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Cloning, expression, properties, and functional amino acid residues of new trehalose synthase from *Thermomonospora curvata* DSM 43183

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ABSTRACT

A new *trehalose synthase* (*TreS*) gene from *Thermomonospora curvata* DSM 43183 was cloned and expressed in *Escherichia coli* XL10-Gold. The purified recombinant enzyme (TreS-T.C) could catalyze the reversible interconversion of maltose and trehalose of sucrose into trehalulose without other disaccharides including isomaltulose at an optimum temperature of 35 °C and a pH of 6.5. The K_m of TreS-T.C for maltose (96 mM) was lower than those for trehalose (198 mM) and sucrose (164 mM), suggesting that maltose is the optimum substrate. The maximum trehalose and trehalulose yields were 70% and >80%, respectively. Active TreS-T.C is a trimer comprising three identical 60 kDa subunits. Homology modeling analysis revealed that TreS-T.C had a GH13-typical $(\beta/\alpha)_8$ barrel catalytic domain. Two sites, one determining substrate specificity (L116) and the other affecting product formation (E330), were found near the active center by homology modeling combined with site-directed mutagenesis. TreS-T.C may be used effectively as a potential biocatalyst for the production of trehalose and trehalulose from maltose and sucrose in a one-step reaction, respectively. This study also provides a feasible and effective method for studying functional amino acid residues around TreS without performing crystal structure analysis and high-throughput screening.

1. Introduction

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is a non-reducing disaccharide widely found in various organisms such as bacteria, archaea, yeast, fungi, insects, non-mammalian animals, and plants [1–5]. Trehalose is known as reserve energy that is a carbon source similar to glycogen and starch [6] as well as an active preservative of proteins, biomass, pharmaceutical preparations, and even organs for transplantation [7,8]. Therefore, trehalose is widely used in the food, cosmetics, and pharmaceutical industries [3,9], thus attracting increasing interest among researchers. On the other hand, trehalulose (1-O-α-D-glucosylpyranosyl-β-D-fructose) is a structural isomer of sucrose (α-D-glucosylpyranosyl-1,2-β-D-fructofuranoside) and a reducing disaccharide naturally present in honey in small quantities [10,11]. Trehalulose is non-cariogenic, exhibits a slower rate of monosaccharide release into the blood, and has high solubility [12]. Relative to trehalose, fewer studies have been conducted on trehalulose. However, both have broad application prospects in the food, cosmetics, and pharmaceutical industries, thus the extensive interest of numerous researchers.

Five main enzymatic routes are involved in trehalose biosynthesis: (1) trehalose-6-phosphate synthetase and trehalose-6-phosphate phosphatase [13–15], (2) maltooligosyltrehalose trehalohydrolase and maltooligosyltrehalose synthase [15–18], (3) trehalose phosphorylase [14,15,19,20], (4) trehalose glycosyltransferring synthase (TreT) [20–22], and (5) trehalose synthase (TreS) [23–25]. Among these routes, the conversion of maltose into trehalose by TreS is a simple, fast, and low-cost method for the industrial production of trehalose. Thus, an increasing number of TreS or *TreS* genes have been purified or heterologously expressed [23–29]. However, most studies on TreS have been confined to gene cloning and enzymatic property determination; few have reported on its catalytic mechanism or on functional amino acid residues. The chemical synthesis of trehalulose is extremely difficult. Trehalulose is industrially produced exclusively from sucrose using immobilized microorganisms. Through sucrose hydrolysis, several organisms and enzymes, including trehalulose synthase, isomaltulose synthase, and sucrose isomerase, can convert sucrose into trehalulose (a mixture of isomaltulose and trehalulose) to produce glucose and fructose in residual amounts. Depending on the enzyme, the composition of enzyme products varies from mainly isomaltulose at 66–91% [30–33] to predominantly trehalulose at

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~90% [34]. However, isomaltulose and trehalulose have similar physical and chemical properties, such that the presence of isomaltulose significantly increases the difficulty in the production and purification of trehalulose. In particular, sucrose isomerase from whiteflies converts sucrose into mainly trehalulose without isomaltulose [35]. However, this paper does not report trehalulose yield.

In this study, a new *TreS* gene was cloned and expressed from *Thermomonospora curvata*. The expressed recombinant enzyme has a novel action on sucrose that differs from that of other reported enzymes, such as trehalulose synthase, isomaltulose synthase, and sucrose isomerase. Two functional amino acid residues, which determine substrate specificity and affect product formation, were identified.

