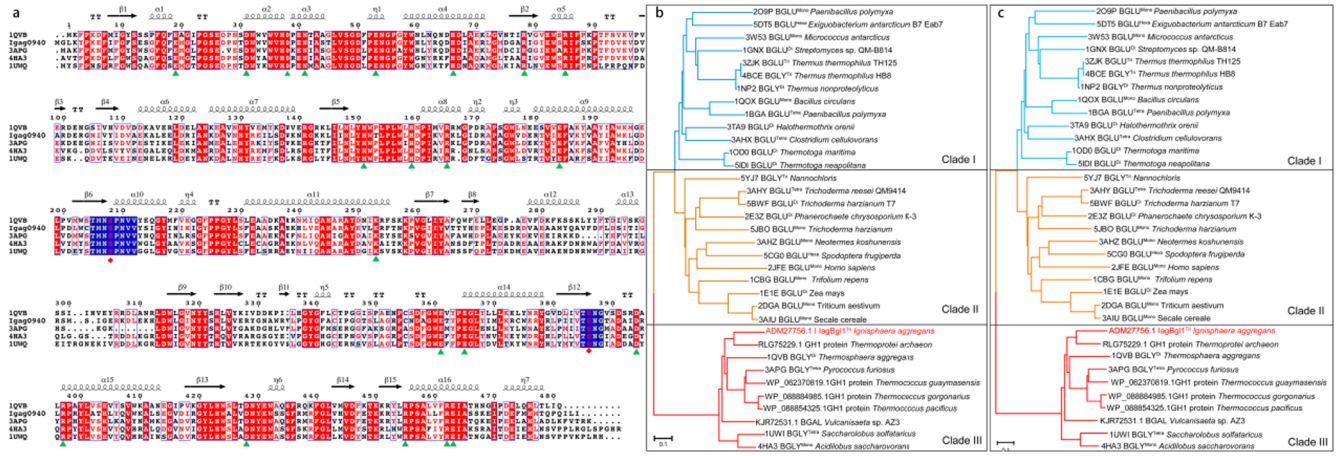


Fig. 3. The sequences comparison of IagBgl1 with family 1 glycoside hydrolases by multi-alignment and the analysis of evolutionary relationship between IagBgl1 and 34 other GH1family proteins. a) Multiple sequence alignment among IagBgl1 and other four GH 1 family enzymes. The PDB IDs and the species names of glycoside hydrolases from GH1 family were as follows: *Ignisphaera aggregans* glycosidase (Igag0940), *Thermosphaera aggregans* glycosidase (1QVB), *Pyrococcus furiosus* glucosidase (3APG), *Acidilobus saccharovorans* 345-15 (4HA3), *Saccharolobus solfataricus* (1UWQ). The original figure was generated by Clustal X1.9 and ESPript 3.0. The yellow font with blue background indicated the conserved sequences around catalytic active center. The conserved residue amino acids with red background and green triangles indicate the as substrate binding sites for constituting catalytic domain. The red diamond below the conserved sequences highlighted by red font, indicate the residue amino acid as the catalytic active site. b) The phylogenic trees generated from analysis result of 35 GH family 1 protein amino acid sequences using neighbor-Joining (NJ) method. The bootstrap values were 1000 replicates. c) The phylogenic trees generated from analysis result of 35 GH family 1 protein amino acid sequences using Maximum-Likelihood (ML) method. The superscript text Mono, Dim, Trim, Tetra and Hexa represented that the natural protein existed as monomer, dimer, trimer, tetramer, hexamer form. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

under routine conditions. To the best of our knowledge, this is the first trimeric β-glucosidase identified from archaea. The closest trimeric β-glucosidase is from *Thermus thermophilus* HB8 [34], which only shares 28% sequence similarity with IagBgl1. At present, the multimerization regularity of the protein has not been elucidated systematically; therefore, this special multimerization of the trimeric β-glucosidase IagBgl1 may provide new interaction forms between protein subunits of the GH1 family, after further investigation by protein crystallography.

3.3. Substrate specificity of the recombinant IagBgl1

The results for the catalytic activity of recombinant IagBgl1 with different artificial substrates are summarized in Table 1. The recombinant IagBgl1 exhibited the highest hydrolytic activity on *p*NPG of 92.47 U/mg, followed by *p*NPGal of 26.16 U/mg, *p*NPX of 13.93 U/mg and *p*NPArap of 8.95 U/mg, which indicated that it was a multifunctional β-glucosidase with broad substrate specificity. However, it could not hydrolyze *p*NPArf, which indicates that IagBgl1 has high selectivity for pyranose-type glycosides rather than for furanose-type glycosides. Otherwise, the catalytic activities of β-xylosidase and α-arabinopyranoside are rarely found in the GH 1 family.

