Gene Cloning, Expression, and Biochemical Characterization of a Recombinant Trehalose Synthase from *Picrophilus torridus* in *Escherichia coli*

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A trehalose synthase (TSase) gene from a hyperacidophilic, thermophilic archaea, *Picrophilus torridus*, was synthesized using overlap extension PCR and transformed into *Escherichia coli* for expression. The purified recombinant *P. torridus* TSase (PTTS) showed an optimum pH and temperature of 6.0 and 45 °C, respectively, and the enzyme maintained high activity at pH 5.0 and 60 °C. Kinetic analysis showed that the enzyme has a 2.5-fold higher catalytic efficiency (k_{cat}/K_M) for maltose than for trehalose, indicating maltose as the preferred substrate. The maximum conversion rate of maltose into trehalose by the enzyme was independent of the substrate concentration, tended to increase at lower temperatures, and reached ≈71% at 20 °C. Enzyme activity was inhibited by Hg^{2+}, Al^{3+}, and SDS. Five amino acid residues that are important for α-amylase family enzyme catalysis were shown to be conserved in PTTS (Asp^{203}, Glu^{245}, Asp^{311}, His^{106}, and His^{310}) and required for its activity, suggesting this enzyme might employ a similar hydrolysis mechanism.

KEYWORDS: **High performance liquid chromatography; maltose;** *Picrophilus torridus***; trehalose; trehalose synthase**

INTRODUCTION

Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a naturally occurring nonreducing disaccharide in which the two glucose molecules are linked through a 1,1-glycosidic bond. Although three anomers of trehalose exist, that is, α,α-1,1-, α,β-1,1,- and β,β-1,1-trehalose, only the α,α-1,1-form is widespread in nature and present in a large number of organisms, including bacteria, yeast, fungi, insects, invertebrates, and plants (*1*). Trehalose has important biological functions in organisms, including serving as an energy and carbon source, bacterial cell wall component and signaling molecule, and helping organisms acquire tolerance to various stresses, such as cold, heat, desiccation, dehydration, and osmotic and oxidative stress (*2*−*10*). It is also very important for industrial applications in fields such as food, cosmetic, and pharmaceutical industries (*11*).

Three main pathways specifying the biosynthesis of trehalose have been identified in various organisms (*11*). The first pathway utilizes trehalose-phosphate (P) synthase (EC 2.4.1.15) (OtsA in *Escherichia coli*) that catalyzes the transfer of glucose from UDP-glucose to glucose-6-P to form trehalose-P and UDP. The phosphate is then removed by trehalose-P phosphatase (EC 3.1.3.12) (OtsB in *E. coli*) to give free trehalose (*12*). The second pathway also involves two enzymes called maltooligosyl trehalose synthase (EC 5.4.99.15) and maltooligosyl trehalose trehalohydrolase (EC 3.2.1.141). The former enzyme first converts the α-1,4-linkage in the reducing end of the malto-oligosaccharide chain into an α-1,1-linkage, and then the latter enzyme hydrolyzes the reducing-end disaccharide to release one molecule of trehalose (*13*−*15*). The third pathway, catalyzed by trehalose synthase (TSase) (EC 5.4.99.16), involves the direct conversion of maltose into trehalose by an intramolecular rearrangement of the α-1,4-linkage of maltose to the α-1,1-linkage of trehalose (*16*). Because this pathway allows one-step formation of trehalose and an inexpensive substrate, maltose, is employed, this pathway is capable of industrial manufacture of trehalose. Up to now, about six TSases have been reported from different species and characterized for their biochemical properties (*17*−*22*). However, the three TSases

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from *Pimelobacter* sp. R48, *Thermobifida fusca*, and *Pseudomonas stutzeri* CJ38 are thermolabile, and the one from *Thermus aquaticus* is highly thermostable, possessing the problem of low enzyme yield in the original organism. Furthermore, these enzymes prefer to work at a pH that is near or above neutral.

In this study, we report the biochemical properties and proposed catalytic mechanism of a recombinant TSase (PTTS) from *Picrophilus torridus*, a hyperacidophilic, thermophilic, heterotrophic, and absolutely aerobic archaea that grows optimally at 60 °C and pH 0.7 (*23*). The unique property of this enzyme suggests that it is potentially useful for industrial production of trehalose.

MATERIALS AND METHODS

Materials. All saccharides were purchased from Sigma Chemical Co. The column for protein purification was obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). Acetonitrile was from Tedia Co. Inc. All other chemicals and reagents were of analytical grade.

Bacterial Strains and Plasmids. The expression vector pET-23a-(+) (Novagen, Madison, WI) was used and transformed into the *E. coli* strains DH5α and Rosetta-gami B (DE3) (Novagen) for cloning and expression, respectively. *E. coli* strains were cultured in Luria−Bertani (LB) broth and on LB agar supplemented with 100 μg/mL ampicillin (LB-Amp) for cloning host or in combination with 15 μg/mL kanamycin, 12.5 μg/mL tetracycline, and 34 μg/mL chloramphenicol (LB-Amp-Kan-Tet-Chl) for expression host. All *E. coli* strains were cultured at 37 °C and in an orbital shaker at a speed of 225 rpm unless otherwise mentioned.

