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Enhancing soluble expression of sucrose phosphorylase in *Escherichia coli* by molecular chaperones

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ABSTRACT

Sucrose phosphorylase (SPase, EC 2.4.1.7) has a wide range of application in food, cosmetics, and pharmaceutical industries because of its broad substrate specificity. However, low SPase yields produced by wild-type strains cannot meet industrial requirements due to their complex metabolic regulation mechanisms. In this study, *spase* gene from *Thermoanaerobacterium thermosaccharolyticum* was cloned and expressed in *Escherichia coli* BL21 (DE3), leading to 7.05 U/mL (3.71 U/mg) of *T. thermosaccharolyticum* SPase (*Tt*SPase) under optimum conditions. Co-expression of molecular chaperone teams pGro7 (GroES-GroEL), pG-KJE8 (DnaK-DnaJ-GrpE and GroES-GroEL), and pG-TF2 (GroES-GroEL-Tig) significantly enhanced the *Tt*SPase activities to 18.5 U/mg (59.2 U/mL), 9.52 U/mg (28.6 U/mL), and 25.7 U/mg (64.5 U/mL), respectively. Results suggested that GroES-GroEL chaperone combination could regulate protein folding processes and protect misfolded proteins from aggregation. The enzymatic characterization results showed that *Tt*SPase had an optimal temperature of 60 °C and optimal pH of 6.5. In particular, it had high thermostability of T_{50}^{30} = 67 °C and half-life ($t_{1/2}$ at 70 °C) of 19 min. Furthermore, purified *Tt*SPase was used for hydroquinone transglycosylation and 21% of molar production yield of α-arbutin was obtained. This study provides a *Tt*SPase with high thermostability for potential industrial applications, and develops an effective strategy for improving soluble *Tt*SPase production in *E. coli*.

1. Introduction

Sucrose phosphorylase (SPase, EC 2.4.1.7), belonging to the glycoside hydrolase 13 family [1–3], was first discovered from *Leuconostoc mesenteroides* and subsequently found in various microorganisms (e.g. *Pseudomonas saccharophila*, *P. putrefaciens*, *Clostridium pasteurianum*, *Acetobacter xylinum*, *Pullularia pullulans*) [2]. SPase specifically catalyzes the transfer of glycoside bonds and sucrose phosphates, and is widely applied in food, cosmetics, and pharmaceutical industries. For instance, it can be used for the synthesis of oligosaccharides [3], 2-*O*-D-glucopyranosyl-L-ascorbic acid (AA-2G) [4], and α-arbutin [5], which are often used as skin care elements and pharmaceutical additives. Although SPase shows great application potential, its poor thermostability and low expression levels limit its industrial applications. SPase from *Thermoanaerobacterium thermosaccharolyticum* (*Tt*SPase) was reported to have great thermostability but low production [6,7]. Therefore, it is of great importance to improve the heterologous expression of *Tt*SPase.

Generally, *Escherichia coli* is one of the most commonly used host for recombinant protein expression due to its clear genetic characteristics, simple culture requirements, and short growth cycle [8]. Most SPases from different organisms have been expressed in *E. coli*, but this process often produces inclusion bodies, greatly limiting their production [9]. Currently, efforts have been focused on improving the soluble expression of SPase in *E. coli*. For instance, the production of soluble SPase expressed in *E. coli* was improved by 1.85-fold by optimizing several induction conditions, such as initial cell density, IPTG concentration, induction time, and induction temperature [10]. Enhanced production of extracellular soluble SPase was realized by co-expression with phospholipid C [11]. Selective introduction of synonymous rare codons could weaken translation at specific points and separate the translation of peptide chain segments and coordinate their co-translational folding, which enhances the soluble expression of SPase [6]. Recently, a constitutive expression system was used to improve the soluble expression

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of SPase in low-well plates [12].

In addition, several chaperones are usually utilized for decreasing the aggregated proteins and increasing the soluble expression of the target protein in *E. coli* [13], such as GroES, GroEL, DnaK, DnaJ, GrpE, and trigger factor (TF). TF plays an important role in facilitating the solubility of model target protein, which is the first chaperone to combine newly synthesized polypeptides and assist their folding [14]. DnaK not only assists in folding newly prepared polypeptides but may also alternatively act during co- and post-translation to repair misfolded proteins after the completion of their synthesis. In contrast to TF and DnaK, the GroEL system is only associated with newly prepared peptides in a post-translational manner. These chaperones have an obvious impact on correct folding, aggregation, and assembly of model proteins *in vitro* [15,16]. For example, co-expression with different molecular chaperone teams, including GroES-GroEL and DnaK-DnaJ-GrpE-GroES-GroEL, increases the soluble expression of TrSOX [17]. TF greatly enhances the expression of soluble fCRM197 in recombinant *E. coli* [18].

In this study, we successfully expressed *Tt*SPase in *E. coli* BL21 (DE3). To enhance the soluble expression of *Tt*SPase, several chaperones plasmids such as pG-KJE8 (DnaK-DnaJ-GrpE-GroES-GroEL), pGro7 (GroES-GroEL), pKJE7 (DnaK-DnaJ-GrpE), and pG-TF2 (GroES-GroEL-Tig) were co-expressed and their co-expression conditions were optimized. Finally, the enzymatic characteristics of *Tt*SPase were investigated.

2. Materials and methods

2.1. Bacterial strains, plasmids, and materials

The *spase* gene from *T. thermosaccharolyticum* (NCBI Accession No: WP_094046414.1) was synthesized by Synbio Technologies (Shanghai, China) with codon optimization, and cloned in pUC18 vector within the restriction sites *Nco*I and *Xho*I. *E. coli* JM109 and BL21 (DE3) strains were used as the hosts for plasmid construction and *Tt*SPase expression, respectively. The four chaperone plasmids pG-KJE8, pGro7, pKJE7, and pG-TF2 were purchased from TaKaRa (Dalian, China). The main characteristics of plasmids and strains in this study were listed in Table S1.