3.4. The effects of pH and temperature on recombinant IagBgl1

The biochemical properties of purified IagBgl1 were determined by using *p*NPG as a substrate, and the results are summarized in Fig. 5. The recombinant IagBgl1 exhibited an optimal pH of 6.5, as shown in Fig. 5a. Meanwhile, the activity of IagBgl1 was more than 50 percent of the maximum activity in the pH range of 4.0–8.0, which indicated that it had a broad pH range for its efficient catalytic reaction and was similar to other thermostable GH family 1 protein [15,36–38]. IagBgl1 was stable within a pH range of 4.0–7.5 after incubation at 90 °C for 2 h, while it was more stable at the neutral pH (Fig. 5b).

Usually, enzymes from extreme thermophiles have high optimal reaction temperatures and excellent thermostability. The greatest hydrolysis activity of IagBgl1 was exhibited at 95 °C, and its catalytic activity was more than 50 percent of the maximum activity in the temperature range from 75 °C to 100 °C (Fig. 5c). Thermostability is an important property for the industrial utilization of enzymes, due to its advantages of viscosity reduction, a fast mass transfer rate, high substrate solubility and a lowered risk of contamination [22]. The residual activity of IagBgl1 was greater than 70% after incubation at 90 °C for 4 h, and it retained more than 90% of its activity after being incubated at 70 °C for 4 h (Fig. 5d). Compared with some other GH1 thermostable β-glucosidases, such as the rBglA from *Thermotoga neapolitana* with a 3.6 h half-life at 100 °C [38], that from *Sulfolobus acidocaldarius* with a 0.2 h half-life at 90 °C [39] and the DTGH from *Dictyoglomus*

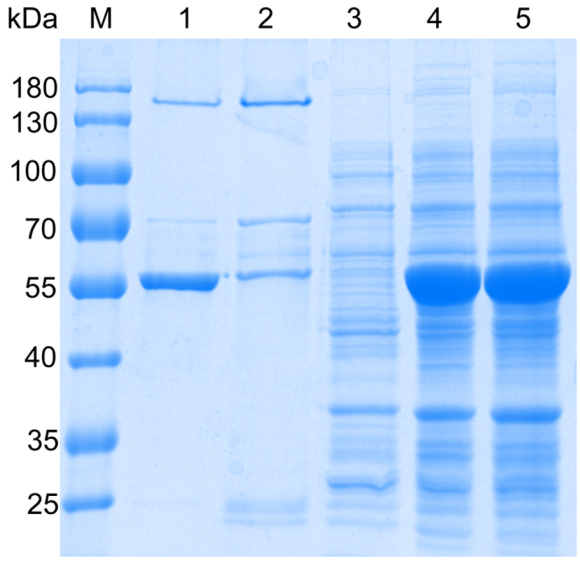


Fig. 4. SDS-PAGE analysis of recombinant IagBgl1 expressed in *E. coli* BL21 (DE3). Lane M: protein molecular mass ladder, lane 1: IagBgl1 purified by size-exclusion chromatography; lane 2: IagBgl1 purified by Ni-NTA resin affinity chromatography; lane 3: 2: the crude extracts of E. coli BL21 (DE3) harboring PET-*IagBgl1*; lane 4: the insoluble protein of E. coli BL21 (DE3) harboring PET-*IagBgl1*; the total cell of E. coli BL21 (DE3) harboring PET-*IagBgl1*.