Synthesis of Trehalose Synthase Gene. According to the published DNA sequence of the TSase gene of *P. torridus* (DSM 9790) (GenBank accession number AE017261) (*PTTS*), 36 oligonucleotides were designed accounting for both strands of the full-length ORF of *PTTS* (1677 bp). Each oligonucleotide contained 70 base pairs and overlapped with adjacent ones by 20 base pairs. *Nde*I and *Eco*RI cutting sites were introduced into the 5′ and 3′ ends of the gene, respectively, and the three *Nde*I cutting sites inside the sequence were modified according to the *E. coli* codon usage database (at http://www.kazusa.or.jp/codon) without changing the amino acid sequence. The stop codon of *PTTS* was eliminated to in-frame read a His (*6*)-tag on the C terminus of the protein for one-step purification. Overlap extension PCR was used to assemble the 36 oligonucleotides to obtain the full-length PTTS gene. The synthesized PTTS gene was then digested with restriction enzymes *Nde*I and *Eco*RI and ligated into vector pET-23a(+). The resulting plasmid was transformed into Rosetta-gami B (DE3) for expression.

Protein Purification. The strain harboring the plasmid was cultivated overnight in LB-Amp-Kan-Tet-Chl medium and refreshed in a ratio of 1:40 with the same medium. Protein expression was performed by using basal-level expression, and the refreshed culture was cultivated at 27 °C and in an orbital shaker at a speed of 225 rpm for 2 days without the addition of IPTG. The culture broth was centrifuged at 4000 rpm for 10 min at 4 °C and the supernatant decanted. The resulting cell pellet was resuspended in 20 mM sodium phosphate buffer (pH 7.0), and cells were lysed using a sonicator (Misonix, model XL-2020) set to a power of 5% for 20 times of 30 s bursts, with a 30 s intermission between bursts. After centrifugation at 12000 rpm for 20 min, the supernatant containing crude enzyme was collected and then purified according to the following procedures: 20 mM sodium phosphate (pH 7.0) buffer containing 2.5 M NaCl and 25 mM imidazole was added to the crude extract to reach a final concentration of 0.5 M NaCl and 5 mM imidazole. It was then loaded onto a HiTrap Chelating HP column (1 mL) equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl and 5 mM imidazole on a fast protein liquid chromatography system (Pharmacia). After the column was washed, the PTTS was eluted with a linear gradient of 5−500 mM imidazole in the same buffer. Active fractions were pooled, concentrated, and buffer exchanged using Centricon PL-30 (Amicon, Beverly, MA). The purified enzyme was stored in 20 mM sodium phosphate buffer (pH 7.0) and was analyzed on 12% SDS-PAGE.
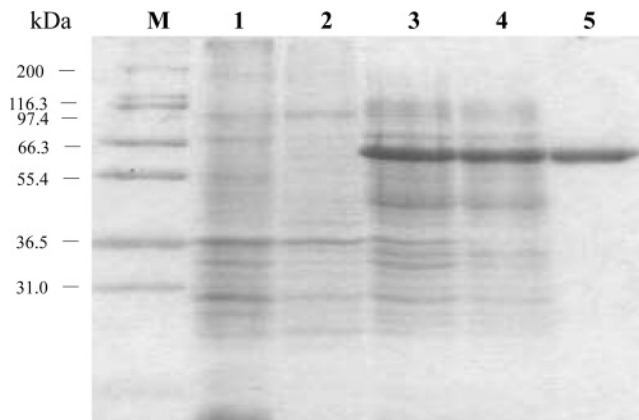

Figure 1. 12% SDS-PAGE analysis for the purification of recombinant PTTS: (lanes 1 and 2) total cell lysate and soluble fraction, respectively, obtained from a non-expression control of Rosetta-gami B transformed with pET-23a(+)-PTTS plasmid; (lanes 3 and 4) total cell lysate and soluble fraction, respectively, obtained from Rosetta-gami B(DE3) transformed with pET-23a(+)-PTTS; (lane 5) purified recombinant PTTS after Ni column purification; (lane M) protein molecular mass markers indicated in kDa.

Protein Assay. Protein concentration was measured according to the method of Bradford using a protein assay kit purchased from Bio-Rad Laboratories (Hercules, CA) with bovine serum albumin as standard.