2. Materials and methods

2.1. Bacterial strains, plasmids, and reagents

T. curvata DSM 43183 was obtained from DSMZ (Germany). The plasmid pSE380 (Invitrogen, WI, USA) was used as expression vector for TreS. *E. coli* XL10-Gold was used for routine cloning and gene expression. *T. curvata* was grown under aerobic conditions in a medium containing 30 g/l starch, 5 g/l peptone, 5 g/l yeast extract, 0.5 g/l $CaCl_2$, 0.5 g/l $MgCl_2{\cdot}7H_2O$, 1 g/l K_2HPO_4, and 0.5 g/l $MnCl_2{\cdot}4H_2O$ at pH 7.0. The medium was incubated in a rotary shaker at 55 °C and 200 rpm for 48 h. The *E. coli* and recombinant *E. coli* strains were routinely grown at 37 °C and 200 rpm in Luria–Bertani medium alone or with 100 μg/ml ampicillin. Restriction enzymes, ligase, LA Taq DNA polymerase, and PrimeSTAR HS DNA polymerase, were all obtained from Takara (Shiga, NJ, Japan). Protein mixture molecular mass markers were purchased from Biosciences (Amersham, CA, UK). Trehalulose was obtained from Wako (Osaka, MO, Japan). All other saccharides were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. TreS gene cloning and expression vector construction

Genomic DNA was extracted according to a previously described method [36]. Based on the published coding DNA sequence of TreS from *T. curvata* (GenBank accession no. YP_003301157), the primers amplifying the *TreS-T.C* gene were designed with corresponding restriction recognition sites (underlined) and His (*6*)-tag (bold and italic bases). His-tagged primers were designed to facilitate one-step purification of resultant TreS-T.C. The sense primer was 5′-CTGAATTCATG***CACCATCATCATCATCAT***CAGATGACCGGGGACC-3′, and the antisense primer was 5′-ACAAAGCTTTCACTTCGCCGTCTGCC-3′ (*Eco*RI and *Hind*III restriction sites were underlined). The amplification conditions with LA Taq DNA polymerase were as follows: 4 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 60 °C, and 3 min at 72 °C and a final extension of 10 min at 72 °C. Polymerase chain reaction (PCR) products were analyzed using 0.8% agarose gel electrophoresis. PCR fragment digested with corresponding restriction enzyme was inserted into pSE380, which was linearized by the same enzymes. The fidelity of inserted DNA sequence was confirmed by sequencing. Constructed vectors were used to transform competent *E. coli* XL10-Gold cells.

2.3. Enzyme isolation, purification, and PAGE analysis

When the cell density (A_{600}) of the incubated recombinant cells reached approximately 0.6, isopropyl-beta-D-thiogalactopyranoside was added to a final concentration of 1 mM to induce gene expression. The cells were harvested by centrifugation at 8000 × *g* for 10 min, washed twice with 100 mM potassium phosphate buffer (pH 7.0), resuspended in the same buffer, and then disrupted by sonication on ice for 10 min after 10 h induction at 37 °C. Cell debris was removed through centrifugation at 12,000 × *g* for 20 min. The recombinant protein was purified using nickel–nitrilotriacetic acid agarose resin (Qiagen, Hilden, CA, Germany) according to manufacturer's instructions. Enzyme protein content was determined through the Bradford method, with bovine serum albumin as standard. The purified protein was analyzed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE.

2.4. Enzyme assay

Standard reaction was conducted by adding 0.5 mg purified enzyme to a 500 μl reaction mixture containing 100 mM phosphate buffer (pH 6.5) and 1% substrate followed by incubation at 30 °C for 30 min. A volume of distilled water equal to that of the purified enzyme was added to the reaction mixture as control. The identity of substrate products obtained from TreS-T.C catalysis was confirmed through high-performance liquid chromatography (HPLC), performed at 30 °C under a pressure of 96 bar using an Agilent 1100 series column (Alltima Amino 100A 5u, 250 mm × 4.6 mm) and an Alltech 2000ES evaporative light scattering detector. Acetonitrile/water (79:21) was used as solvent at a flow rate of 1 ml min^{-1}. With the control as the original substrate content, enzyme activity was assayed by measuring the amount of consumed substrate through HPLC. One unit of enzyme activity was defined as the amount of enzyme consuming 1 μmol of substrate per minute.

Kinetic analysis was performed at optimum temperature and pH. The experiment was conducted for 30 min in a 100 mM phosphate buffer with substrate at various concentrations. K_m and V_{max} values were obtained through a Lineweaver–Burk plot.

2.5. Construction and analysis of protein models for TreS-T.C

Models were built through an online Automatic Modeling Mode server at http://swissmodel.expasy.org. Obtained models were analyzed through Swiss-Pdb Viewer and PyMOL.

2.6. Site-directed mutagenesis of TreS-T.C

Mutants were constructed through a fast cloning method [30] with mutation primers containing mutation bases and 15–17 complementary bases (forward and reverse primers) at the 5′-ends. Linear mutation recombinant vectors were amplified by PCR with PrimeSTAR HS DNA polymerase with TreS-T.C recombinant plasmids as parent DNA templates. Briefly, after gel confirmation of PCR products, remaining unpurified PCR reactions were digested with *Dpn*I for 1 h at 37 °C to remove the templates. The digested products were then directly transformed into competent *E. coli* XL10-Gold cells to obtain mutation recombinant plasmids.