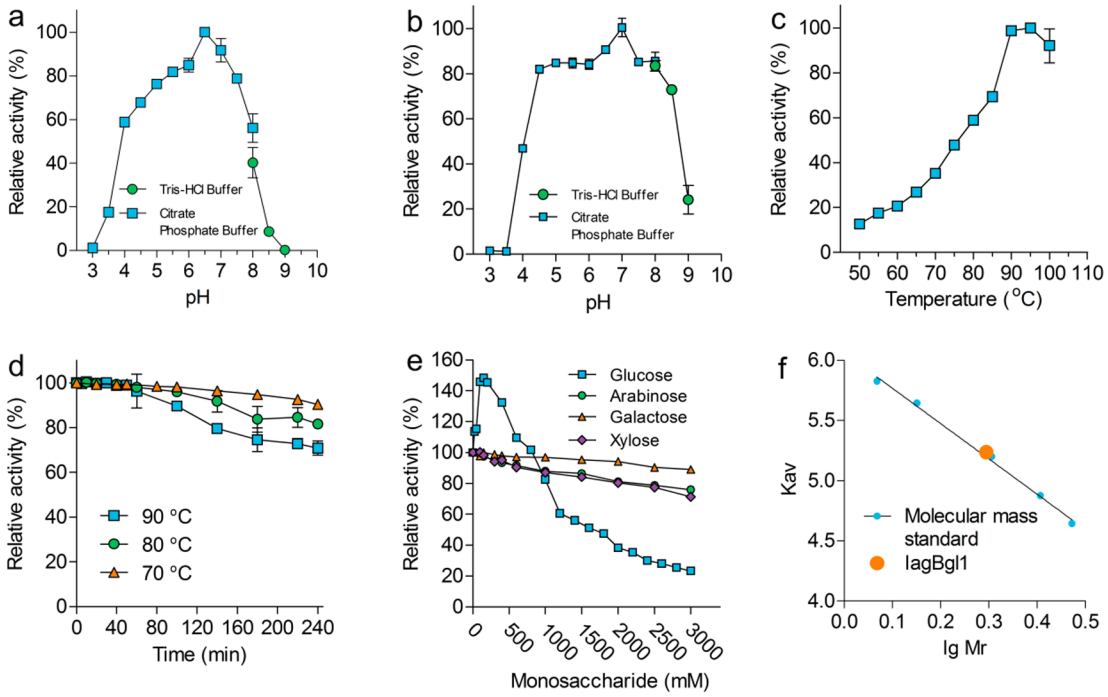


Fig. 5. The biochemical properties of recombinant IagBgl1. a) The optimal reaction pH of recombinant IagBgl1, b) The pH stability of recombinant IagBgl1, c) The optimal reaction temperature of recombinant IagBgl1, d) The thermostability of recombinant IagBgl1, e) The sugar tolerance of recombinant IagBgl1, f) The molecular mass determination of native recombinant IagBgl1. These activities were expressed as relative values. All measurements were repeated three times at the same condition. The data represent the mean values of three experiments, and the error bars represent the standard deviation.

Table 1
Substrate specificity of the recombinant protein IagBgl1.

Substrate[a]	Specific activity (U/mg)
p-Nitrophenyl-β-D-glucopyranoside	92.47 ± 6.44
p-Nitrophenyl-β-D-galactopyranoside	26.16 ± 1.73
p- Nitrophenyl-β-D-xylopyranoside	13.93 ± 0.36
p-Nitrophenyl-α-L-arabinopyranoside	8.89 ± 0.13
p- Nitrophenyl-α-L-rhamnopyranoside	ND [b]
p-Nitrophenyl-α-L-arabinofuranoside	ND
Cellobiose (10 mM)	5.53 ± 0.95
Sucrose (10 mM)	ND
Lactose (10 mM)	1.78 ± 0.23

Data represents the means of three experiments, and the SD represents the standard deviation.
[a] Final concentration of artificial substrate was 1.0 mM.
[b] Not detected, specific activity is not detected by the analytical methods used in this study.

Table 2
Effects of metal cations chemical reagents on the activity of recombinant protein IagBgl1.

Cation of reagent	Relative activity [b] (100%)	
	1 mM [a]	5 mM [a]
Control	100 ± 0.4	100 ± 0.58
Zn^{2+}	48.78 ± 0.4	29.55 ± 0.33
Ca^{2+}	97.41 ± 1.08	96.72 ± 0.4
Co^{2+}	89.53 ± 1.06	76.30 ± 0.88
Mg^{2+}	101.11 ± 0.66	101.02 ± 0.76
Ba^{2+}	96.46 ± 0.4	94.01 ± 8.99
Ni^{2+}	92.12 ± 0.32	84.43 ± 1.05
Cu^{2+}	108.93 ± 0.88	76.40 ± 1.12
Li^{+}	98.5 ± 1.3	92.67 ± 0.91
EDTA	96.62 ± 0.4	99.20 ± 0.74

[a] Final concentration of cation reagent in the mixture was 1 mM and 5 mM.
[b] Data represents the means of three experiments, and the SD represents the standard deviation.

thermophilum with a 5.0 h half-life at 90 °C[37], IagBgl1 exhibited better thermostability, with a 7.7 h half-life at 90 °C.

3.5. The effects of metal cations, organic solvent and monosaccharides on recombinant IagBgl1

The effects of metal cations and chemical reagents on the activity of IagBgl1were also investigated in various assays, and the results are summarized in Tables 1 and 2. The activity of IagBgl1 was highly inhibited after adding Zn^{2+} with final concentrations of 1 mM and 5 mM. However, most of the other metal cations did not significantly influence the activity of IagBgl1. Moreover, it was not inhibited by EDTA, which indicated that the catalytic activity of this enzyme does not depend on cofactors such as metal ions. This was similar to other GH 1 family enzymes [40–42].

The tolerance of organic solvent is an important property for the application of hydrolysis enzymes in transformation and for obtaining more valuable compounds from natural compounds with poor water solubility. The residual activities of IagBgl1 were greater than 90% with concentrations of methanol below 15% and more than 70% with concentrations of ethanol and DMSO below 15% (Table 3). Moreover, the activity of IagBgl1 was activated by the addition of low concentrations of methanol and ethanol. This has seldom been reported for GH 1 family enzymes, except the β-glucosidase *Dtur*-Glu from *Dictyoglomus turgidum* [40] and β-glucosidase TtBGL1 from *Thermotoga thermarum* [21]. Otherwise, the tolerance of organic solvent of some β-glucosidases was modified through the regulation of key amino acid residues and motifs [43–45]; however, further investigation is still required to reveal the mechanism of organic solvent tolerance. Moreover, the enzyme also displays application potential in biotransformation in the presence of organic solvents.