Enzyme Assay. The activity of TSase was assayed by measuring the amount of trehalose produced from maltose. The standard reaction was performed by adding 1.5 μL of purified enzyme into 50 μL of reaction solution containing 50 mM sodium phosphate (pH 6.0) and 150 mM maltose and incubating in a 45 °C water bath for 25 min. The assay time was under the linear range of enzyme reaction. The reaction was terminated by heating the mixture in boiling water for 15 min. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of trehalose per minute. Kinetic analysis was performed under conditions of pH 6.0 and 45 °C for 5 min in 50 mM sodium phosphate buffer containing substrate (maltose, trehalose, or maltose plus a constant 10 mM glucose) at various concentrations. The resulting data were analyzed off-line with Origin 6.0 software (Microcal, Northampton, MA). All experiments were carried out in duplicate or triplicate.

Carbohydrates Analysis. The amount of trehalose, glucose, and maltose after each enzymatic reaction was measured using a high-performance liquid chromatography (HPLC) (SFD 2100) system equipped with an RI detector (Schambeck SFD, RI 2000) at a flow rate of 0.9 mL/min. A carbohydrate analysis column (Hypersil-100 Amino, Thermo Hypersil-Keystone) equilibrated with 75% acetonitrile, 24% Milli-Q water, and 1% formic acid was used. The retention times of glucose, maltose, and trehalose were 8.0, 11.2, and 12.5 min, respectively.

RESULTS

Purification of Recombinant PTTS. The production of recombinant PTTS constituted a very high percentage (≈28%) of the total cell protein (data not shown). Protein purification was carried out by Ni column; the enzyme was purified 3.6-fold with a yield of 63%, and the specific activity was 80 units mg^{-1} of protein (data not shown). SDS-PAGE analysis of the purified enzyme showed a single protein band around 65 kDa in size (**Figure 1**).

Effects of pH and Temperature on the Activity and Stability of Recombinant PTTS. The pH dependence of PTTS was studied at 45 °C in 50 mM acetate buffer (pH 3.5−6.0) or 50 mM phosphate buffer (pH 6.0−8.5) using 150 mM maltose as a substrate. The optimum pH for PTTS was 6.0, but the

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Table 1. Kinetic Parameters of PTTS

substrate	K_M (mM)	$V_{max}{}^a$ (μmol/min·μmol)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
maltose	42.4 ± 1.6	5699.4 ± 61.4	95.0 ± 1.0	2238.1 ± 108.1
trehalose	210.3 ± 3.9	11257.3 ± 295.1	187.6 ± 5.0	892.1 ± 6.9
maltose + 10 mM glucose	137.8 ± 4.0	5870.1 ± 120.7	97.8 ± 2.0	709.8 ± 6.4

[a] Kinetics analyses were carried out under conditions of pH 6.0 and 45 °C for 5 min with maltose or trehalose of various concentrations. The concentration of purified PTTS used was 0.38 μM. The theoretical molecular weight of the recombinant PTTS is 68016.
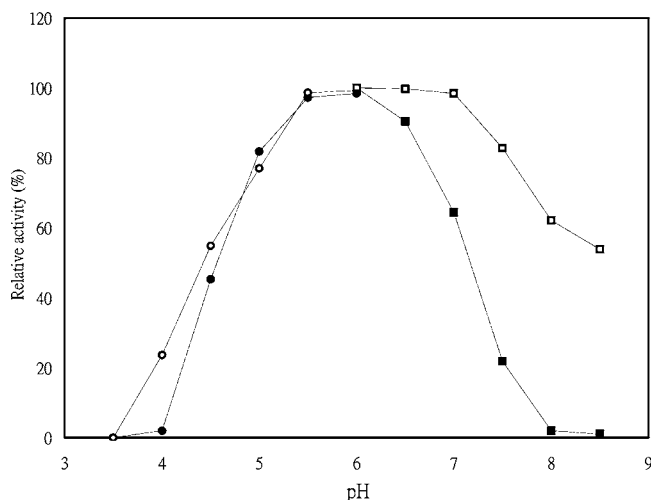

Figure 2. Effects of pH on the activity and stability of PTTS. The enzyme activities at various pH values were examined at the maltose concentration of 150 mM and 45 °C for 25 min. The pH stability of enzyme was examined by measuring the residual activity of enzyme at pH 6.0 after preincubation in various pH values at 45 °C for 20 min. The solid square (■) and circle (●) indicate the enzyme activity under 50 mM sodium phosphate buffer (pH 6.0–8.5) and 50 mM acetate buffer (pH 3.5–6.0), respectively. The open square (□) and circle (○) represent the pH stability under 50 mM sodium phosphate buffer (pH 6.0–8.5) and 50 mM acetate buffer (pH 3.5–6.0), respectively.

enzyme maintained high activity at pH 5.0. pH stability, examined by preincubating the purified enzyme at various pH values for 20 min and then assaying the remaining enzyme activity under standard assay condition, showed a highly retained activity within a pH range of 5.0−7.5 (**Figure 2**). The effects of temperature on PTTS activity and stability are depicted in **Figure 3**. The optimum temperature was 45 °C, and the enzyme maintained stability up to 60 °C at pH 6.0 for 20 min.

