

Gene cloning, expression, and characterization of a novel trehalose synthase from *Arthrobacter aurescens*

Wu Xiuli · Ding Hongbiao · Yue Ming · Qiao Yu

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Abstract Trehalose synthase (TreS) is an intramolecular transglycosylase. It specially catalyzes the conversion of maltose and trehalose. In this study, a novel *treS* gene, which had a length of 1,797 bp and encoded 598 amino acids, was cloned from *Arthrobacter aurescens* CGMCC 1.1892 and expressed in *Escherichia coli*. Thin layer chromatography results indicated that it could catalyze the conversion between maltose and trehalose in one step. However, the ion chromatography results showed that, as a byproduct, about 13% glucose was also produced. The purified recombinant enzyme had a molecular weight of 68 kDa and showed its optimal activity at 35 °C and pH 6.5. This enzyme was not thermostable, and its activity was increased by 1 mM Mg²⁺, Mn²⁺, and Ca²⁺ while strongly inhibited by 5 mM Cu²⁺ and SDS.

Keywords Trehalose synthase · Trehalose · *Arthrobacter aurescens* · *Escherichia coli*

Introduction

Trehalose (α -D-glucopyranosyl α -D-glucopyranoside), a non-reducing disaccharide, is widely distributed in nature (Elbein et al. 2003; Richards et al. 2002). It has been reported that this heat- and acid-stable sugar protects tissues of some plants and animals against environmental stresses such as freezing, desiccation, heat shock, and osmotic stress (Elbein et al. 2003; Richards et al. 2002). As an additive or

stabilizer, trehalose can be used in foods, cosmetics, and medicines (Richards et al. 2002; Schiraldi et al. 2002).

Trehalose synthase (TreS) catalyzes the reversible interconversion of trehalose and maltose (Koh et al. 2003). It would provide a prospective biochemical method for the trehalose production. Recently, the complete genome of *Arthrobacter aurescens* TC1 was sequenced (Mongodin et al. 2006), but the gene noted as “*treS*” has not been identified yet. In this study, we cloned the *treS* gene from *A. aurescens* CGMCC 1.1892, identified its biochemical function, and also characterized its main enzyme features. This is the first TreS report from *Arthrobacter* genus, and it could afford some useful information for the further study on TreS.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1.

Growth media and conditions

Escherichia coli strains were routinely grown at 37 °C in LB media. Final concentrations of 50 μ g/ml kanamycin (Kan), 50 μ g/ml ampicillin (Amp), and 34 μ g/ml chloramphenicol (Cam) were used for the selection of transformed bacteria in LB plates. *A. aurescens* was grown in nutrient broth medium (pepton 1%, beef extract 0.3%, NaCl 0.5%) at 30 °C.

Gene cloning and expression vector construction

Genomic DNA from *A. aurescens* CGMCC 1.1892 was prepared as described (Sambrook et al. 1989). Based on the

W. Xiuli · D. Hongbiao (✉) · Y. Ming · Q. Yu
Feed Research Institute,
Chinese Academy of Agricultural Sciences,
Beijing 100081, China
e-mail: dinghongbiao@mail.caas.net.cn

Table 1 Bacterial strains and plasmids used in this work

Strains or plasmids	Genotype/phenotype	Source/reference
Bacterial strains		
<i>Arthrobacter aurescens</i>		CGMCC1.1892
<i>E. coli</i> Top10	Cloning host, no antibiotic resistance	Sambrook et al.
<i>E. coli</i> BL21(DE3) pLysS	Expression host, Cam ^r	Novagen, Germany
Plasmids		
pGEM-T	Amp ^r , <i>LacZ</i> α ⁺	Promega, USA
pET-30a(+)	Kan ^r , 6 \times His-tag, T7 promoter	Novagen, Germany

ORF (gi=119951278) of *treS* in GenBank, we designed the sense primer AaTreSF 5'-AGATCTGAGTTTTTCTCCG CAGAACC-3' and antisense primer AaTreSR 5'-CTCGAG TCATCCTTTGGAGGTCAC-3' (the *Bgl* II and *Xho* I restriction sites are underlined, respectively). The PCR products were ligated with pGEM-T cloning vector. After sequence analysis, the *treS* gene was digested by *Bgl* II and *Xho* I and inserted into pET-30a (+) vector to generate the recombinant vector pET-30a-*treS*. A His (6)-tag was added to the N-terminus of the recombinant protein to facilitate a one-step purification. The purified pET-30a-*treS* plasmid was transformed into BL21(DE3) pLysS for expression.