On the other hand, the feedback inhibition of the terminal product was a normal phenomenon in the self-regulation of biological

Table 3
Effects of organic solvent on the activity of recombinant protein IgaBgl1.

Final concentration of organic solvent [a]	Relative activity [b] (%)		
	Methanol	Ethanol	DMSO
0	100.0 ± 0.6	100.0 ± 0.6	100.0 ± 0.6
2	105.6 ± 3.0	122.2 ± 1.1	103.1 ± 2.6
5	110.3 ± 1.0	135.7 ± 1.3	93.0 ± 0.5
10	111.9 ± 2.1	142.6 ± 1.6	79.0 ± 1.2
15	102.8 ± 2.4	91.6 ± 1.6	70.8 ± 2.7

[a] Final concentration of substrate was 1.0 mM.
[b] Data represents the means of three experiments, and the SD represents the standard deviation.

metabolism. However, it is undesirable for the application of enzymes, especially for monosaccharide preparation from biomass. Based on previous reports, β-glucosidases from GH family 1 usually exhibit better monosaccharide tolerance than GH family 3 enzymes, because their catalytic pockets are deep and narrow, and their active sites are located at the bottom of the catalytic cavity [46]. IagBgl1 exhibited a high glucose tolerance (*Ki*) of approximately 1600 mM (Fig. 5e), which is higher than that of the famous glucose-tolerant β-glucosidase from *T. thermosaccharolyticum* with a *Ki* of 600 mM [15], the β-glucosidase from *A. oryzae* with a *Ki* of 1066 mM [47] and the β-glucosidase from *Debaryomyces vanrijiae* with a *Ki* of 668 mM [48]. Moreover, high concentrations of arabinose, galactose and xylose did not significantly inhibit its activity with *p*NPG in the reaction mixture. In addition, the catalytic activity of IagBgl1 was activated at a glucose concentration range from 0 mM to 800 mM. The highest activation of enzyme activity was approximately 1.5 times greater than that under standard conditions, which was measured at a glucose concentration of 150 mM (Fig. 5e). These outstanding properties implied that monosaccharide-tolerant IagBgl1 had high catalytic efficiency in complex reaction systems with high substrate concentrations of different glycosides.

Enzyme kinetic studies with *p*NPG, *p*NPGal and *p*NPX added as the substrate at the optimum temperature and pH allowed for the determination of Michaelis-Menten parameters (Table 4). The enzyme had an obvious K_m of 0.43 mM, K_{cat} of 134.64 s^{-1} and K_{cat}/K_m of 313.20 $mM{\cdot}s^{-1}$ when using *p*NPG as the substrate. It also exhibited broad substrate specificity with *p*NPGal, *p*NPX and *p*NPAarf, making it a potential candidate for bioconverting natural compounds with different glycosides.

3.6. Enzymatic transformation of icariin into baohuoside I

Baohuoside I is a minor compound of the TEF that exhibits more attractive bioactivities than the major compound icariin. Furthermore, baohuoside I and icariin share the same flavonoid skeleton, except one difference in the glucose residue at the C-6 position. Therefore, icariin was employed as a potential substrate for producing baohuoside I. Commercial enzymes, including β-glucosidase, β-dextranase, cellulose, and snailase, were used with an aim of converting icariin into baohuoside I [14,49]. Among them, β-glucosidase exhibited the best catalytic ability in producing baohuoside I from icariin [14]. As Table 4 shows, the K_m and K_{cat} of recombinant IagBgl1 via icariin were 0.89 mM and 43.86 s^{-1}, respectively. It was indicated that the recombinant IagBgl1 has high catalytic activity and great affinity for the hydrolysis of icariin. Based on its excellent thermostability, great sugar tolerance, and high specificity for icariin, recombinant IagBgl1 might be a potential alternative for producing baohuoside I with high productivity.

To verify this hypothesis, single factors were investigated for optimization of the transformation conditions for converting icariin into baohuoside I though recombinant IagBgl1. The reaction temperature and pH had a significant influence on the conversion rate of the enzymatic transformation system. After a 1 h reaction with DMSO as the cosolvent, the optimal reaction temperature and pH for transforming icariin into baohuoside I were 6.5 and 90 °C, respectively (Fig. 6a, 6b). On the other hand, the substrate concentration is also an important factor, as well as substrate inhibition and product inhibition. As different concentrations of icariin were added from 1 g/L to 10 g/L, the yield of baohuoside I was slightly increased after the highest yield was obtained under the enzyme dosage of 500 U/L. This also indicated that recombinant IagBgl1 was not inhibited even when the concentration of icariin was increased into 10 g/L (Fig. 6c). As Fig. 6d shows, the conversion rate was increased for the reaction duration of 1.5 h following the increase in enzyme dosage. The optimal enzyme dosage and optimal reaction time were 800 U/L and 1.5 h, respectively. The time-course analysis of the biotransformation reaction for converting icariin by recombinant IagBgl1 is shown in Fig. 6e and Fig. 6f. When adding 800U/L (approximately 8.9 mg/L) recombinant IagBgl1, 10 g/L icariin was converted into 7.564 g/L baohuoside I after incubation at 90 °C for 2 h, with a molar conversion of 99.48% and productivity of 5.043 g/L/h.