The following culture mediums were used: TB (24 g/L yeast extract; 12 g/L tryptone; 17 mM KH_2PO_4; 72 mM $K_2HPO_4{\cdot}3H_2O$; 4 g/L glycerin), LB (5 g/L yeast extract; 10 g/L NaCl; 10 g/L tryptone), SOB (5 g/L yeast extract; 20 g/L tryptone; 0.5 g/L NaCl; 2.5 mM KCI; 10 mM $MgCl_2$), M9 (5 g/L glucose; 6 g/L $Na_2HPO_4{\cdot}7H_2O$; 3 g/L K_2HP0_4; 0.12 g/L $MgSO_4$; 0.5 g/L NaCl; 1 g/L NH_4Cl), and 2 YT (16 g/L yeast extract; 5 g/L NaCl; 5 g/L tryptone).

2.2. Construction of recombinant strain E. coli BL21(DE3)/pET20b (+)/spase and co-expressed strain with chaperone

The recombinant strain *E. coli* BL21 (DE3)/pET20b (+)/*spase* was constructed as follows: *spase* gene with restriction sites *Nco*I and *Xho*I at the 5′- and 3′-terminal was amplified by PCR with pUC18/*spase* as the template. The amplified DNA fragments and the pET20b (+) vector were digested with *Nco*I and *Xho*I, ligated, and transformed into *E. coli* JM109. Then, colonies were identified by colony PCR and DNA sequencing. The correct plasmid pET20b (+)/*spase* was then transformed into *E. coli* BL21 (DE3) for expression.

The construction of co-expressed strains *E. coli* BL21 (DE3) with plasmids pET20b (+)/*spase* and chaperones were as follows: with the recombinant *E. coli* BL21 (DE3)/pET20b (+)/*spase* as the competent cell, four chaperone plasmids such as pG-KJE8, pGro7, pKJE7, and pG-TF2, were transformed into competent cells to construct the co-expressed strains, which were named as *E. coli* BL21 (DE3)/pG-KJE8/pET20b (+)-*spase*, *E. coli* BL21 (DE3)/pGro7/pET20b (+)-*spase*, *E. coli* BL21 (DE3)/pKJE7/pET20b (+)-*spase*, and *E. coli* BL21 (DE3)/pG-TF2/pET20b (+)-*spase*, respectively.

2.3. Expression of recombinant TtSPase in E. coli BL21(DE3)

The recombinant *E. coli* BL21 (DE3)/pET20b-*spase* were incubated overnight in 25 mL LB medium containing 100 μg/mL ampicillin with shaking at 180 rpm and 37 °C. The seed with 1% (v/v) of inoculum size was inoculated in a 500 mL flask containing 100 mL TB medium and then grown in a shaker at 180 rpm and 37 °C until the OD_{600} reached 0.9. After the addition of IPTG, the incubation was continued for 12 h at 25 °C. Cells were collected via centrifugation (8000 g at 4 °C for 15 min), resuspended in 50 mM PBS buffer (pH 6.5), and sonicated in an ice-water bath for 10 min. The cell debris was discarded by centrifugation (8000 g at 4 °C for 15 min). The crude enzyme was further analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Analysis of recombinant TtSPase activity

SPase activity was measured in accordance with a previously described method [19]. The reaction system included PBS buffer (50 mM, pH 6.5), 1.48 M sucrose, and diluted enzyme. After incubation at 55 °C for 10 min, the concentration of released fructose was determined by using the dinitrosalicylic acid method with fructose as a standard. One unit of SPase activity was defined as the amount of enzyme that liberates 1 μmol fructose or α-D-glucose-1-phosphate from the sucrose per minute.

2.5. Influence of incubation conditions on soluble TtSPase expression

The influence of different culture media (TB, LB, M9, SOB, 2 YT) on the expression of soluble *Tt*SPase was investigated. The initial cell density (0.6–1.1), IPTG concentrations (0.025–0.15), and induction temperature (18 °C, 25 °C, and 30 °C) were also taken into consideration. The enzyme activity was used to assess the expression level.

2.5.1. Influence of induction temperature on recombinant co-expressed strains

To study the influence of induction temperature on soluble *Tt*SPase expression, the cells were incubated at 37 °C until the OD_{600} reached 0.9 and then reduced to different induction temperatures (18 °C, 25 °C, and 30 °C) after the addition of inducers.

2.5.2. Influence of inducer concentrations on recombinant co-expressed strains

E. coli BL21 (DE3) strains harboring pET20b (+)/*spase* and pG-KJE8, pGro7, pKJE7, and pG-TF2 plasmids were inoculated into LB medium containing 100 μg/mL ampicillin in combination with 30 μg/mL chloramphenicol for plasmid selection and 0.5 mg/mL L-arabinose and/or 10 ng/mL tetracycline for induction. Different concentrations of IPTG (0.01, 0.025, 0.05, and 0.1 mM) were added into the culture when the OD_{600} reached 0.9. Cells were then further incubated for 12 h and harvested by centrifugation for 15 min at 8000 rpm and 4 °C. Subsequently, the precipitate was resuspended in 50 mM PBS buffer (pH 6.5) and sonicated in an ice-water bath for 10 min. Cell debris were discarded by centrifugation (8000 g at 4 °C for 15 min). The protein expression in supernatant and the insoluble inclusion body were analyzed by SDS-PAGE.

Similarly, the influences of different tetracycline concentrations (5 and 10 ng/mL) and different L-arabinose concentrations (0.5, 1, and 2 mg/mL) were also investigated.

