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RESEARCH NOTE

Gene Cloning and Characterization of a Trehalose Synthase from *Corynebacterium glutamicum* ATCC13032

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Abstract Trehalose synthase (TreS) is an enzyme which produces trehalose from maltose through intramolecular transglycosylation. In this study, a gene (cg2529) encoding for TreS from *Corynebacterium glutamicum* (CgTS) was cloned and expressed in *Escherichia coli*. The hexahistidine-tagged CgTS showed an optimum temperature and pH of 35°C and pH 7.0, respectively. This enzyme was not thermostable, but stable in a broad pH range from pH 5.0 to 8.5. Its activity slightly increased by 5 mM Mg^{2+} and Fe^{2+}, while it was strongly inhibited by 5 mM sodium dodecyl sulfate (SDS). CgTS catalyzed the conversion from maltose into trehalose, and *vice versa*. Lowering reaction temperature by 5°C from the optimum temperature significantly reduced hydrolysis activity to produce glucose as a by-product compared to transglycosylation activity to produce trehalose, leading to increase in the conversion yields from maltose into trehalose. Consequently, the maximum conversion yield by CgTS reached 69% at 25°C after 9 hr of reaction.

Keywords: trehalose, *Corynebacterium glutamicum*, trehalose synthase, intramolecular transglycosylation

Introduction

Trehalose (α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside) is a naturally occurring nonreducing disaccharide which present in a large number of organisms, including bacteria, yeast, fungi, insects, invertebrates, and plants (1). Trehalose and its derivatives can protect proteins against denaturation due to desiccation and freezing (2,3). In addition, its water-holding capability has been applied to the development of additives, stabilizers, and sweeteners, which are useful in the food, cosmetic, and pharmaceutical industries (4).

Due to its desirable physical and chemical characteristics, investigations have been focused on searching for efficient synthetic processes and abundant raw sources for the production of trehalose (1). To date various enzymatic pathways to synthesize trehalose in bacteria have been reported; i) trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase (Tps-Tpp) from UDP-glucose and glucose 6-phosphate as the substrates (5), ii) maltooligosyltrehalose synthase and maltooligosyltrehalose hydrolase (MTS-MTH) from starch (6), iii) trehalose synthase (TreS) from maltose (7-9), iv) glucosyltransferase from ADP-glucose and glucose (10), and v) a reversible trehalose phosphorylase pathway from glucose and glucose-1-phosphate (11).

Corynebacterium glutamicum ATCC13032, which has been used extensively for the industrial production of vitamins and numerous L-amino acids for foodstuffs (12), excretes significant amounts of trehalose into the culture media. On the basis of its genome sequence (13) *C. glutamicum* ATCC13032 possesses 3 pathways for trehalose synthesis (14): the Tps-Tpp pathway, the MTS-MTH pathway, and the TreS pathway. To our best knowledge, the former 2 pathways have been extensively studied (5,14,15), but TreS from *C. glutamicum* ATCC13032 (CgTS) has not been characterized yet. In this study, the gene (cg2529) encoding CgTS was cloned and investigated the biochemical functions of CgTS.

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Materials and Methods

Cloning and expression of the gene encoding CgTS Genomic DNA of *Corynebacterium glutamicum* ATCC13032 was isolated using a QIAmp tissue kit (Qiagen, Hilden, Germany). The target gene (cg2529) was amplified by polymerization chain reaction with Pwo polymerase (Roche Molecular Biochemicals, Mannheim, Germany) using the chromosomal DNA of *C. glutamicum* as a template and the 2 synthetic primers cg2529-Nd-fw (5'-TCTATCCTGGCACATATGACTGATACCTCT-3') and cg2529-Nt-rv (5'-TGTGTTGGCTGCGGCCGCTTCCATA TCGTCCTT-3'). The polymerase chain reaction (PCR) product was digested with NdeI and NotI and ligated with pTKNd6xH (16) that had been digested with the corresponding restriction enzymes. After sequence analysis, one clone without any PCR error was chosen and designated as pTKCgTS6xH and was used for the production of hexahistidine-tagged CgTS. The analysis of DNA sequences was carried out by Bioneer Co. (Daejeon, Korea).