Protein expression and purification

The recombinant strain was cultured in 1 l LB medium containing 50 μ g/ml kanamycin and 34 μ g/ml chloramphenicol with vigorous shaking until the OD₆₀₀ reached 0.6. To maximize the amount of soluble recombinant TreS, cultures were incubated at 25 °C for 6 h with 0.4 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation at 4,000 \times g for 15 min at 4 °C and suspended in 250 ml of 100 mM potassium phosphate buffer (pH 7.5). The cells were disrupted by sonication and insoluble cell debris were removed by centrifugation at 10,000 \times g for 20 min at 4 °C. The supernatant was filtered through a 0.45- μ m filter and loaded onto a Ni-NTA column according to the manufacturer's purification protocol manual (Novagen, Ni-NTA His•Bind Resins). The purified enzyme was analyzed by 12% SDS-PAGE and protein concentration was determined by the method of Bradford using BSA as a standard.

Function identification and enzyme activity assay

The function of the recombinant protein was identified by TLC, using maltose and trehalose as a substrate, respectively. A reaction mixture containing the enzyme solution and 90 mM maltose or trehalose in 100 mM phosphate buffer (pH 7.5) was incubated at 35 °C for 4 h. Then the reaction mixture was heated in boiling water for 10 min to stop the reaction. After removal of the insoluble fraction, the product mixtures were separated on silica gel plates

(Merck, Darmstadt, Germany) using a solvent system of 1-butanol/pyridine/water (4:5:1, v/v). Products were visualized by heating TLC plates at 100 °C for 5 min after spraying with 50% (v/v) sulfuric acid in ethanol. The recombinant trehalose synthase activity was measured by the amount of trehalose produced from maltose. One unit (U) of trehalose synthase was defined as the amount of enzyme required to produce 1 mmol trehalose per minute under the specified conditions. The amount of trehalose was quantified by IC using a Dionex 2500 system equipped with a CarboPacPA-2 column.

Nucleotide sequence accession number

The nucleotide and amino acid sequences of TreS in *A. aurescens* CGMCC 1.1892 are deposited in GenBank with accession number FJ545264.

Results

Gene and amino acid sequence analysis of TreS

We used the software Clustalx1.83 to analyze all the TreS sequences published online and found several highly conserved amino acid motifs (Table 2). These conserved motifs were identified in all TreS proteins reported so far (Pan et al. 2004; Wei et al. 2004; Lee et al. 2005; Chen et al. 2006). According to the published genome sequence of *A. aurescens* TC1 (gi=119947346), there are two assumed *treS* genes in it. One is 1,797 bp (gi=119951278), which has all the conserved motifs, but the other (2,136 bp, gi=119947556) has not. In this study, we cloned the first *treS* gene from *A. aurescens* CGMCC 1.1892, which is more similar to the other known trehalose synthases, into *E. coli* to identify its biochemical function and for enzyme characterization. Using the primer pairs AaTreSF and AaTreSR, we got one 1,797 bp gene, which encoded 598 amino acids. The sequencing result showed that its amino sequence was 98.16% identical to the TreS online (gi=119951278), which was higher than the corresponding nucleotide sequence homology of 90.14%. The result of amino acid sequence analysis by SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) showed that there

Table 2 Comparisons of conserved amino acid sequences in the TreS

Origin strains	Region 1	Region 2	Region 3	Region 4	Region 5
	NHTSDQH	HQPDLN	NHDELTLE	GIRRRRLAPL	YYGDEIGMGDN
<i>Arthrobacter aurescens</i> CGMCC 1.1892	128	193	334	366	399
<i>Arthrobacter aurescens</i> TC1 (gi=119951278)	NHTSDQH	HQPDLN	NHDELTLE	GIRRRRLAPL	YYGDEIGMGDN
<i>Picrophilus torridus</i> DSM9790 (gi=48429789)	128	193	334	366	399
	NHTSDQH	HQPDLN	NHDELTLE	GIRRRRLAPL	YYGDEIGMGDN
<i>Thermus aquaticus</i> ATCC33923 (gi=20140415)	105	173	309	341	374
	NHVSDQH	SQPDLN	NHDELTLE	GIRRRRLAPL	YYGDEIGMGDN
<i>Pimelobacter</i> sp. R48 (gi=1536814)	100	164	304	336	369
	NHTSIDH	HQPDLN	NHDELTLE	GIRRRRLMPL	YYGDEIGMGDN
<i>Corynebacterium glutamicum</i> ATCC13032 (gi=23308924)	112	177	325	357	390
	NHTSDAH	HQPDLN	NHDELTLE	GIRRRRLAPL	YYGDEIGMGDN
<i>Pseudomonas putida</i> KE2440 (gi=26990760)	149	214	356	388	421
	NHTSDQH	HQPDLN	NHDELTLE	GIRRRRLSPL	YYGDELGMGDN
<i>Mycobacterium tuberculosis</i> H37Rv (gi=15607268)	113	179	320	352	385
	NHTSDQH	HQPDLN	NHDELTLE	GIRRRRLAPL	YYGDELGMGDN
	140	205	348	380	413
	NHTSESH	HQPDLN	NHDELTLE	GIRRRRLAPL	YYGDEIGMGDV