Kinetics Analysis. Kinetic parameters of PTTS were investigated at pH 6.0 and 45 °C for 5 min using maltose or trehalose as substrate (**Table 1**). The results showed that this enzyme has a much higher affinity for maltose than trehalose because the K_M for trehalose was ≈5 times that for maltose. Although a higher k_{cat} for trehalose was seen, PTTS had a 2.5-fold higher enzyme efficiency (k_{cat}/K_M) toward maltose than trehalose. Moreover, it was found that the addition of glucose into the reaction mixture would retard trehalose formation, and this effect was proportional to the glucose concentration (data not shown). In the presence of 10 mM glucose, PTTS showed a 3.3-fold increase in K_M and a nearly unchanged V_{max} for maltose, implying that glucose is a competitive inhibitor of TSase (**Table 1**).

Effects of Metal Ions and Reagents on PTTS Activity. The effects of metal ions and reagents were determined by examining enzyme activity in the presence of 1 and 10 mM metal ions or reagents under standard assay conditions (**Table 2**). The results
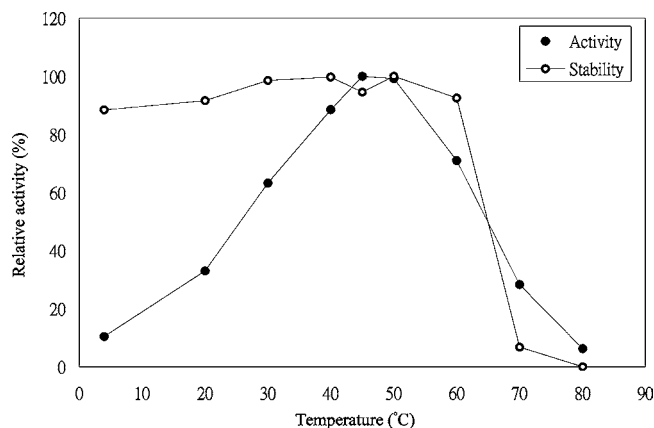

Figure 3. Effects of temperature on the activity and stability of PTTS. The enzyme activities at various temperatures were examined at the maltose concentration of 150 mM and pH 6.0 for 25 min. To examine temperature stability, the residual activity of the enzyme was measured at 45 °C after preincubation of the purified enzyme at different temperatures (4–100 °C) and pH 6.0 for 20 min. The solid circle (●) and open circle (○) denote the enzyme activity and temperature stability, respectively, under various temperatures.

Table 2. Effects of Metal Ions and Reagents on the Activity of PTTS

	relative activity[a] (%)			relative activity[a] (%)	
reagent	1 mM	10 mM	reagent	1 mM	10 mM
none	100	100	CdSO$_4$	64	0
MnCl$_2$	108	0	CuSO$_4$	62	0
ZnSO$_4$	102	0	PbCl$_2$	60	62
MgCl$_2$	99	7	HgCl$_2$	0	0
SrCl$_2$	98	4	Al$_2$(SO$_4$)$_3$	0	0
CaCl$_2$	97	4	DTT	96	85
CoCl$_2$	96	1	EDTA	93	85
BaSO$_4$	93	4	Tris	67	8
NiCl$_2$	90	3	SDS	0	0
FeSO$_4$	84	0			

[a] Enzyme activity was measured in the presence of 1 and 10 mM metal ions or reagents under assay conditions of temperature 45 °C, 50 mM sodium phosphate buffer (pH 6.0), and 150 mM maltose for 25 min. Relative activity is expressed as a percentage of the enzyme activity in the absence of metal ions and reagents.

indicated that the enzyme activity was inhibited strongly by Hg^{2+}, Al^{3+}, and SDS and moderately by Cd^{2+}, Cu^{2+}, Pb^{2+}, and Tris at concentrations of 1 mM. However, at a concentration of 10 mM, almost all metal ions and reagents, except DTT and EDTA, inhibited the enzyme activity.