3. Results

3.1. Purification and molecular mass analysis of TreS

TreS-T.C expressed in recombinant *E. coli* was isolated and purified. The result of SDS-PAGE analysis showed a single protein band approximately 60 kDa (Fig. 1A, lane 3). On the other hand, active enzymes had a molecular mass of approximately 180 kDa (Fig. 1B, lane 4) according to native PAGE analysis, which suggested that the native enzyme was a trimer of three identical 60 kDa subunits. The purified enzyme was stored at 4 °C for more than one year in imidazole elution buffer with no apparent loss in activity.

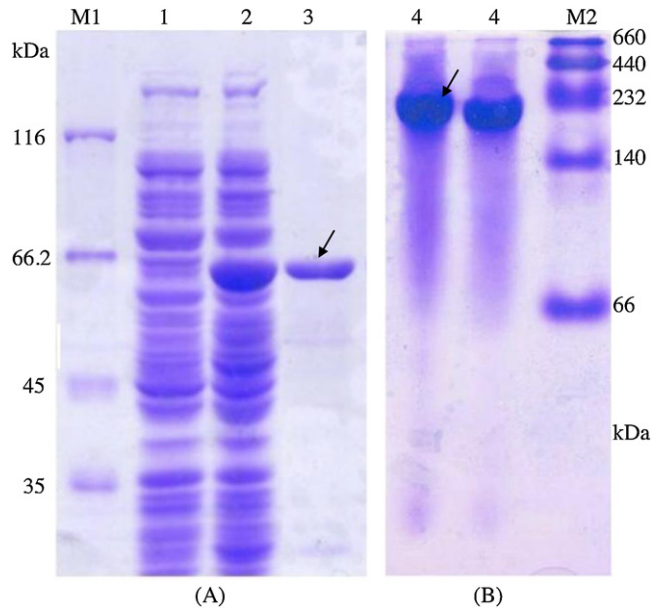

Fig. 1. Purification and molecular mass analysis of TreS-T.C. (A) SDS-PAGE analysis using 10% denaturing acrylamide separating gel and 4% stacking gel without SDS; (B) NATIVE-PAGE analysis using 5.0–12.5% native gradient acrylamide separating gel and 4.0% stacking gel without SDS. Lanes M1 and M2 are protein molecular weight markers. Lane 1 is the crude extract of the recombinant strain *E. coli* XL10-Gold with pSE380. Lane 2 is the crude extract of the recombinant strain *E. coli* XL10-Gold with pSE380-TreS-T.C. Lane 3 is the recombinant enzyme TreS-T.C purified using nickel ion affinity chromatography. Lane 4 is the active purified recombinant enzyme TreS-T.C.

3.2. Effects of pH, temperature, and metal ions on TreS-Sgris activity

The optimum pH for TreS-T.C was 6.5. The enzyme maintained high activity from pH 6.0 to 8.0 (Fig. 2A). The optimum temperature was 35 °C. Consequently, the enzyme maintained high activity when reaction temperature ranged from 25 °C to 50 °C. However, relative activity quickly decreased when temperature was above 50 °C or below 25 °C (Fig. 2B). The effects of metal ions (KCl, $FeCl_3$, $MnCl_2$, $CaCl_2$, $ZnCl_2$, $CuCl_2$, $BaCl_2$, and $FeCl_2$) were determined by examining enzyme activity in the presence of 5 mM of these substances under optimum reaction conditions. Enzyme activity was inhibited partly by Cu^{2+}, Zn^{2+}, and Fe^{2+} (about 85% relative activity), whereas other metal ions had no significant effect on enzyme activity (graphic ignored).

3.3. Substrate specificity of recombinant enzyme

Different 1% sugars, including trehalose, lactose, sucrose, cellobiose, isomaltose, isomaltulose, trehalulose, maltotriose, maltotetrose, sorbitol, and dextrin, were used to detect the substrate specificity of TreS-T.C under optimal pH and temperature. As in the enzyme assay, a series of controls was designed. Each reaction sample was analyzed by HPLC after 1 h or 5 h of incubation. TreS-T.C specifically catalyzed the reversible conversion of maltose and trehalose and acted on sucrose-catalyzed trehalulose formation without other disaccharides, releasing small amounts of glucose and fructose as by-products (Fig. 3). Other sugars were not catalyzed (graphic ignored).

Finally, with sucrose as substrate, the optimum pH value, reaction temperature, and influence of metal ions were consistent.