was no obvious signal peptide, which indicated that the TreS of *A. aurescens* CGMCC 1.1892 was an intracellular enzyme.

Expression and purification of recombinant protein

The recombinant expression vector pET-30a-treS was transformed into the BL21 (DE3) pLysS strain, and then TreS was produced by induction of T7 polymerase. The SDS-PAGE result (Fig. 1) showed that a recombinant protein

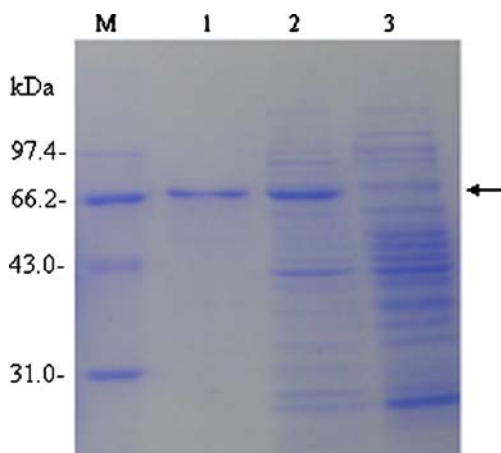


Fig. 1 SDS-PAGE analysis of the recombinant protein. All the protein samples were analyzed on a 12.5% polyacrylamide gel under denaturing conditions. The gel was stained with Coomassie Brilliant Blue R-250. The arrow indicates recombinant TreS. Lane 1 purified recombinant TreS. Lane 2 soluble fraction of BL21(DE3)pLysS transformed with pET-30a-TreS. Lane 3 total cell lysate of BL21 (DE3)pLysS transformed with pET-30a-TreS. Lane M, molecular weight standards (97.4, 66.2, 43.0, 31.0 kDa)

about 68 kDa, which was in accordance with the predicted value, was expressed. Expression studies indicated that the induction with 0.4 mM IPTG at 25 °C for 5 h was the best condition for the expression of soluble TreS. Higher temperature or IPTG concentrations lead to a higher rate of inclusion bodies, whereas a temperature below 20 °C and lower IPTG concentrations decreased the total amount of TreS (data not shown).

To determine whether the gene encoded a functional TreS, we purified the recombinant protein from the supernatant of cell lysate as shown by the single band in Fig. 1, lane A. Crude protein concentration in the supernatant of the cell lysate was 3.04 mg/ml, and the purified TreS concentration was 295 µg/ml.

Identification of the recombinant protein

To identify the function of the recombinant protein, we incubated the purified enzyme with trehalose and maltose, respectively. The reaction mixture was analyzed by standard TLC. TLC results (data not shown) showed that the enzyme could catalyze the conversion of maltose and trehalose, which meant this recombinant protein was TreS.

Effects of pH and temperature on the activity and stability of TreS

The pH dependence of TreS was studied at various pH values ranging from 4.5 to 10.0. To determine the pH stability, the recombinant enzymes were preincubated at various pH values (pH 4.5–10.0) for 20 min before the residual activity was measured at pH 6.5, immediately. The