Reports on the enzymatic hydrolysis of icariin are classified in Table 5. When the substrate concentration is lower than 20 g/L, the baohuoside I productivity of IagBgl1 is significantly higher than that of cellulase, acommercial β-glucosidase and the β-glucosidase from *Trichoderma viride*. When the substrate concentration was increased to 50 g/L, the biphasic biotransformation system providedhigh baohuoside I productivity of 18.639 g/L/h; however, its enzyme dosage was much higher than that of IagBgl1. Thus, it can be seen that the recombinant IagBgl1 has better prospects in producing natural medicines, such as baohuoside I.

3.7. Enzymatic production of baohuoside I from the TFE

Except for icariin, the major compounds of TEF, including epimedin C, epimedin B, and epimedin A, were also potential candidate substrates for producing the minor compound baohuoside I, due to their high structural similarity in the flavonoid skeleton (Fig. 1).

Based on the sugar residues of major compounds of TEF (Fig. 1), the production of baohuoside I from TEF might be accomplished by using β-D-glucosidase, α-L-rhamnoside and β-D-xylosidase, theoretically. Two thermostable enzymes from a hyperthermophile, α-L-rhamnoside TpeRha and β-D-xylosidase TpeXyl3, were recently reported as efficient candidates for converting epimedin C and major ginsenosides into their corresponding minor active compounds. To obtain a feasible multiple

Table 4
Michaelis–Menten kinetics of recombinant protein IagBgl1 on different substrate.

Substrate	Product	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($mM^{-1}{\cdot}s^{-1}$)
p-Nitrophenyl-β-D-glucopyranoside	*p*-Nitrophenyl	0.43 ± 0.02	134.64 ± 0.92	313.20 ± 13.60
p-Nitrophenyl-β-D-galactopyranoside	*p*-Nitrophenyl	1.27 ± 0.11	55.91 ± 2.29	44.25 ± 2.06
p-Nitrophenyl-β-D-xylopyranoside	*p*-Nitrophenyl	3.71 ± 0.26	249.01 ± 10.81	67.11 ± 1.87
Epimedin A	Sagittatoside A	0.021 ± 0.003	19.51 ± 0.11	919.10 ± 28.63
Epimedin B	Sagittatoside B	0.041 ± 0.004	2.40 ± 0.16	57.84 ± 5.67
Epimedin C	Sagittatoside C	0.101 ± 0.006	41.34 ± 0.57	409.87 ± 12.47
Icariin	Baohuoside I	0.089 ± 0.003	43.68 ± 0.28	488.19 ± 13.04