2.5.3. Influence of the addition order of different inducers on recombinant co-expressed strains

The influences on the addition order of different inducers (IPTG, L-arabinose, and tetracycline) were also taken into consideration. “Prior” indicates that the inducers of chaperone (L-arabinose and/or tetracycline) were added at OD_{600} of 0.6 before IPTG addition at OD_{600} of

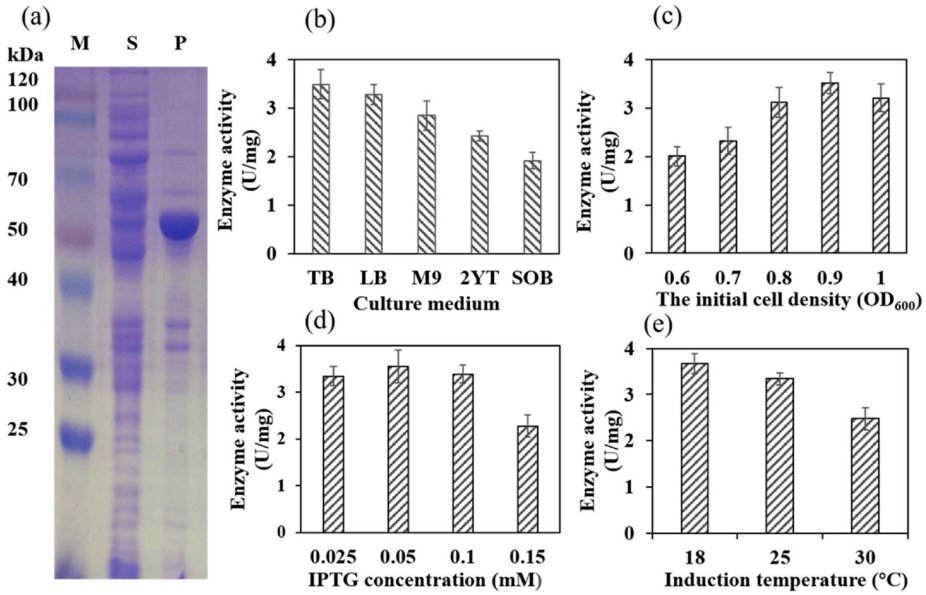

Fig. 1. Expression of recombinant *Tt*SPase in *E. coli* BL21 (DE3). (a) SDS-PAGE analysis of recombinant *Tt*SPase in *E. coli* BL21 (DE3). M, protein marker; Lane S, the soluble fraction of *Tt*SPase; Lane P, the insoluble fraction of *Tt*SPase; (b) Effect of different culture mediums on *Tt*SPase expression; (c) Effect of the initial cell density on *Tt*SPase expression; (d) Effect of IPTG concentrations on *Tt*SPase expression; (e) Effect of different induction temperatures on *Tt*SPase expression. Each value represents the mean of three independent measurements.

0.9. "Simultaneously" represents that IPTG, L-arabinose, and/or tetracycline were added simultaneously when the OD_{600} reached 0.9. "Postpone" means that L-arabinose and/or tetracycline were added at OD_{600} of 1.2 after IPTG addition at OD_{600} of 0.9.

2.6. Purification of recombinant TtSPase and SDS-PAGE

Crude enzyme solution was obtained via centrifugation (8000 g at 4 °C for 10 min) to remove cell debris, incubated at 60 °C for 1 h to partly remove impure protein, and subsequently purified by Ni-NTA affinity chromatography. HisTrap FF column with the loading crude enzyme was washed by the buffer with 50 mM imidazole to remove the impure protein initially. Then, *Tt*SPase was eluted using elution buffer with 150 mM imidazole. The collected eluents were added in a HiTrap Desalting column to remove the residual imidazole. Purified *Tt*SPase was verified by SDS-PAGE and stored at −80 °C with 20% (v/v) glycerol for enzyme characterization.

The collected cells were suspended in 50 mM PBS buffer with pH of 6.5. The suspension was lysed by ultrasonication (3 s pulse, total 600 s at 45% amplitude) in an ice-water bath. The cell debris was discarded by centrifugation (8000 g at 4 °C for 15 min). The supernatant and pellets were mixed with SDS loading buffer at 3:1 ratio. After heat treatment at 100 °C for 10 min, the samples were analyzed via SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R-250 and analyzed by Gel-Pro Analyzer 3.

2.7. Characterization of recombinant TtSPase

The optimal temperature was estimated by measuring the *Tt*SPase activity at various temperatures ranging from 30 °C to 70 °C in 50 mM PBS buffer (pH 6.5). The optimum pH was estimated by measuring the enzyme activity at various pH from 5.5 to 8 in 50 mM PBS buffer at 60 °C. The stability of pH was determined by measuring *Tt*SPase activity after 1 h incubation at different pH (5.5, 6.0, 6.5, 7.0, 7.5, and 8.0). Similarly, the half-life and T_{50}^{30} value of the enzyme were also investigated to determine the thermal stability of the enzyme. *Tt*SPase was incubated at different temperatures (from 55 °C to 85 °C with increments of 10 °C) for 30 min; then, the activities of the samples were analyzed. Moreover, *Tt*SPase were incubated at 70 °C and 80 °C for different times to measure the half-life of thermal inactivation.

The kinetic analysis was measured as previously described [7]. The Michaelis–Menten kinetic parameters K_m, k_{cat}, and V_{max} were determined by using different concentrations of sucrose (5, 10, 25, 50, 100, 250, 500 and 1000 mM) as substrates. All samples were determined in triplicate. The result was determined by using Michaelis–Menten kinetic equations with the Origin 8.0 software.