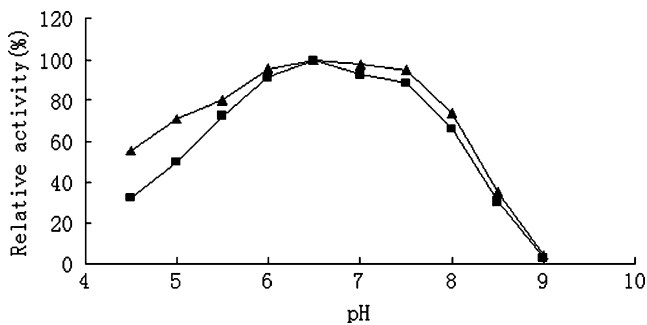


Fig. 2 Effects of pH on the activity and stability of the recombinant TreS. The enzyme activity of TreS at various pH was studied at 35 °C in 100 mM phosphate buffer (pH 4.5–10.0) for 30 min, using 90 mM maltose as a substrate. To examine the stability of TreS, the enzymes were preincubated at various pH values (pH 4.5–10.0) for 20 min at 35 °C. The residual activities were measured at pH 6.5. The *square* (■) represents effects of pH on the activity of TreS; the *triangle* (▲) represents effects of pH on the stability of TreS

optimum pH for TreS was 6.5, but it remained highly stable within a pH range from 5.5 to 7.5 (Fig. 2).

The effects of temperature on TreS activity were determined at various temperatures ranging from 20 to 60 °C. To determine the stability against thermal denaturation, the recombinant enzymes were preincubated at various temperatures (20–60 °C) for 20 min and then cooled immediately to assay the residual activity at 35 °C. The optimum temperature was 35 °C, and the enzyme remained stable up to 40 °C (Fig. 3).

Conversion profile of maltose to trehalose by TreS

The purified enzyme was incubated in 100 mM phosphate buffer (pH 6.5) at 35 °C for 0–10 h, using 90 mM maltose

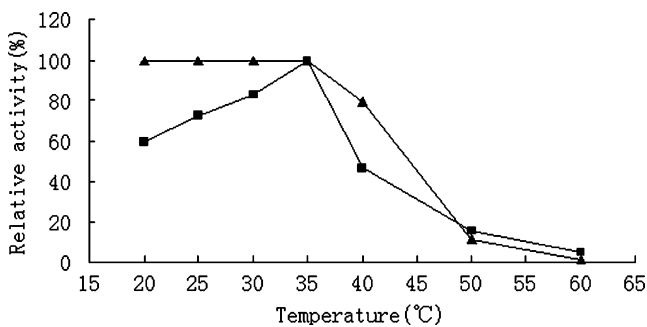


Fig. 3 Effects of temperature on the activity and stability of the recombinant TreS. The enzyme activity of TreS at various temperatures was studied at pH 6.5 in 100 mM phosphate buffers (pH 4.5–10.0) for 30 min, using 90 mM maltose as a substrate. To examine the thermal stability of TreS, the enzymes were preincubated at various temperatures (20–60 °C) for 20 min at pH 6.5. The residual activities were measured at 35 °C. The *square* (■) represents effects of temperature on the activity of TreS; the *triangle* (▲) represents effects of temperature on the stability of TreS

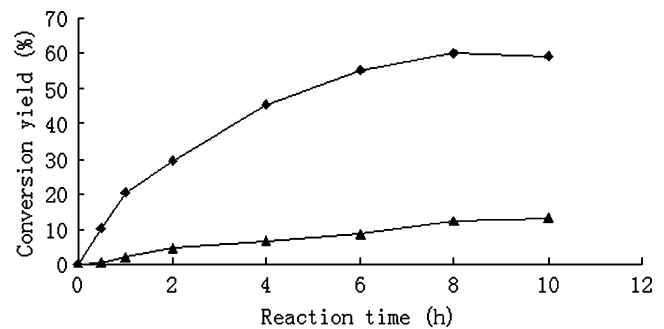


Fig. 4 Conversion profile of maltose to trehalose by TreS. The conversion profile was obtained by incubating the enzyme (0.5 mg/ml) at 30 °C, pH 6.5 for 0–10 h, using 90 mM maltose as a substrate. Then, the reaction mixture was analyzed by IC, as described in the ‘Materials and methods’ section. *Square* (■): trehalose; *triangle* (▲): glucose

as a substrate. All the reactions were stopped by boiling them for 10 min before the samples were analyzed by IC. The retention time for trehalose, glucose, and maltose were 1.4, 2.1, and 4.6 min, respectively (data not shown). After 8 h of reaction, the conversion rates of trehalose and glucose were 59.5% and 13.2%, respectively (Fig. 4).