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

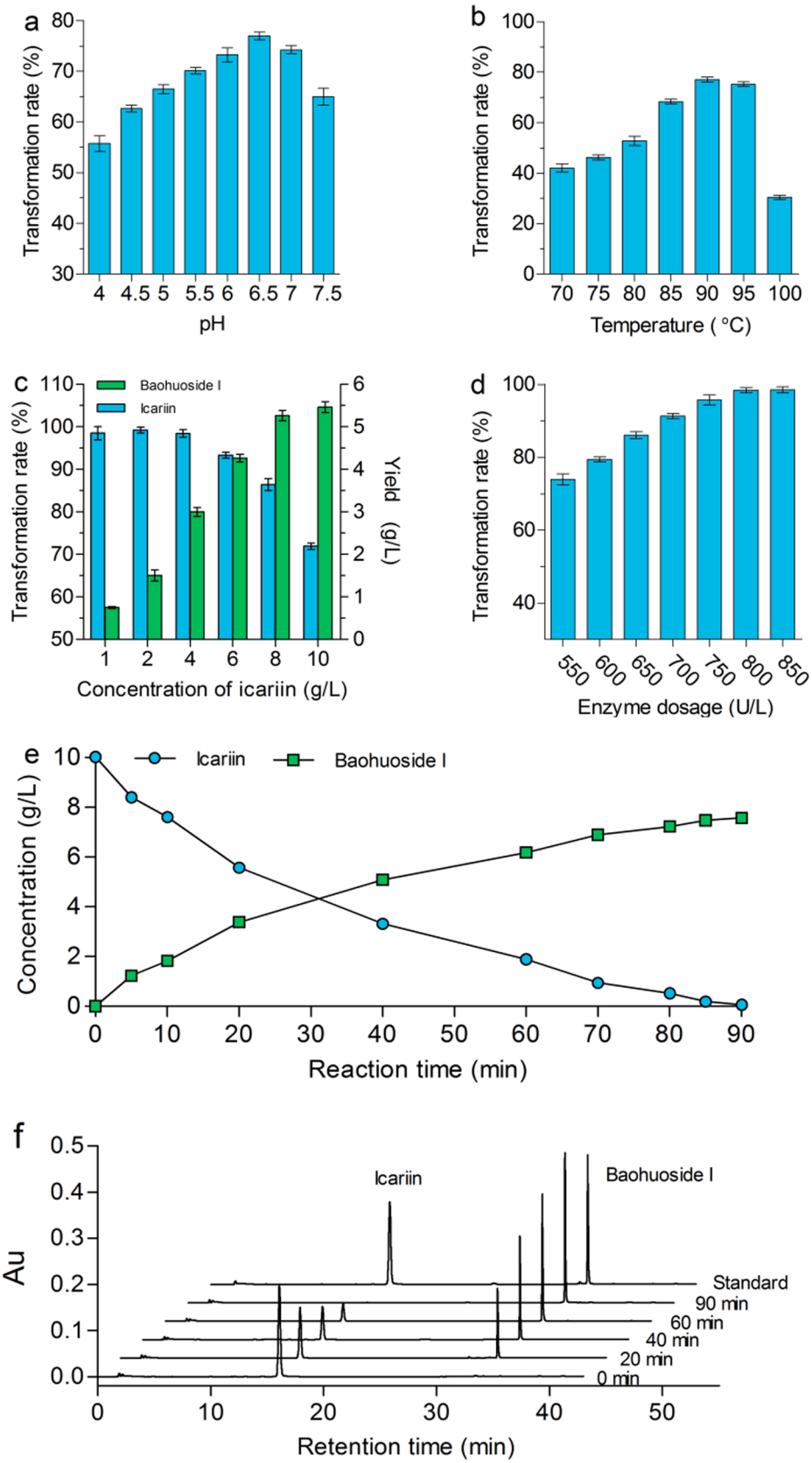


Fig. 6. The optimal transformation condition of baohuoside I production from icariin. a) Effect of pH on the hydrolysis of icariin by IagBgl1; b) Effect of temperature on the hydrolysis of icariin by IagBgl1; c) Effect of substrate concentration on the hydrolysis of icariin by IagBgl1; d) Effect of enzyme dosage on the hydrolysis of icariin by IagBgl1; e) HPLC analysis of icariin hydrolysis by IagBgl1. The reaction mixture was containing 50 mM citrate phosphate buffer pH 6.5 and 800 U/L of purified IagBgl1, 10 g/L icariin was incubated at 90 °C for 5, 10, 20, 40 min, 60, 70, 80, 85, 90 min, respectively. f) HPLC Spectrogram of the time-course curve on bioconversion of icariin by IagBgl1.

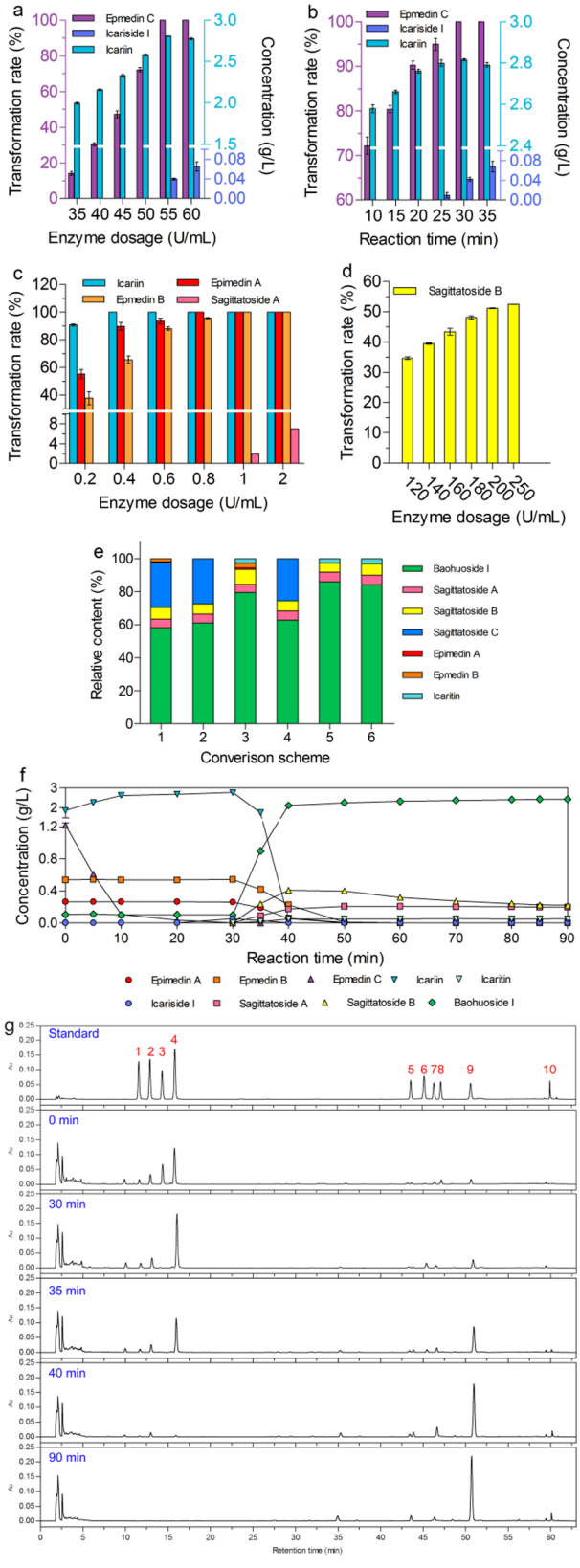
enzymatic conversion scheme for producing baohuoside I from TFE with high efficiency, a comparison of enzymatic properties among Iagbgl1, TpeRha, and TpeXyl3 was performed, and six designed conversion schemes were implemented with product distribution analysis. With a comprehensive analysis of the results (Supplementary Fig. 2 and Fig. 7e), four relative insights were acquired: first, a reaction temperature of 90 °C was a suitable condition for this biotransformation system, because all three enzymes exhibited optimal catalytic abilities