Substrate Specificity of Recombinant PTTS. The recombinant PTTS acted on maltose and trehalose and converted them to each other with the production of a small amount of glucose. It also acted on sucrose and catalyzed the formation of glucose, fructose, and trehalulose [α-D-glucopyranosyl-(1,1)-D-fructofuranose]. However, the activity was quite low (data not shown).
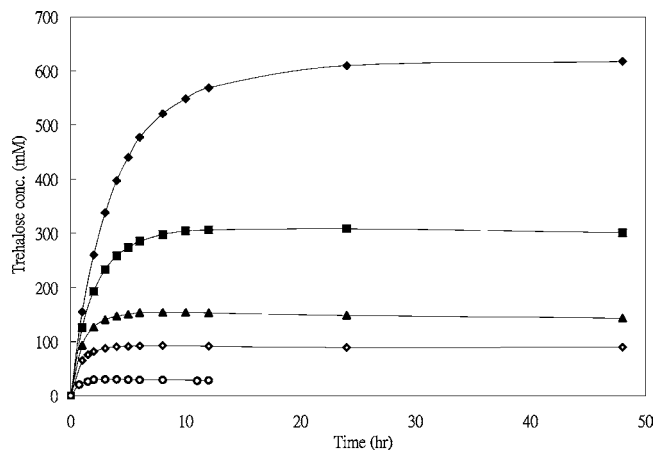
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Figure 4. Effects of substrate concentration on the maximum yield of trehalose by PTTS. Purified enzyme was incubated with different concentrations of maltose in 50 mM sodium phosphate buffer (pH 6.0) at 45 °C. Samples were collected at various intervals of reaction time and analyzed by HPLC. The symbols, which indicate different concentrations of maltose, are ◆, 1 M; ■, 500 mM; ▲, 250 mM; ◇, 150 mM; and ○, 50 mM.

In addition, it converted the α,β-1,1-trehalose to maltose, although only ≈7%, just as with the α,α-1,1-trehalose. No detectable activities were seen with glucose, fructose, galactose, β,β-1,1-trehalose, lactose, mannose, sorbitol, isomaltulose, maltotriose, maltotetraose, maltopentose, starch, or methyl cellulose as substrates under the standard assay conditions described under Materials and Methods (data not shown).

Effects of Substrate Concentration and Temperature on the Maximum Yield of Trehalose. The effects of the substrate concentration on the yield of trehalose by PTTS were examined at pH 6.0, 45 °C, and various maltose concentrations. The final yield of trehalose increased as the substrate concentration increased. The maximum conversions of maltose into trehalose were all ≈60%, implying that the enzyme conversion was independent of the substrate concentration and that higher maltose concentration did not have any adverse effect on the trehalose productivity (**Figure 4**). For the effects of temperature, reaction mixtures containing 150 mM maltose were incubated under pH 6.0 at 20, 30, 45, and 60 °C for 3 days, and the maximum conversions of maltose into trehalose were 71, 68, 61, and 50% with maltose and glucose contents of 25.4 and 3.6%, 27.6 and 4.3%, 31.2 and 7.8%, and 31.3 and 19.2%, respectively (**Figure 5**). Although a faster catalytic rate can be achieved at higher temperature, a slightly lower conversion was displayed due to more glucose generated. Therefore, a higher maximum yield was gained at a lower temperature.

Site-Directed Mutagenesis. Domain architectures analysis using the SMART program (http://smart.embl-heidelberg.de) revealed that PTTS contains an α-amylase domain spanning residues 16−413, with an *E* value of 2.10E−10[7]. In alignment with the amino acid sequences of two structure-resolved glycosyl hydrolase family 13 (α-amylase family) enzymes, oligo-1,6-glucosidase from *Bacillus cereus* and isomaltulose synthase from *Klebsiella* sp. LX3, three putative active sites (Asp[203], Glu[245], and Asp[311]) and two substrate-binding sites (His[106] and His[310]) of PTTS were deduced (*24*, *25*). To verify the importance of these residues, site-directed mutagenesis was used to replace the five residues individually with Ala, and each mutant recombinant protein was purified by Ni column. The drastic reduction in enzyme activity of all mutants suggested that these five residues might play essential roles in PTTS catalysis (**Table 3**).
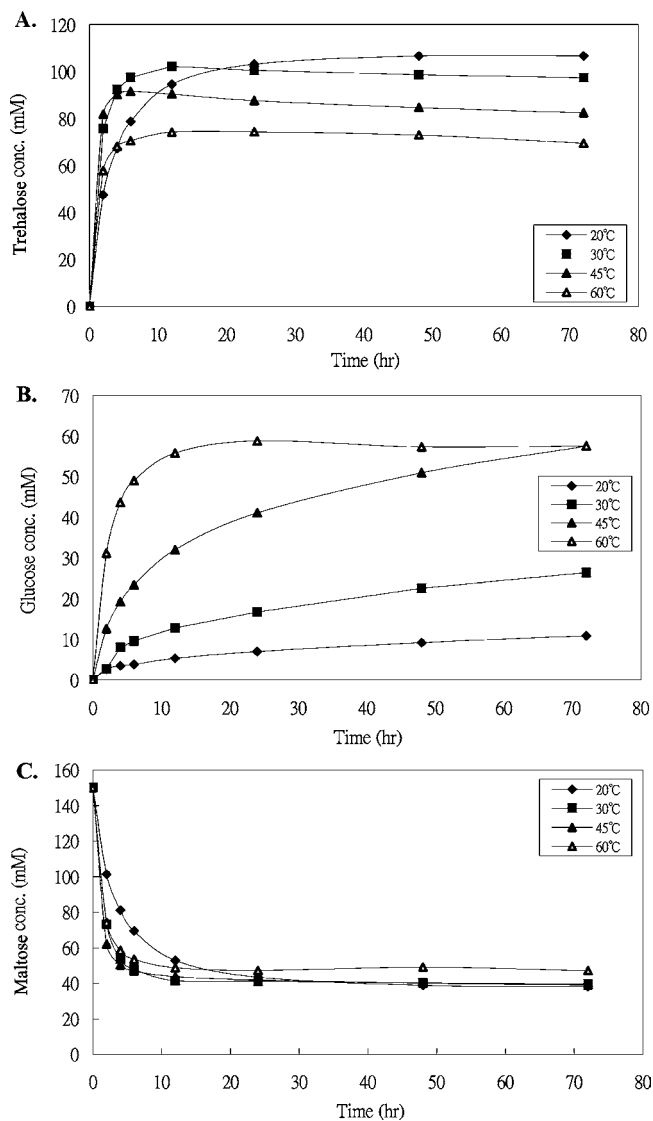