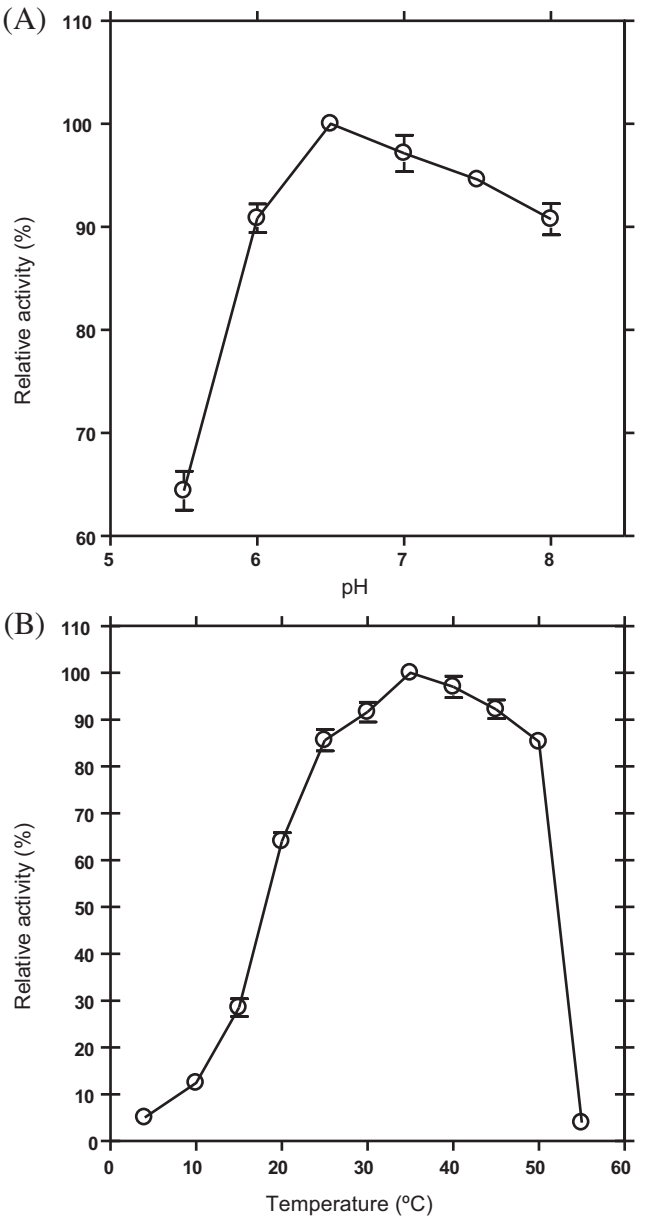

Fig. 2. Effects of pH and temperature on activity. (A) Reactions using 1% maltose as substrate (*w*/*v*) were assayed at 30 °C with different pH values (5.5–8.0) for 30 min. (B) Reactions were set at different temperatures, ranging from 5 °C to 55 °C to detect the effects of temperature on enzyme activity with pH 6.5. Other reaction conditions were the same as in (A). All enzyme activity was measured by monitoring the amount of consumed substrate by HPLC. A 100% relative activity was denoted as the highest conversion rate of maltose.

3.4. Kinetic analysis of TreS-T.C

The results of the kinetics analysis on TreS-T.C are shown (see Table 1). The K_m value for maltose is approximately two degrees

Table 1
Kinetic analysis of TreS-T.C.

Substrate	K_m (mmol/l)	V_{max} [a] (U/mg)	Product	Byproduct(s)
Maltose	96 ± 5	1549 ± 20	Trehalose	Glucose
Trehalose	198 ± 4	708 ± 30	Maltose	Glucose
Sucrose	164 ± 5	911 ± 20	Trehalulose	Glucose and fructose

Note: Data represent the mean values and standard deviations of three independent experiments.

[a] Amount of substrates.