Table 5
The comparison of conversion conditions and productivity on transforming icariin into baohuoside I by different enzymes.

Icariin concentration (g/L)	Enzyme (source)	Enzyme dosage [a] (g/L)	Reaction condition (pH, temperature)	Reaction time (h)	Conversion rate (%)	Baohuoside I productivity (g/L/h)	Reference
20	Cellulase (commercial)	12 g/L	5.0, 50 °C	12	98.2	1.245	[50]
50	β-glucosidase (commercial)	160 g/L	6.0, 50 °C	2	98	18.639	[15]
0.2	β-glucosidase (commercial)	0.2 g/L	6.0, 50 °C	5	65.42[b]	0.020	[14]
1	β-glucosidase (Trichoderma viride)	9.8 U/mL	4.0, 41 °C	1	95.03	0.723	[52]
1	β-glucosidase (commercial)	2 g/L	5.0, 50 °C	1.5	100	0.507	[53]
10	IagBgl1 (recombiant)	800 U/L (8.89 mg/L)	6.5, 90 °C	1.5	99.48	5.043	This study

[a] The values were obtained by calculation based on the ratio of icariin/enzyme from the references.
[b] The value was obtained by numerical conversion based on the baohuoside I yield from the references.

at this temperature (Supplementary Fig. 2). Second, the baohuoside I yield of the staged reaction was higher than that of a one-pot reaction by these three enzymes. After detecting the products of one-pot reactions under a pH of 4.5 and 6.0 (conversion schemes 1 and 2, respectively), the results indicated that the generation of the intermediate



(caption on next page)

Fig. 7. Analysis of transformation factors for co-converting the TEF into baohuoside I through three thermostable enzymes. (a) The Enzyme dosage of TpeRha required for transforming the TEF. In the first conversion stage, the epimedin C of the TEF was converted into icariin by TpeRha at different concentrations. (b) The reaction time required for transforming the TEF with TpeRha. In the first stage on conversion of epimedin C from the TEF, the optimum time factor was estimated after addition of 55 U/mL of TpeRha with incubation for different duration. (c) The dosage of IagBgl1 required for transforming the TEF. (d) The dosage of TpeXyl3 required for transforming the TEF. (e) Product distribution of the reaction mixture on converting the TEF by six different conversion schemes. The details of each conversion schemes are described in Fig. 2. (b) time-course curve of production of baohuoside I from the TEF by two-step bio-transformation system. (g) HPLC Spectrogram for analysis of the products of two-step bio-transformation system of the TEF into baohuoside I mediated by TpeRha, IagBgl1 and TpeXyl3. The standards were followed by 1. epimedin A, 2. epimedin b, 3. epimedin c, 4. icariin, 5. sagittatoside A, 6. sagittatoside b, 7. icariside I, 8. sagittatoside c, 9. baohuoside I, 10. icaritin.