Figure 5. Effects of temperature on the formation of trehalose (**A**) and glucose (**B**) from maltose (**C**) by PTTS. Reaction mixtures containing purified PTTS, 150 mM maltose, and pH 6.0 sodium phosphate buffer were incubated at various temperatures for 72 h. Samples were collected at various intervals and analyzed by HPLC. The symbols, which indicate different reaction temperatures, are ◆, 20 °C; ■, 30 °C; ▲, 45 °C; and △, 60 °C.

Table 3. Relative Specific Activity of Wild-Type PTTS and Its Mutant Enzymes

mutation	relative specific activity[a] (%)	mutation	relative specific activity[a] (%)
wild type	100	E245A	0.26 ± 0.007
H106A	0.73 ± 0.115	H310A	10.95 ± 0.144
D203A	0.21 ± 0.005	D311A	0.20 ± 0.034

[a] Relative activities are represented as the ratio of mutants to wild type. The specific activity of wild-type PTTS was 86 units/mg.

Moreover, similar conservations of active sites and substrate-binding sites were observed in two other TSases, *Pimelobacter* TSase and *Thermus aquaticus* TSase (**Figure 6**), further supporting the catalytic importance of these residues in TSase activity.

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PTTS   LVLNHVSDQHPWFIESRSSIDNPKRDWFIWSDTPEKFKEARIIFIDTEKSNWTYLPETKQYYFHRFYSSQ 171
PSTS   FVMNHTSDAHPWFQASRSDPDGPYGDFYVWSDTDELYQDARVIFVDTEPSNWTWDQTRGQYYWHRFFHHQ 178
TATS   LVLNHTSIDHPWFQEARK-PNSPMRDWYVWSDTPEKYKGVRVIFKDFETSNWTFDPVAKAYYWHRFYWHQ 165
BSOG   LVVNHTSDEHAWFAESRKSKDNPYRDYYLWKDPKPDGSEPNNWGSIFSGSAWTYLEGTGQYYLHYFSKKQ 167
BCOG   LVVNHTSDEHNWFIESRKSKDNKYRDYYIWRPGK-EGKEPNNWGAAFSGSAWQYDEMTDEYYLHLFSKKQ 167
PalI   VVINHTSDQHPWFIQSKSDKNNPYRDYYFWRDGK-DNQPPNNYPSFFGGSAWQKLAKSGQYYLHYFARQQ 209

PTTS   PDLNYDNPDVRNEVKKVIRYWLDLGLDGFRADAVPYLFKRE----------------NTNCENLPETHNFF 226
PSTS   PDLNFDNPKVQDAMLEAMAFWLDMGLDGFRLDAVPYLYERP----------------GTNGENLPETHEML 233
TATS   PDLNWDSPEVEKAIHQVMFFWADLGVDGFRLDAIPYLYERE----------------GTSCENLPETIEAV 220
BSOG   PDLNWENEAVRREVYDVMRFWMDRGVDGWRMDVIGSISKYTDFPDYETDHSRSYIVGRYHSNGPRLHEFI 237
BCOG   PDLNWDNEKVRQDVYEMKFWLEKGIDGFRMDVINFISKEEGLPTVETEEEGYVSGHKHFMNGPNIHKYL 237
PalI   PDLNWDNPKVREDLYAMLRFWLDKGVSGMRFDTVATYSKIPGFPNLTPEQQ--KNFAEQYTMGPNIHRYI 277

                  *
PTTS   KEIRKMMDEDY-PGTILLAEANQWPTETKAYFGN----------GDEFHMAFNFPLMPRIFIALA------ 280
PSTS   KRVRRFVDDNY-PDRVLLYEANQWPTDVVEYFGPEEREDGTVVGPESHMAFHDPVMPRIFMAVR------ 296
TATS   KRLRKALEERYGPGKILLAEVNMWPEETLPYFGD------G----DGVHMAYNFPLMPRIFMALR------ 275
BSOG   QEMNREVLSHY--DCMTVGEANGSDIEEAKKYTDASR--------QELNMIFTFEHMDIDKEQNSPNGKWQ 298
BCOG   HEMNEEVLSHY--DIMTVGEMPGVTTEEAKLYTGEER--------KELQMVFQFEHMDLDSGEG---GKWD 295
PalI   QEMNRKVLSRY--DVATAGEIFGVPLDRSSQFFDRRR--------HELNMAFMFDLIRLDRDSN----ERWR 335