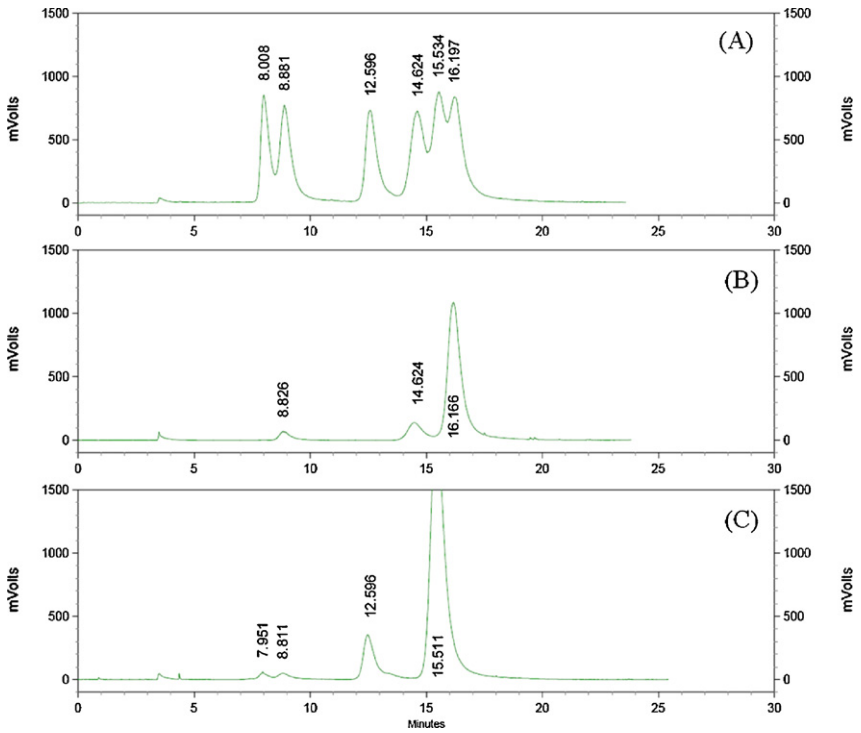

Fig. 3. HPLC results for maltose and sucrose production. Reaction samples were analyzed by HPLC after 1 h or 5 h incubation under other standard reaction conditions. Sample concentration was diluted to approximately 0.1%, and 20 μl was loaded onto the column. (A) Standards for fructose (8.008), glucose (8.881), sucrose (12.596), maltose (14.624), trehalulose (15.534), and trehalose (16.197) shown as peaks; (B) peaks of reacted sample (maltose incubated 1 h) and of products [glucose (8.826), maltose (14.624), and trehalose (116.166)]; (C) peaks of reacted sample (sucrose incubated 5 h) and of products [fructose (7.951), glucose (8.811), sucrose (12.596), and trehalulose (15.511)].

lower than those of trehalose and sucrose, indicating that TreS-T.C preferred maltose as substrate instead of trehalose and sucrose. Furthermore, under optimal reaction conditions, TreS-T.C has a relatively higher catalytic rate with maltose as substrate compared with trehalose and sucrose.

3.5. Ranges of trehalose and trehalulose yields

TreS-T.C yield was different under different incubation times. Maximum trehalose yield was 70% with glucose content of 8% on maltose under optimum reaction conditions (Fig. 4). However, glucose yield increased when the reaction was continued after maximum trehalose yield was reached. Results were the same with different substrate concentrations (from 2% to 80%), but not under the same reaction times (data not shown). With sucrose as substrate, the conversion rate into trehalulose was also independent of substrate concentration. Maximum trehalulose yield from sucrose exceeded 80% but required longer reaction time (Fig. 4B).

3.6. Homology model building and structure analysis

Using trehalulose synthase catalyzing sucrose into trehalulose as template (PDB ID: 2pwg), a TreS-T.C model was built through SWISS-MODEL. The sequence identity of TreS-T.C and trehalulose synthase was 29%, but both belonged to the GH13 family and had the same substrate and product. The model and template had a typical $(\beta/\alpha)_8$ barrel catalytic domain (Fig. 5A). Both trehalulose synthase and TreS-T.C had five conserved key amino acids constituting a catalytic pocket: H104, D200, E254, H326, and D327 [31] and H120, D217, E259, H328, and D329, respectively. Three-dimensional structures showed that all conserved amino acids were in the center of the barrel catalytic domain (Fig. 5B). The catalytic pocket had a deep groove on one side for binding substrates. We speculated that the deep groove served as an entry point of substrate into the catalytic center and as an export site of products. Activity and substrate specificity were also directly related to the central catalytic area of an enzyme [32–34]. Thus, each amino acid was chosen to detect its function within 3.5 Å of active sites. These amino acid residues included H120, D217, E259, H328, D329, E330, L116, L32, L68, R408, N327, Y80, V117, M118, N119, T121, L216, A218, V219, Y392, A260, N261, P220, P186, V411, R412, Y85, Q185, E30, R215, and A258 (Fig. 5C).

3.7. Site-directed mutagenesis and saturation mutagenesis

Based on the opposite characteristics of amino acid, we designed mutation primers to obtain various mutants, including H120A, D217A, E259A, H328A, D329A, E330A, L116F, L32S, L68S, R408D, N327S, Y80A, V117S, M118S, N119A, T121A, L216S, A218S, V219S, Y392A, A260S, N261A, P220S, P186S, V411S, R412D, Y85A, Q185A, E30H, R215D, and A258S.

Enzyme assays were performed at pH 6.5 and 35 °C with maltose and sucrose as substrates. The results of enzymatic properties showed that H120A, D217A, E259A, D329A, H328A, R408D, Y80A, A218S, Y392A, P220S, P186S, R412D, N119A, N327S, V219S, Q185A, E30H, R215D, and L68S had little to no enzyme activity on maltose and sucrose compared with the wild type. Mutant L116Y showed 2.1-fold higher activity than the wild type on sucrose, but only 0.43-fold on maltose. On the other hand, mutant E330A only had hydrolytic activity and no intermolecular transglycosylation activity on maltose and sucrose. Only 32% of mutations (V117S, M118S, V411S, L32S, T121A, L216S, Y85A, A260S, N261A, and A258S) had no significant effect on enzyme activity and substrate specificity. Most amino acids near the active site significantly affected enzyme activity or substrate specificity.