2.8. Application of TtSPase for α-arbutin synthesis

The reaction system for α-arbutin synthesis by *Tt*SPase was done as follows: the reaction mixture (0.5 mL) including 15 mM sucrose, 5 mM hydroquinone and *Tt*SPase (1.5 units) in PBS buffer (50 mM, pH 6.5) was incubated in 25 °C for 16 h. Samples were analyzed by high performance liquid chromatography (HPLC). HPLC analysis was performed using an Agilent 1260 Infinity HPLC system with an Aglient 1260 WVD-detector, and equipping with a C18 chromatography column (4.6 × 250 mm, Diamonsil®) at 25 °C. A mobile phase consisted of methanol and phosphate solution (0.05 mM, pH 6.0) at a ratio of 90/10 (v/v), and samples were detected at 254 nm at a flow rate of 0.8 mL min^{-1}. The molar production yield of α-arbutin was calculated according to the following formulas:

$$\text{Molar production yield} = \frac{\textit{molar amout of synthesized } \alpha - \textit{arbutin}}{\textit{molar amout of initial hydroquinone}} \times 100\%$$

3. Results and discussion

3.1. TtSPase expression in recombinant E. coli BL21(DE3)

The crude enzyme expressed by *E. coli* BL21 (DE3)/pET-20b (+)*/spase* was analyzed by SDS-PAGE. As shown in Fig. 1a, a significant band was observed at approximately 56 kDa, which correlates well with the size of SPase [3,20]. However, the majority of *Tt*SPase protein were recognized as insoluble aggregates. Hence, increasing soluble *Tt*SPase expression seems quite necessary.

3.2. Effect of induction conditions on soluble TtSPase expression

To improve the expression of soluble *Tt*SPase, different induction conditions were investigated. As shown in Fig. 1b, compared with other culture media (LB, M9, 2 YT, and SOB), TB medium showed the highest activity of recombinant *Tt*SPase. Five different initial cell densities (OD_{600}) for induction were evaluated to determine the optimal time for IPTG addition. Fig. 1c shows that the highest enzyme activity was achieved when the initial cell density (OD_{600}) was 0.9. IPTG generally has a significant effect on the expression rate, solubility, and enzyme

activity of recombinant protein [21]. According to literature, appropriate amount of IPTG could reduce the metabolic burden of *E. coli* and increase the production of target protein. However, excess IPTG would inhibit the growth of *E. coli* and cause the target protein to form inclusion bodies [22,23]. As shown in Fig. 1d, the highest enzyme activity of *Tt*SPase was obtained at the final concentration of 0.05 mM IPTG. Moreover, as the IPTG concentrations increased, the enzyme activity gradually decreased. The effect of induction temperatures on the soluble *Tt*SPase expression was also investigated. Generally, temperature not only affects the growth of host cells but also regulates the synthesis efficiency of heterologous proteins. High temperature usually accelerates the synthesis rate of heterologous proteins and leads to protein misfolding for inclusion body formation [24,25]. Therefore, the induction temperature is one key factor that regulates the soluble expression of heterologous protein in *E. coli*. Cells are generally cultured at 37 °C in the pre-fermentation phase until OD_{600} reached 0.9 and then reduced to different temperatures (18 °C, 25 °C, and 30 °C) after IPTG addition. As shown in Fig. 1e, low temperature (18 °C) slightly improved the enzyme activity of *Tt*SPase. Finally, *Tt*SPase with activity of 7.05 U/mL (3.71 U/mg) was obtained under optimum conditions. However, most *Tt*SPase still presented as inclusion bodies.

3.3. Co-expression of TtSPase and chaperones of pG-KJE8, pKJE7, pGro7, and pG-TF2

To further improve the soluble expression of *Tt*SPase, four chaperone teams (pG-KJE8, pKJE7, pGro7, and pG-TF2) co-expressed with *Tt*SPase were investigated. As shown in Fig. 2, SDS-PAGE results indicated that four kinds of chaperone teams were successfully expressed. The co-expression of pG-KJE8, pGro7, and pG-TF2 teams significantly improved the soluble expression of *Tt*SPase and reduced inclusion body formation. It has been reported that a majority (90%) of the expressed *Tt*SPase was inclusion bodies [6]. The positive chaperone teams (pG-KJE8, pGro7, and pG-TF2) included two similar chaperones (GroES and GroEL), revealing that the GroES-GroEL chaperone team plays an important role on the soluble expression of *Tt*SPase. It was reported that the internal cavity of GroEL could bind to denatured polypeptides, and the latter were released in an ATP-dependent reaction regulated by the cofactor GroES. GroES and GroEL acted as a foldase to interact with polypeptides and assist in refolding correctly [26]. For instance, GroES-GroEL could function as mRNA stabilizer to increase the production of protein [27]. Compared with pGro7, pG-KJE8 co-expression showed more soluble *Tt*SPase (Fig. 2). The reason may be that DnaK-DnaJ-GrpE accompanied with GroES-GroEL behave well in assisting the folding of *Tt*SPase. DnaK also stabilizes the proteins for subsequent folding by GroEL [28]. GroES-GroEL and DnaK-DnaJ-GrpE have complementary functions in folding and assembly of most proteins [29]. As shown in Fig. 2, co-expression with pG-TF2 led to the highest soluble *Tt*SPase and less inclusion bodies. Studies showed that TF could recognize aromatic and basic amino acid residues in peptide substrates and then bind to peptides [30]. It can further avoid misfolding, protect nascent chains from digestion by proteases, and cooperate with other chaperones to assist folding. Therefore, GroES-GroEL accompanied by TF (pG-TF2) positively influenced *Tt*SPase *in vivo*. Meanwhile, we found that DnaK-DnaJ-GrpE chaperone protein team (pKJE7) was not beneficial to soluble *Tt*SPase expression, although it was recognized to improve the soluble expression of *r-XynB* in *E. coli* [31]. The results demonstrated that same chaperone teams have diverse efficiency to the expression of different proteins. Therefore, co-expression with pG-KJE8, pGro7, and pG-TF2 plasmids was further investigated.