                         **
PTTS   ---RSDYYPIMDIIKQTLPIPDNCDWCIFLRNHDELTLEMVTDSER------DIMYREYAKIPKMRLNLG 341
PSTS   ---RESRFPISEIMEQTPAIPEGCQWGIFLRNHDELTLEMVTDEDR-------DYMWGEYAKDPRMKANIG 357
TATS   ---REDRGPIETMLKEAEGIPETAQWALFLRNHDELTLEKVTEEER-------EFMYEAYAPDPKFRINLG 336
BSOG   IKPFDLIALKKTMTRWQTGLMNVGWNTLYFENHDQPRVISRWGNDR-KLRKECAKAFATVLHGMKGTPFI 367
BCOG   VKPCSLLTLKENLTKWQKALEHTGWNSLYWNNHDQPRVVSRFGNDG-MYRIESAKMLATVLHMMKGTPYI 364
PalI   HKSWSLSQFRQIISKMDVTVGKYGWNTFFLDNHDNPRAVSHFGDDRPQWREASAKALATITLTQRATPFI 405
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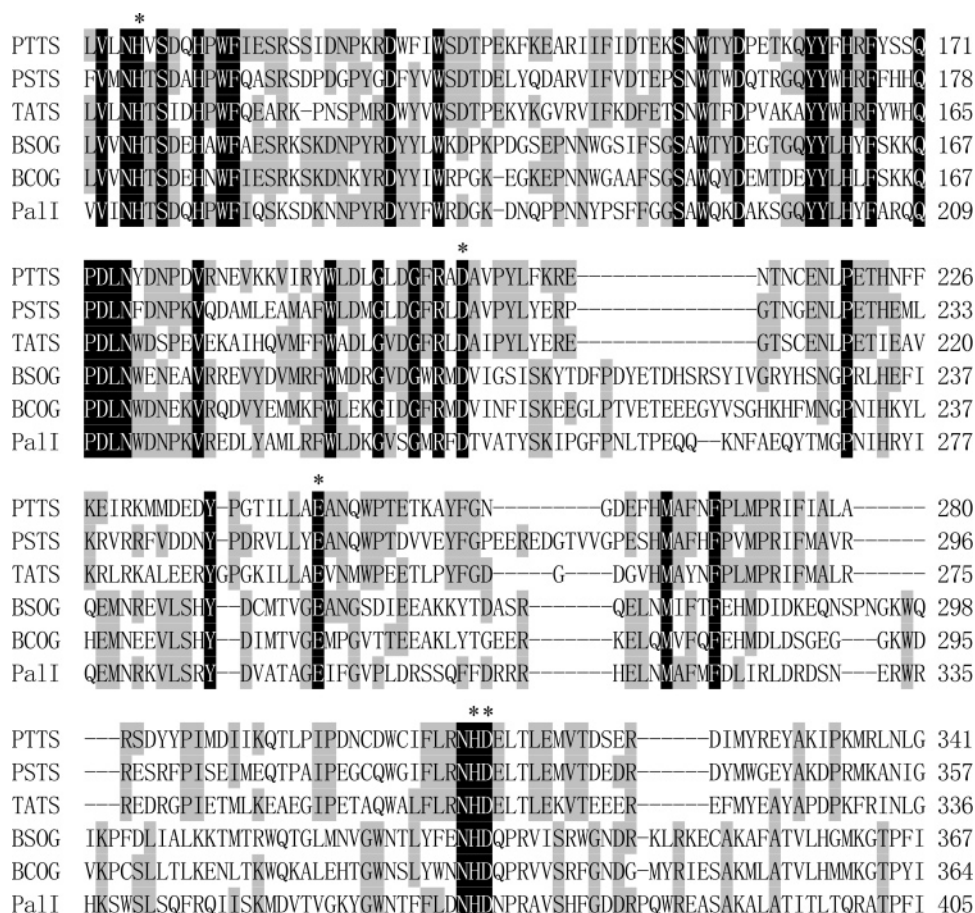
Figure 6. Alignment of amino acid sequences of trehalose synthases from *P. torridus* (PTTS), *Pimelobacter* sp. R48 (PSTS), and *T. aquaticus* (TATS); oligo-1,6-glucosidases from *B. subtilis* (BSOG) and *B. cereus* (BCOG); and isomaltulose synthase from *Klebsiella* sp. LX3 (*PalI*). The conserved active sites and substrate-binding sites are denoted by asterisks. Protein sequence alignment was performed using Vector NTI suite 7.0 (Informax Inc., Bethesda, MD).