Given that site L116 significantly affects substrate specificity and that E330 is vital to product formation, saturation mutagenesis was performed. All mutants were purified to assay the enzymatic

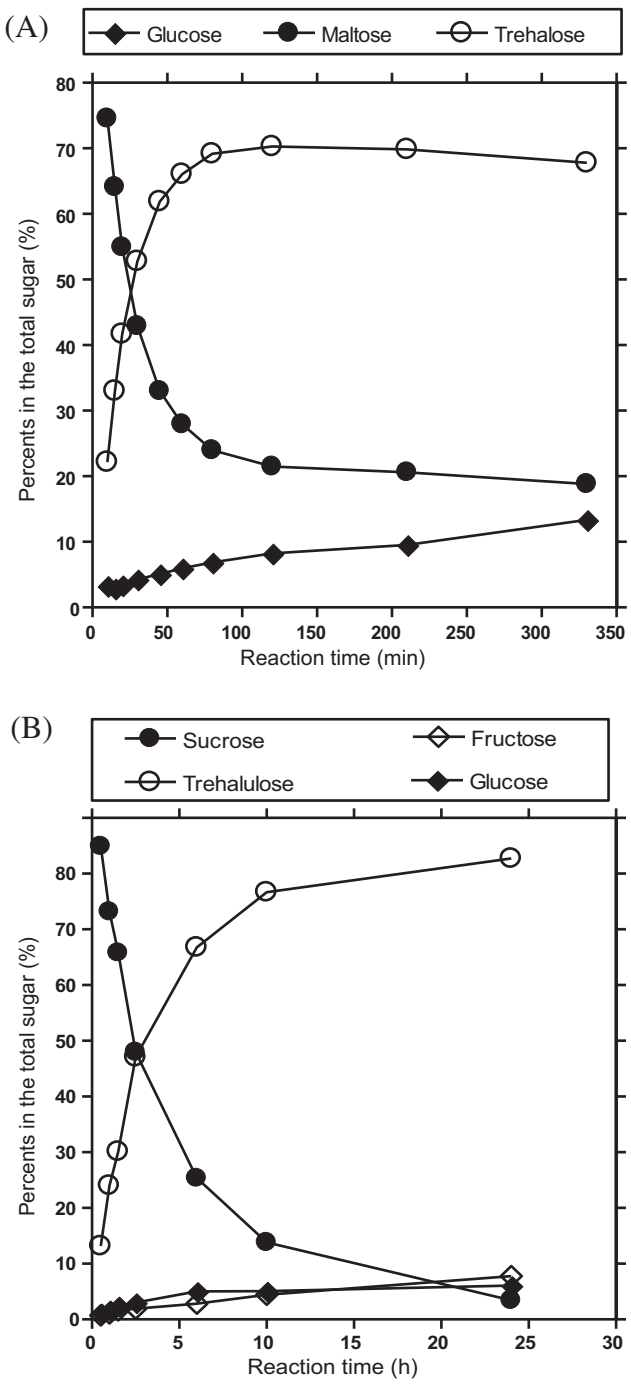

Fig. 4. Effects of reaction time on maximum product yield with maltose or sucrose as substrate. Percentages of substrate and products in total sugars were monitored by HPLC under different incubation times. (A) Maltose as substrate; (B) sucrose as substrate. All data were average values from at least triplicates.

properties. All 18 E330 mutants had only hydrolytic activity against trehalose and sucrose. Compared with the wild type, almost all 18 L116 mutants had different catalytic activities and changes with maltose or sucrose (Table 2). Although mutants L116G, L116K, L116A, L116Y, and L116H had higher activity against sucrose, they had lower activity against maltose. By contrast, the activity of L116V, L116I, L116Q, L116T, L116M, and L116E decreased with sucrose as substrate, but had minimal effect on maltose. Thus, these two sites are important in determining substrate specificity and formatting products.

4. Discussion

A trehalose synthase (TreS-T.C) from *T. curvata* was cloned and expressed in *E. coli* XL10-Gold. TreS-T.C has some distinctive characteristics: it exists in the form of a trimer of three identical 60 kDa subunits, has a new reaction on sucrose, and is stored at 4 °C for more than one year with no apparent loss of activity. In previous papers, an active TreS purified from the cytosol of *Mycobacterium smegmatis* is a hexamer of six identical 68 kDa subunits [23] and from *Thermobifida fusca* is a tetramer of four identical 61 kDa subunits (data measured in the laboratory but not published). TreS has a complex structure in active state. TreS from *M. smegmatis* [23] and *Thermus caldophilus* GK24 [37] was also previously reported to have unstable activity. However, TreS-T.C could be stored with very stable activity at 4 °C for more than one year. Thus, TreS-T.C may ideally be studied for its crystal structure.