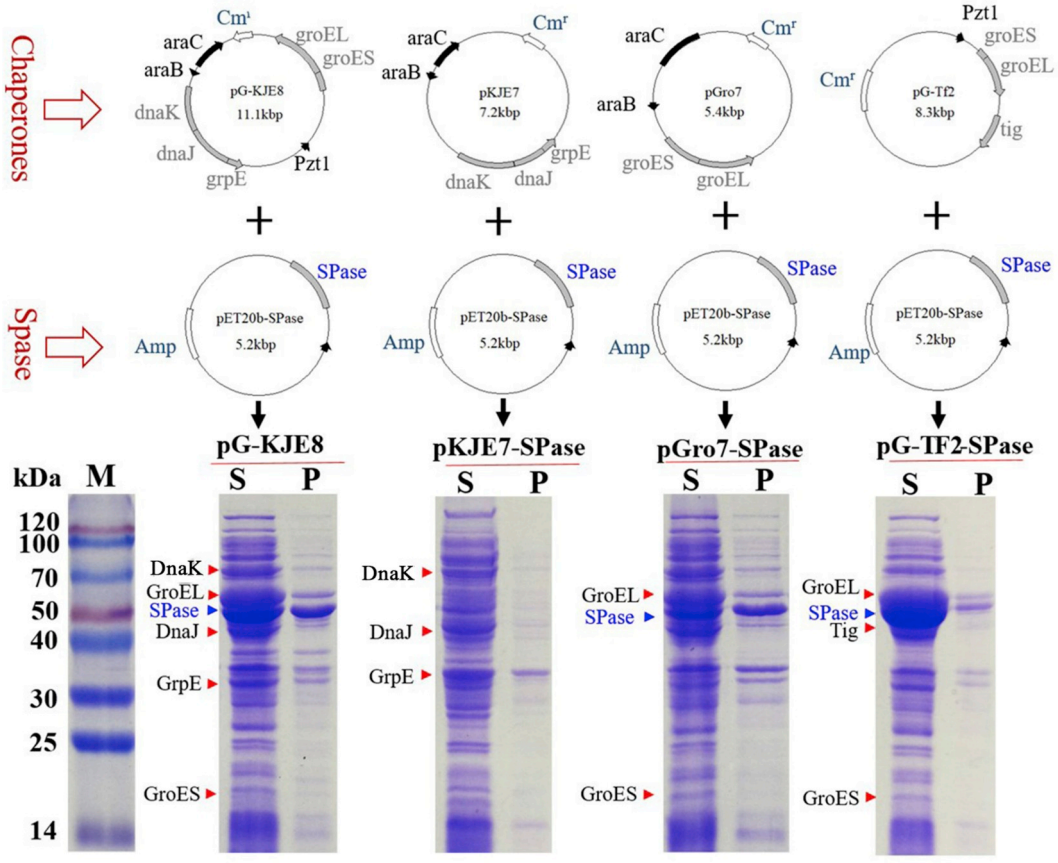

Fig. 2. SDS-PAGE analysis of four chaperone teams (pG-KJE8, pKJE7, pGro7, and pG-TF2) and their co-expression with *Tt*SPase.

Table 1
Effect of different IPTG concentrations on *Tt*SPase activity.

IPTG concentrations (mM)	Enzyme activity (U/mg)			
	SPase	pG-KJE8-SPase[a]	pGro7-SPase[b]	pG-TF2-SPase[c]
0.01	3.13 ± 0.26	14.6 ± 1.34	7.69 ± 0.91	21.8 ± 0.19
0.025	3.34 ± 0.19	15.3 ± 2.26	8.93 ± 0.71	22.6 ± 0.31
0.05	3.71 ± 0.28	17.3 ± 1.95	9.52 ± 0.12	25.7 ± 0.55
0.1	3.43 ± 0.15	7.25 ± 0.22	25.7 ± 0.55	20.9 ± 0.12

Each value represents the mean of three independent measurements.
[a] : pG-KJE8-SPase was induced by IPTG, 0.5 mg/mL L-arabinose and 10 ng/mL tetracycline concentration.
[b] pGro7-SPase was induced by IPTG and 0.5 mg/mL L-arabinose.
[c] pG-TF2-SPase was induced by IPTG and 10 ng/mL tetracycline concentration.

3.4. Effect of induction conditions on the co-expression of pG-KJE8-SPase, pGro7-SPase and pG-TF2-SPase

3.4.1. Effect of inducer concentration on soluble TtSPase expression

The effects of IPTG concentration on the *Tt*SPase activities are summarized in Table 1. The optimal IPTG concentration for all co-expression stains was 0.05 mM. *Tt*SPase specific activities of pGro7, pG-KJE8, and pG-TF2 co-expression strains were 2.57-, 4.66-, and 6.92-fold higher than that without chaperone co-expression. On the basis of 0.05 mM IPTG concentration, the effect of chaperone inducer concentration on the soluble *Tt*SPase expression was also investigated. As shown in Table 2, pG-KJE8 co-expression strain showed the highest *Tt*SPase activity of 18.5 U/mg (59.2 U/mL) at 0.5 mg/mL L-arabinose and 5 ng/mL tetracycline. pGro7 co-expression strain showed the highest *Tt*SPase activity of 9.52 U/mg (28.6 U/mL) at 0.5 mg/mL L-arabinose. At higher L-arabinose or tetracycline concentrations, both co-expression strains of pG-KJE8 and pGro7 showed decreased *Tt*SPase activities. This result may be attributed to the overexpression of molecular chaperones and increased growth pressure of cells caused by high L-arabinose or tetracycline concentration, which are not conducive to the secretion of soluble enzymes. On the contrary, pG-TF2 co-expression strain showed the highest *Tt*SPase activity of 25.7 U/mg (64.5 U/mL) at 10 ng/mL tetracycline concentration.