products sagittatoside C and sagittatoside B significantly reduced the final productivity of baohuoside I. Based on a report for TpeRha [50], the imbalance of catalytic efficiency between TpeRha (epimedin C - icariin) and IagBgl1 (icariin - baohuoside I) causes the transformation pathway to have low efficiency (epimedin C - sagittatoside C – baohuoside I); therefore, sagittatoside C accumulated in the reaction mixtures treated by conversion schemes 1 and 2. Almost all the epimedin C of the TEF was converted into the target product baohuoside I by the staged reaction schemes (Fig. 7e). Third, the efficiency and baohuoside I productivity could be improved by adjusting the pH condition of the different transformation stages, based on the enzyme properties. TpeRha displayed an optimal reaction pH and the best pH stability at 4.5, while IagBgl1 and TpeXyl3 exhibited batter catalytic ability at pH 6.0 (Supplementary Fig. 2). Fourth, comparing the results of treatment with the two-stage reaction (conversion scheme 5) and the three-stage reaction (conversion scheme 6), it can be found that the accumulation of sagittatoside B cannot be reduced by a three-stage reaction. Although TpeXyl3 exhibited high specific activity of xylosidase and IagBgl1 displayed xylosidase activity, the special β-1,2-glycosidic bond between the outer xylose and inner glucoside residue on epimedin B was hardly cleaved by both TpeXyl3 and IagBgl1, which differed from the transformation of epimedin C by TpeRha. Therefore, conversion scheme 5 with two-step transformation is more suitable than other schemes. Furthermore, the scheme 3 exhibited the potential to further enhance baohuoside I productivity by optimization of the transformation conditions.

To further verify the likelihood of converting the TEF into baohuoside I by the three thermostable enzymes, the transformation conditions were optimized under a two-step conversion scheme, and HPLC analysis was employed for a time-course experiment on the transformation process. The results are shown in Fig. 7. TpeRha was reported to have catalytic abilities on both of inner and outer rhamnose residues of epimedin C; therefore, two key factors (TpeRha dosage and reaction time) were optimized to reduce the concentration of the byproduct icariside I at the first transformation stage. The major compound epimedin C of the TEF was converted into icariin as much as possible by adding TpeRha with a final concentration of 55 U/mL after incubation for 30 min (Fig. 7a, 7b). Then, the optimal enzyme dosage of IagBgl1 and TpeXyl3 was obtained, and the results are shown in Fig. 7c and 7d. In the second transformation stage, all the icariin and nearly half of the epimedin B were converted into the target product baohuoside I after addition of 1 U/mL IagBgl1 and 200 U/mL TpeXyl3 and incubation for 90 min. Moreover, the time-course experiment of the first transformation stage revealed that epimedin C was rapidly converted into icariin at the beginning of the reaction. Before epimedin C was completely converted into icariin, a trace of the byproduct icariside I was detected after incubation for 30 min. At the second transformation stage, all the icariin of the reaction mixture was efficiently converted into the target product baohuoside I after incubation for 50 min. Meanwhile, the epimedin B was converted into sagittatoside B, and then more than half of the sagittatoside B was converted into baohuoside I after incubation for 60 min with TpeXyl3 at the second transformation stage. Finally, after two-stage biotransformation under the optimum conversion conditions, 2.432 g/L baohuoside I was obtained from 10 g/L TEF, and the productivity was calculated to be 1.621 g/L/h. Though all the epimedin A and nearly half of the epimedin B of the TEF were transformed into sagittatoside A (0.198 g/L) and sagittatoside B (0.220 g/L), almost all the other major epimedium flavonoids of TEF were converted into baohuoside I. Based on the reports, the baohuoside I yield of this multiple enzymatic conversion system via TEF is significantly higher than that of the transformation method with a single enzyme [51]. Thus, our study provides a useful two-stage transformation system for producing baohuoside I from TEF by three thermostable enzymes.

4. Conclusion

A novel thermostable β-glucosidase, IagBgl1, from *Ignisphaera aggregans* DSM17230 was overexpressed in *E. coli* BL21 (DE3) and sufficiently purified. Its quaternary structure was identified as a special trimetric form, which differed from those of other reported archaea GH 1 family enzymes. IagBgl1 has multifunctional activity for hydrolyzing glucopyranose, galactopyranose, xylopyranose and arabinopyranose moieties from artificial substrates and natural components with β-1,4-, β-1,6- and α-1,6-type glycosidic bonds, respectively. Moreover, it exhibited high catalytic activity and stability at elevated temperatures in a wide pH range and showed good tolerance to sugar and organic solvents. Under optimal conditions, IagBgl1 had an efficient catalytic ability for transforming 10 g/L icariin into 5.043 g/L baohuoside I with a molar conversion rate of 99.48% and productivity of 5.043 g/L/h. On the other hand, a two-step multiple enzymatic conversion system was built by combination with IagBgl1 and other two thermostable enzymes. baohuoside I at 2.432 g/L was obtained from 10 g/L TEF by transformation under the conversion system within 90 min. In summary, the recombinant IagBgl1 was demonstrated to have high efficiency in the production of baohuoside I. The excellent enzymatic properties of IagBgl1 and the multiple enzymatic conversion system suggested the feasibility and application potential of preparing baohuoside I from TEF through enzymatic transformation, which provides an effective pathway for improving the utilization rate of epimedium resources.

Declaration of Competing Interest

The authors declare that there is not conflict of interest.

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Author statement

JJ JX conceived and designed the experiments. JX HX XT TW performed the experiments. JX NZ JY JZ analyzed the data. HX JY JZ MW contributed to reagents, materials and analysis tools. JX wrote the manuscript. JJ JX HX revised and approved the final version of the paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2020.104296.

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