Moreover, TreS-T.C had a new reaction: it could convert substrate sucrose into only trehalulose without other disaccharides, including isomaltulose, in contrast to other enzymes synthesizing trehalulose, including trehalulose synthase, isomaltulose synthase, and sucrose isomerase. All TreS reported previously could specifically catalyze the reversible interconversion of maltose and trehalose, but only a few acted on sucrose. A TreS obtained from *Thermus aquaticus* also converted substrate sucrose into trehalulose; however, the resulting by-products were glucose, fructose, and palatinose, with only very low trehalulose-forming activity [38]. As noted above, trehalulose synthase, isomaltulose synthase, and sucrose isomerase also can convert sucrose into trehalulose to produce glucose and fructose in residual amounts, but not on maltose. However, the product was a mixture of trehalulose and isomaltulose. Although TreS-T.C and those enzymes have some similar characteristics, their amino acid sequence identities are very low. The identities of TreS-T.C toward trehalose synthase (*T. aquaticus*), trehalulose synthase (*Pseudomonas mesoacidophila* MX-45), isomaltulose synthase (*Klebsiella* sp. LX3), and sucrose isomerase (*Erwinia rhapontici*) are 32%, 29%, 29%, and 28%, respectively. These enzymes have some similar characteristics, but protein molecules are extremely different. Their origin and evolution need further exploration by researchers.

In this paper, maximum product yield from maltose and sucrose was 70% and exceed 80%, respectively, under optimum conditions. However, some TreS from other microorganisms have lower product yield under optimum conditions, such as those from *Picrophilus torridus* (61%) [26], *Meiothermus rubber* (47.2%) [39], *Arthrobacter aurescens* (59.5%) [29], *Thermus thermophilus* (60%) [40], and *Pseudomonas putida* (~45%) [27]. The formation of other minor products also reduces the yield of major products about trehalulose synthase, isomaltulose synthase, and sucrose isomerase. Indeed, the product yield of TreS is associated with reaction temperature, pH, and enzyme stability. According to the laws of thermodynamics and the free energies of the hydrolysis of trehalose and maltose, the calculation of equilibrium position between maltose and trehalose is allowed to be 82% in favor of trehalose [41]. Thus, all TreS, given sufficient time, should give 82% trehalose under optimum conditions.

The crystal structure of TreS has not been obtained. Native and carbohydrate complex structures of trehalulose synthase have been studied to provide insight into sucrose isomerization [42]. Considering amino acid sequence identity with TreS-T.C, trehalulose synthase is higher than isomaltulose synthase, sucrose isomerase, and trehalose glycosyltransferring synthase (TreT) [43]. Similar to all GH13 enzymes, they both use a two-step double-displacement mechanism involving the formation and breakdown of a covalent glucosyl-enzyme intermediate [44–46]. Thus, we chose trehalulose synthase as the template of TreS-T.C to build a 3D model using SWISS-MODEL. Five conserved amino acids (H120, D217,