3.4.2. Effect of induction temperature on soluble TtSPase expression

As shown in Fig. 3, the highest soluble *Tt*SPase expression of co-expression strains pGro7-SPase, pG-KJE8-SPase, and pG-TF2-SPase were obtained at induction temperature of 18 °C. As the temperature increased, the *Tt*SPase activities gradually decreased, which is consistent with the results of recombinant *Tt*SPase without chaperonin co-expression (Fig. 1e) but different from SPases from other organisms such as *Streptococcus mutans* (induction at 27 °C) [32] and *L mesenteroides* (induction at 28 °C) [33]. Most protein expression systems showed increased inclusion body formation as the temperature increased, owing to the faster protein synthesis, aggregation, and interaction between nascent polypeptides [34].

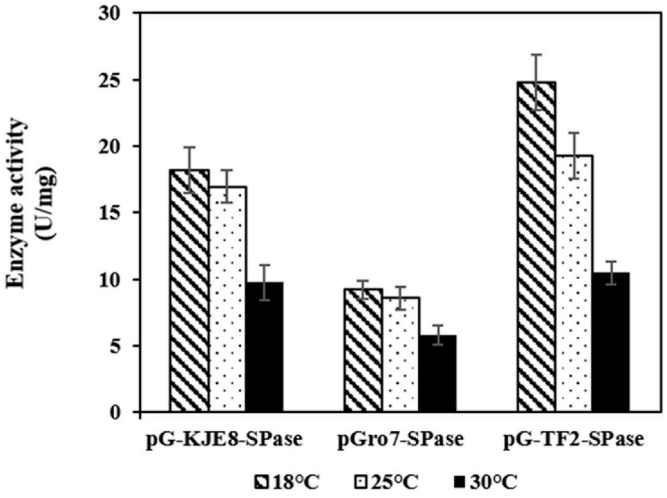

Fig. 3. Effect of induction temperatures on the co-expressions of pG-KJE8-SPase, pGro7-SPase, and pG-TF2-SPase. Each value represents the mean of three independent measurements.

3.4.3. Effect of addition order of IPTG and L-arabinose (or tetracycline) on the soluble TtSPase expression

In this study, the effect of addition order of IPTG and L-arabinose (or tetracycline) on *Tt*SPase activity was also investigated. The induction of pG-KJE8, pGro7, and pG-TF2 prior to *Tt*SPase caused lower total enzyme activity than that of IPTG (Table 3). Moreover, when inducers of chaperones (L-arabinose and/or tetracycline) were added simultaneously with *Tt*SPase inducer (IPTG), maximum activities of *Tt*SPases from co-expression strains were observed, which were 18.5, 9.53, and 25.3 U/mg for pG-KJE8, pGro7, and pG-TF2 co-expression respectively.

Table 2
Effect of molecular chaperones induction on *Tt*SPase activity.

IPTG concentration (mM)	L-ara concentration (mg/mL)	Tetracycline concentration (ng/mL)	pG-KJE8-SPase enzyme activity (U/mg)	pGro7-SPase enzyme activity (U/mg)	pG-TF2-SPase enzyme activity (U/mg)
0.05	0.5	5	18.5 ± 2.41	–	–
0.05	0.5	10	17.3 ± 1.95	–	–
0.05	1	5	17.6 ± 1.41	–	–
0.05	1	10	14.7 ± 1.73	–	
0.05	2	5	13.9 ± 0.94	–	
0.05	2	10	12.5 ± 0.41	–	–
0.05	0.5	0	–	9.52 ± 0.12	–
0.05	1	0	–	8.91 ± 0.62	–
0.05	2	0	–	7.92 ± 0.75	–
0.05	0	5	–	–	20.2 ± 0.61
0.05	0	10	–	–	25.7 ± 0.55

Each value represents the mean of three independent measurements.

Table 3
Effect of addition order of IPTG and L-arabinose (or tetracycline) on *Tt*SPase activity.

Induction condition	pG-KJE8-SPase enzyme activity (U/mg)	pGro7-SPase enzyme activity (U/mg)	pG-TF2-SPase enzyme activity (U/mg)
Prior [a]	16.7 ± 1.61	8.60 ± 0.33	21.1 ± 0.92
Simultaneously [b]	18.5 ± 2.12	9.53 ± 0.91	25.3 ± 1.91
Postpone [c]	17.3 ± 1.73	8.92 ± 0.64	22.3 ± 2.30

Each value represents the mean of three independent measurements.
[a] "Prior" represents L-arabinose and/or tetracycline added prior to inducer IPTG.
[b] "Simultaneously" means L-arabinose and/or tetracycline added simultaneously with inducer IPTG.
[c] "Postpone" indicates L-arabinose and/or tetracycline added after inducer IPTG. pG-KJE8-SPase was induced by 0.05 mM IPTG concentration, 0.5 mg/mL L-arabinose and 5 ng/mL tetracycline. pGro7-SPase was induced by 0.05 mM IPTG concentration and 0.5 mg/mL L-arabinose. pG-TF2-SPase was induced by 0.05 mM IPTG concentration and 10 ng/mL tetracycline.

3.5. Properties and characteristics of purified TtSPase

Crude *Tt*SPase was purified by affinity chromatography with 150 mmol/L imidazole concentration elution. As shown in Fig.S1, more than 90% purity of *Tt*SPase was obtained after affinity chromatography, and the purification fold and recovery efficiency were 2.01 and 70.4%, respectively (Table S2).

The kinetic analysis of purified *Tt*SPase were subsequently investigated. Based on the Linewaver-Burk plots graph (Fig. S2), kinetic parameters of *Tt*SPase were determined. As shown in Table 4, with sucrose as the substrate, the K_m value of *Tt*SPase was 55.6 mM, which was far higher than that of SPases from other microorganisms (e.g. *L. mesenteroides* [35–38], *Bifidobacterium adolescentis* [39]) (Table 4). It suggested that the substrate affinity of *Tt*SPase to sucrose was lower than that of other microorganisms SPases. The catalytic efficiency (k_{cat}/K_m) of *Tt*SPase was 1.80 s^{-1} mM^{-1}, which was lower than that from *L. mesenteroides* [35–38] but higher than that from *B. adolescentis* [39]. The kinetics characterizations of *Tt*SPase in this study was very similar with the reported sucrose 6′-phosphate phosphorylase from *T. thermosaccharolyticum* [7], which had a higher affinity with sucrose 6′-phosphate phosphorylase than sucrose.