DISCUSSION

The recombinant PTTS reported here had an optimal pH of 6.0 and optimal temperature of 45 °C and maintained high activity and stability up to pH 5.0 and 60 °C. In comparison with other previously reported trehalose synthases, this recombinant PTTS has the most acidic optimum pH. Moreover, the three well-characterized TSases from *Pimelobacter*, *T. aquatics*, and *Mycobacterium smegmatis* showed dramatic decreases in enzyme activity at pH below 5.0 and retained <30% of activity (*17*, *18*, *20*). However, the PTTS could maintain 80% of its activity at pH 5.0. Therefore, it is more acid-tolerant than other TSases reported so far. As the Maillard reaction, a nonenzymatic protein glycation that often leads to protein denaturation and inactivation, is enhanced in the alkaline pH region, using enzymes that can tolerate acidic environment could help to reduce the chemical reaction between maltose and proteins at high temperature (*26*, *27*).

The specific activity of this enzyme toward maltose was estimated to be ≈80 units/mg of protein, which was ≈4.7-fold higher than that of *Pimelobacter* TSase, ≈2.8-fold higher than that of *M. smegmatis* TSase, and roughly equal to that of *P. stutzeri* CJ38 TSase and reached ≈60% of that of *T. aquaticus* TSase (*17*, *18*, *20*, *22*). Kinetics analysis showed that the recombinant PTTS had much greater affinity (5-fold) and catalytic efficiency (2.5-fold) for maltose than trehalose. However, the k_{cat} for trehalose was 2-fold higher than that for maltose (**Table 2**). Because no trehalase gene or other trehalose-

hydrolyzing genes in the *P. torridus* genome were predicted (NCBI), PTTS might play a regulatory role in controlling the intracellular amount of trehalose itself in *P. torridus*.

The biochemical properties of PTTS without the His-tag were the same as those with the His-tag (data not shown). Nevertheless, the specific activity of native PTTS was ≈1.3-fold higher than the recombinant His-tag fusion PTTS, perhaps due to the steric hindrance exerted by the His-tag that blocks the entrance of substrate into the active site. Although the His-tag might have an adverse effect on enzyme activity, it facilitates protein purification and simplifies the procedures. The purification yield of PTTS with the His-tag was 63% (data not shown), which was ≈4-fold higher than that of native protein (data not shown). Therefore, for applications that require purified enzyme, enzyme with the His-tag will be more useful. For applications that use crude enzyme, the enzyme without the His-tag should be considered.

Enzymes belonging to the α-amylase family contain a common structural feature and conserved residues for catalysis and substrate binding (*28*). Although TSase has been classified as a member of this family and proposed to contain a similar hydrolysis mechanism, no direct evidence can prove this speculation (*29*). Our study showed that residues of catalytic importance in α-amylase family enzymes were also conserved in PTTS (**Figure 6**), and the importance of these residues in PTTS catalysis was revealed in a mutagenesis study (**Table 3**). This finding is consistent with previous suggestions and further

Characterization of Recombinant *P. torridus* Trehalose Synthase

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supported the assumption that PTTS employs a similar hydrolysis mechanism as other α-amylase family enzymes to cleave the α-1,4-glycosidic linkage of maltose. A double displacement might proceed through the reaction; Glu[245] might act as a proton donor, and Asp[203] might act as a nucleophile that attacks the bonded anomeric carbon of the maltose.

More glucose was released when the reaction was performed under a higher temperature (**Figure 5**). This phenomenon is thought to be due to the weak hydrolytic property possessed by trehalose synthase itself, which increases as the temperature rises (*18*, *19*). However, this could also be explained from the viewpoint of mechanism. Koh et al. (*29*) proposed that glucose was generated due to the entry of a water molecule into the active site to hydrolyze the enzyme−glucose intermediate prior to the formation of the glycosidic bond. Hence, it is possible that a temperature rise would increase the flexibility of the protein structure, making the active site more accessible to water molecules to attack the split glucose before the formation of the α,α-1,1-glycosidic bond.

In conclusion, the recombinant PTTS reported here is thermostable and more acid-resistant than any others reported so far. Because a high-temperature, acidic environment is unfavorable for the growth of many organisms, the recombinant PTTS can be used to reduce the possibility of contamination. Besides, as high reaction temperature also increases the solubility and fluidity of substrate and no influence was seen on the trehalose conversion by PTTS in high maltose concentration, this enzyme could be applied more economically for the industrial manufacture of trehalose. In addition, our previous paper has described an enzymatic method that can produce high-maltose syrup and high-protein product simultaneously from raw material such as rice or corn (*30*, *31*). Hence, from a combination of PTTS with this invention a method that enables efficient and economical production of high-value trehalose from low-price crops could be developed.

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