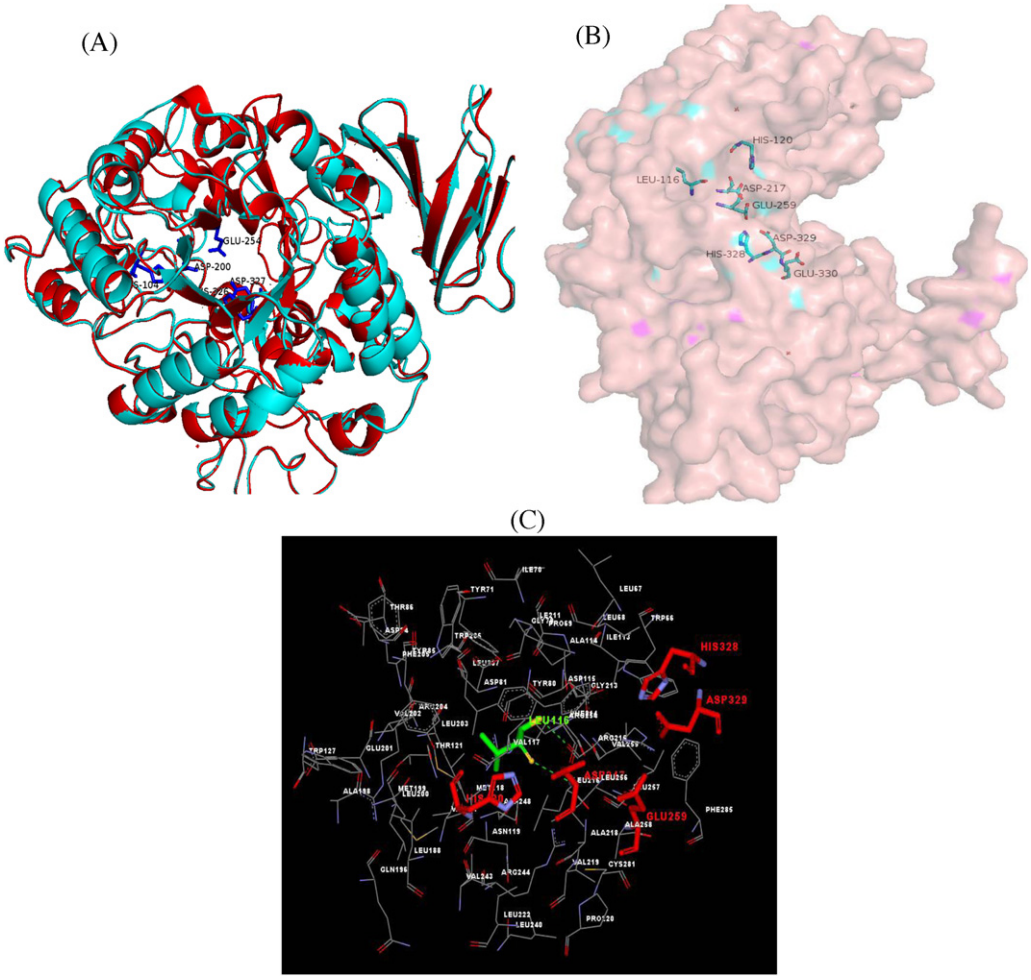

Fig. 5. Comparative modeling of TreS-T.C based on known trehalulose synthase template. (A) Model of TreS-T.C (cyan) constructed using online SWISS-MODEL was superimposed with 2pwg (red). Five key amino acids (H104, D200, E254, H326, and D327) in the active center of 2pwg are indicated by blue marked sticks inside the $(\beta/\alpha)_8$ barrel catalytic domain. (B) Side view of the surface model for TreS-T.C and its five conserved amino acids in the active center. The key amino acids are labeled with sticks and name of residue. (C) All amino acids residues within 3.5 Å of the active sites for TreS-T.C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

E259, H328, and D329) that possibly construct the catalytic pocket structure were decided and done in directed mutagenesis. The mutants H120A, D217A, E259A, D329A, and H328A had little or no enzyme activity against maltose and sucrose compared with the wild type. Thus, the five conserved amino acids significantly affect the catalytic activity of TreS-T.C. Furthermore, several conserved amino acid residues and their functions have already been identified about some GH13 enzymes. Five amino acid residues, namely, H133 (not mentioned in paper), D230, E272, H341, and D342, are highly conserved. D230 is a catalytic nucleophilic residue, E272 is a general acid/base catalyst, and H341 and D342 are conserved carboxylic acids [47]. The same conserved amino acids were found on oligo-1,6-glucosidase from *Bacillus cereus* (Asp199, Glu255, Asp329, His103, and His328) [48], amylosucrase from *Neisseria polysaccharareais* (Asp286, Glu328, Asp393, His187, and His392) [49], trehalulose synthase from *P. mesoacidophila* MX-45 (Asp200,

Table 2
Summary of saturation mutagenesis results.

Variants	Relative activity (%)		Variants	Relative activity (%)	
	Sucrose	Maltose		Sucrose	Maltose
L116	100.00	100.00	L116P	77.20 ± 0.34	81.11 ± 0.07
L116G	143.54 ± 2.60	16.77 ± 0.16	L116K	180.00 ± 1.55	86.72 ± 0.05
L116V	0.91 ± 0.06	63.01 ± 0.45	L116F	130.00 ± 1.04	83.98 ± 0.06
L116W	58.27 ± 0.67	52.37 ± 0.07	L116S	110.32 ± 1.00	39.71 ± 0.04
L116I	1.78 ± 0.02	75.60 ± 0.11	L116M	77.80 ± 0.09	118.20 ± 0.96
L116Q	14.53 ± 0.12	75.60 ± 0.20	L116R	0.00	22.71 ± 0.03
L116T	18.22 ± 0.18	75.12 ± 0.13	L116E	73.56 ± 0.38	101.74 ± 0.34
L116N	115.51 ± 1.15	91.22 ± 0.30	L116H	251.85 ± 1.99	89.43 ± 0.17
L116C	156.64 ± 1.50	92.44 ± 0.22	L116A	256.05 ± 2.86	21.48 ± 0.02
L116Y	210.24 ± 2.26	43.15 ± 0.09	L116D	50.65 ± 0.04	42.61 ± 0.02

Note: Data represent the mean values and standard deviations of three independent experiments.

Glu254, Asp327, His104, and His326) [42]. These amino acids are vital for the catalytic center of enzymes. When mutation range was expanded near the active center, site (L116) determined substrate specificity, and another site (E330) affected product formation. The spatial distance of L116 toward H120 and D217 is very close. A hydrogen bond also exists between L116 and D217 (Fig. 5C). Supposedly, H120 is a substrate binding site, and D217 is a nucleophilic reagent. Therefore, the site of L116 would directly affect activity and substrate specificity, which was also evidenced by the experimental results. On the other hand, E330 mutants affect trehalose formation. These possibly interfere with the second step of the two-step double-displacement mechanism, glucose molecular rearrangement within the enzyme molecule. These findings may provide a theoretical basis for studying catalytic mechanism and the transformation of industrial enzymes on TreS.

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