Temperature is a trade-off between enzyme activity and stability. Higher temperature could increase the interaction between substrate and enzyme, and improve the product conversion rate. On the contrary, lower temperature could result in lower enzyme activity whereas higher stability. As shown in Fig. 4a, the optimum temperature for *Tt*SPase was 60 ˚C, which was higher than that of SPases from other microorganisms, such as *B. longum* SPase (45 ˚C) [10] and *L. mesenteroides* NRRL B-742 SPase (37 ˚C) [33]. *Tt*SPase were incubated at 70 ˚C and 80 ˚C to measure the half-life of thermal inactivation ($t_{1/2}$). The half-life of thermal inactivation ($t_{1/2}$) at 70 ˚C reached approximately 19 min, and almost all of the activity was lost when kept for 1 h, as shown in Fig. 4b. Compared with that at 70 ˚C, the activity of *Tt*SPase was almost exhausted at 80 ˚C for 3 min, and its half-life was only 2.3 min (Fig. 4b). T_{50}^{30} of *Tt*SPase, which is the temperature at which an enzyme loses half of its maximum activity after 30 min incubation, was 67 ˚C (Fig. 4c). The enzyme retained over 80% relative activity at 50 ˚C-65 ˚C. Additionally, the melting temperature was 73 ˚C. These results indicated that *Tt*SPase has great potential in practical industrial applications due to its high thermostability. Although the SPase we used in this work has low affinity with sucrose, it has much better thermostability than SPases from other sources (such as *L. mesenteroides*), and it may have a greater superiority than SPases from other sources in the future industrial application, especially in food industry, because reactions at high temperature will reduce the risk of food spoilage with microorganism breeding.

The optimum pH of the purified *Tt*SPase was 6.5 (Fig. 5), which was generally similar to SPase from other microorganisms, such as *B. adolescentis* DSM20083 [40]. As shown in Fig. 5, when purified *Tt*SPase was incubated in PBS buffer with different pH for 1 h, it retained > 50% of relative activity between pH 6 and 7.5, which was also similar to SPase from *B. longum* [10] and *L. mesenteroides* NRRL B-742 [33].

Table 4
Summary of kinetic parameters of SPase from different microorganisms.

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	Reference
*Tt*SPase [a]	100 ± 3.1	55.6 ± 1.8	1.80	This work
*Lm*SPase [b]	165	9.50	17.3	34
*Lm*SPase [b]	165	5.75	28.7	36
*Lm*SPase [b]	105	3.10	33.8	37
*BL*SPase SJ32 [c]	1.87	3.88	0.48	38

Each value represents the mean of three independent measurements.
[a] : SPase from *Thermoanaerobacterium thermosaccharolyticum*.
[b] SPase from *Leuconostoc mesenteroides*.
[c] SPase from *Bifidobacterium adolescentis* SJ32.

3.6. Application of TtSPase for α-arbutin synthesis

Purified *Tt*SPase was used for α-arbutin synthesis. As shown in Fig. 6, α-arbutin was synthesized with sucrose and hydroquinone as substrates. The retention times of α-arbutin and hydroquinone were 6.997 min and 9.821 min, respectively. Meanwhile, one unknown by-products (18.957 min) were also detected. Fig. 7 showed the α-arbutin production formed during incubation of *Tt*SPase (1.5 units) with sucrose (15 mM) and hydroquinone (5 mM). The highest molar production yield of α-arbutin (21%) was obtained at 16 h. The results indicated that the synthesis of a majority of by-products severely limited the production of α-arbutin. Improving product specificity of α-arbutin by protein engineering strategy seems extremely necessary in our future work.

4. Conclusion

To improve the soluble expression of SPase from *T. thermosaccharolyticum* in *E. coli*, the recombinant strain *E. coli* BL21 (DE3)/pET20b (+)/*spase* was constructed and 3.71 U/mg of *Tt*SPase was obtained under optimum conditions. Furthermore, the co-expressions of different chaperone teams were investigated. Three chaperone teams (pG-KJE8, pGro7, and pG-TF2) could improve the soluble expression of *Tt*SPase. Meanwhile, the effects of inducer concentration, induction temperature and addition order of inducer on soluble protein expression were evaluated. Under optimum conditions, the activity of *Tt*SPase, especially for pG-TF2 co-expression, reached 25.7 U/mg, which was 6.92-fold of that without co-expression. The optimum temperature and pH of *Tt*SPase were 60 ˚C and 6.5, respectively. The purified recombinant *Tt*SPase also had a higher thermostability than SPases from various microorganisms. Transfer reaction of hydroquinone by *Tt*SPase led to 21% of molar production yield of α-arbutin synthesis. This study developed an effective method for enhancing soluble expression of *Tt*SPase in *E. coli*. The recombinant *Tt*SPase with high thermostability is potential for further industrial applications.