Effects of metal ions and reagents on the activity of TreS

The effects of metal ions and reagents were determined by examining enzyme activity in the presence of either 1 or 5 mM of these substances under optimum reaction conditions (Table 3). The results showed that the enzyme activity was inhibited strongly by both 1 mM Cu^{2+} and SDS. However, when the concentration reached 5 mM, Tris could also inhibit most of the TreS activity.

Discussion

In this paper, we confirmed that the gene (gi=119951278) from *A. aurescens* CGMCC 1.1892 encoded a functional

Table 3 Effects of metal ions and reagents on the activity of TreS

Reagent	Relative activity (%)	
	1 mM	5 mM
Control	100	100
CaCl_2	123	98
MgCl_2	118	92
MnCl_2	116	86
CuSO_4	8	0
EDTA	92	86
SDS	0	0
Tris	72	15

enzyme, trehalose synthase, which could catalyze the conversion of maltose to trehalose and vice versa. This enzyme showed its best activity at pH 6.5 and 35 °C. It could convert about 60% maltose to trehalose, accompanied by about 13% glucose as a byproduct. As reported in other studies, most TreS enzymes produce glucose as a byproduct, except the one from *Pseudomonas stutzeri* CJ38 (Lee et al. 2005). Glucose normally inhibits the enzyme activity (Chen et al. 2006) and lowers the conversion rate from maltose to trehalose (Wei et al. 2004). Those trehalose synthases producing less or no glucose have a relatively higher production rate for trehalose of about 70% to 80% (Lee et al. 2005; Chen et al. 2006; Nishimoto et al. 1996a, b). We found no obvious reasons why the enzyme derived from *A. aurescens* CGMCC 1.1892 produced a relatively high amount of glucose.

Till now, five pathways for trehalose synthesis have been identified: (a) TPS–TPP pathway, the most widely reported and best characterized pathway, involving the trehalose-phosphate synthase (TPS) and trehalose-phosphate phosphorylase (TPP) (Tzvetkov et al. 2003; Bell et al. 1998; Avonce et al. 2006). $\text{UDP-glucose} + \text{glucose-6-phosphate} \xrightarrow{\text{TPS}} \text{trehalose-6-phosphate} \xrightarrow{\text{TPP}} \text{trehalose} + \text{phosphate}$. (b) TreP pathway, existing in fungi (Saito et al. 1998a, b; Schwarz et al. 2007); it produces trehalose from α -D-glucose-1-phosphate and D-glucose by trehalose phosphorylase (TreP). (c) TreT pathway, found in extremophilic archaea (Tzvetkov et al. 2003); it converts ADP-glucose and glucose into trehalose by trehalose glycosyltransferring synthase (TreT). However, all these pathways are not suitable in trehalose industrial production due to their expensive substrates. (d) TreY–TreZ pathway; TreY mainly catalyses the formation of trehalosyl dextrans from dextrans by converting the α -1,4-glucosidic linkage at the reducing end to an α -1,1-glucosidic linkage. Then, TreZ cleaves the α -1,4-glucosidic linkage next to the α -1,1-glucosidic linkage of trehalosyl dextrans to produce trehalose and dextrans with lower molecular weight (Mukai et al. 1997; Fang et al. 2004). Trehalose has mainly been manufactured through this pathway since it was discovered in 1994. (e) The last pathway involves the TreS, which could produce trehalose from maltose in one step. Maltose is relatively cheap, and this pathway can be an alternative method for industrial trehalose production. So far, a number of TreS proteins from several bacterial strains (Ma et al. 2006; Lee et al. 2005; Pan et al. 2004; Nishimoto et al. 1996a, b; Chen et al. 2006; Zdziebło and Synowiecki 2006; Wei et al. 2004) have been identified and characterized. All of these TreS proteins are encoded by uninterrupted prokaryotic genes, and the TreS from *Mycobacterium smegmatis* (Pan et al. 2004) proved to be a hexamer of six identical subunits by gel filtration, whereas all others suggested to be monomers by SDS-PAGE. This study provided the charac-

teristics of the TreS from *A. aurescens* CGMCC 1.1892 for the trehalose catalysis metabolism.

There are other kinds of gene sequences, noted as “*treS*”, which do not have the five conserved motifs, and their amino acid sequences are not similar to each other. Till now, none of these sequences have been isolated as an enzyme. In the genome of *A. aurescens* TC1, a *treS* gene like that also exists. It may be a novel trehalose synthase with particular biochemical properties and is worthy of further study.

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