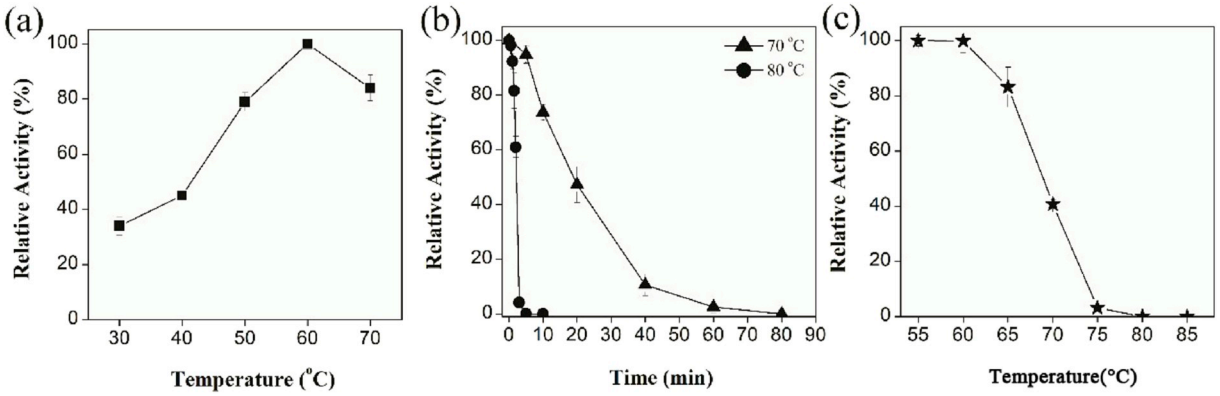

Fig. 4. The optimum temperature and thermostability of the purified *Tt*SPase. The relative activity was calculated as the percentage of activity to the maximum activity. (a) The optimum temperature of the purified *Tt*SPase; (b) The half-life value ($t_{1/2}$) of the purified *Tt*SPase at 70 °C and 80 °C; (c) The T_{50}^{30} value of the purified *Tt*SPase T_{50}^{30} value represents the temperature at which an enzyme is incubated for 30 min, and its reserved activity reached half of the maximum activity. Each value represents the mean of three independent measurements.

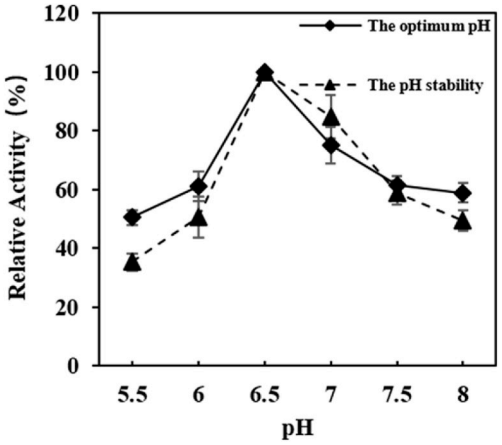

Fig. 5. The optimum pH and pH stabilities of the purified *Tt*SPase. The relative activity in was calculated as the percentage of activity to the maximum activity. Each value represents the mean of three independent measurements.

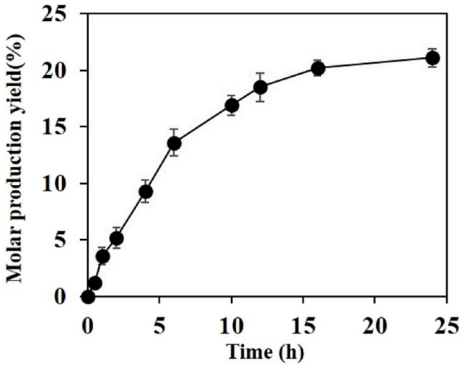

Fig. 7. α-Arbutin production formed during incubation of *Tt*SPase (1.5 units) with sucrose (15 mM) and hydroquinone (5 mM). Each value represents the mean of three independent measurements.

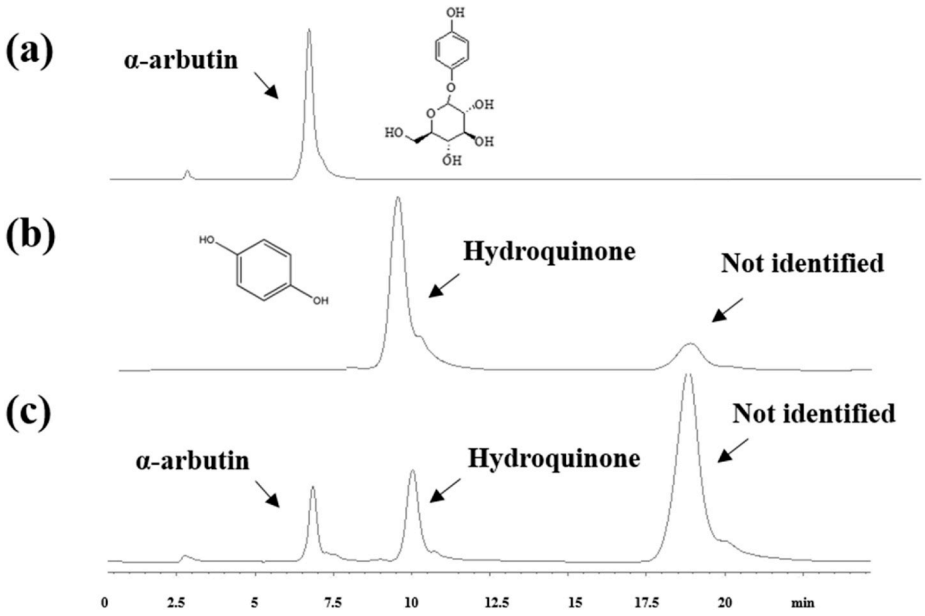

Fig. 6. HPLC chromatogram of transfer reaction of hydroquinone by *Tt*SPase. (a) The standard of α-arbutin. (b) Substrate of hydroquinone. (c) Reaction mixture by *Tt*SPase.

CRediT authorship contribution statement

Dong Yao: Investigation, Writing - original draft, Formal analysis, Data curation. **Jia Fan:** Data curation, Formal analysis. **Ruizhi Han:** Conceptualization, Writing - review & editing, Methodology, Funding acquisition. **Jing Xiao:** Investigation, Formal analysis. **Qian Li:** Methodology, Data curation. **Guochao Xu:** Validation, Data curation. **Jinjun Dong:** Investigation. **Ye Ni:** Supervision, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pep.2020.105571.

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