Production and purification of the recombinant CgTS The recombinant *Escherichia coli* MC1061 [F^-, *araD*139, *recA*13, Δ(*araABC-leu*)7696, *gal*U, *gal*K, *lac*X74, *rpsL*, *thi*, *hsdR*2, *mcrB*] carrying pTKCgTS6xH was cultured overnight in 1 L of Luria-Bertani broth [1%(w/v) Bacto-tryptone, 0.5%(w/v) yeast extract, and 0.5%(w/v) NaCl] supplemented with kanamycin (20 μg/mL). The recombinant CgTS was purified using nickel-nitrilotriacetic acid affinity chromatography (Qiagen) as the manufacturer recommended. The eluted target proteins were dialyzed against 50 mM sodium phosphate buffer (pH 7.0). Protein concentrations were measured according to Bradford method with bovine serum albumin as standard.

Enzyme characterization For convenience, the optimum condition of CgTS was determined by assaying the conversion activity of trehalose into maltose using 0.5%(w/v) trehalose as the substrate. The amount of produced maltose was determined using 3,5-dinitrosalicylic acid (DNS), as described by Miller (17). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of maltose from trehalose/min. To determine the pH stability, the recombinant enzymes were preincubated at various pH values (pH 4.0-10.0) overnight before the residual activity was measured at its optimum condition. The thermal stability was determined by measuring the residual activity after preincubation of the enzyme without the substrate at various temperatures (25 to 55°C) for 30 min.

Analysis of action patterns of CgTS To examine the production of trehalose by the recombinant CgTS, a reaction mixture consisting of 0.5% maltose as the substrate and 9 μU CgTS (0.48 μg) per mg maltose in 50 mM Na phosphate buffer (pH 7.0) was incubated at 35°C for 24 hr. Then 50 μL of aliquot was taken at every sampling time point and heated at 100°C for 10 min to stop the reaction. The trehalose produced was measured by high performance anion exchange chromatography (HPAEC) using a pulsed amperometric detector (ED40; Dionex Co., Sunnyvale, CA, USA). The system was equipped with a CarboPac PA-1 column (0.4×25 cm, 10-μm particle diameter, Dionex Co.) and run with 0.15 M NaOH with a flow rate of 1 mL/min.

Results and Discussion

Molecular cloning of the gene encoding CgTS A DNA fragment containing a gene (cg2529) encoding CgTS was amplified using the primer pairs cg2529-Nd-fw and cg2529-Nt-rev and subcloned into pTKNd6xH vector. The complete nucleotide sequence of the CgTS ORF was 1,812 bp long and encoded 604 amino acid residues with 70 kDa of the molecular mass. The deduced amino acid sequence of CgTS showed 56, 53, and 61% sequence identity to that of *Arthrobacter aurescens* (8), *Deinococcus radiodurans* (7), and *Picrophilus torridus* (9), respectively. The analysis of amino acid sequence using SignalP (http://www.cbs.dtu.dk/services/SignalP/) revealed that there was no obvious signal peptide. CgTS did not have a C-terminal domain which has been found in thermostable TreSs (7,18). In addition, 4 conserved regions of CgTS, which have been found in amylolytic enzymes belonging to glycoside hydrolase family 13, were more similar to those of other TreSs rather than those of the enzymes in the glycoside hydrolase family 13 (Fig. 1).

Characterization of the recombinant CgTS The recombinant CgTS was successfully produced in *E. coli* and purified using nickel-nitrilotriacetic acid affinity chromatography. To identify the function of CgTS, the purified enzyme was incubated with maltose or trehalose as a substrate. HPAEC analysis of the reaction mixtures showed that CgTS catalyzed the conversion of maltose to trehalose, and *vice versa*, suggesting that CgTS is an active TreS (Fig. 2).

The optimum reaction condition was determined at 35°C in Na-phosphate buffer, pH 7.0. The activities of CgTS markedly decreased in 50 mM Tris-HCl buffer from pH 7 to 9 (data not shown), which is consistent with the properties of other TreSs (8,9). This is not surprising because 3 hydroxymethyl groups and 1 primary amine in the Tris molecule can form stable H-bonding with the residues in the active site of glycosidases (19). More than

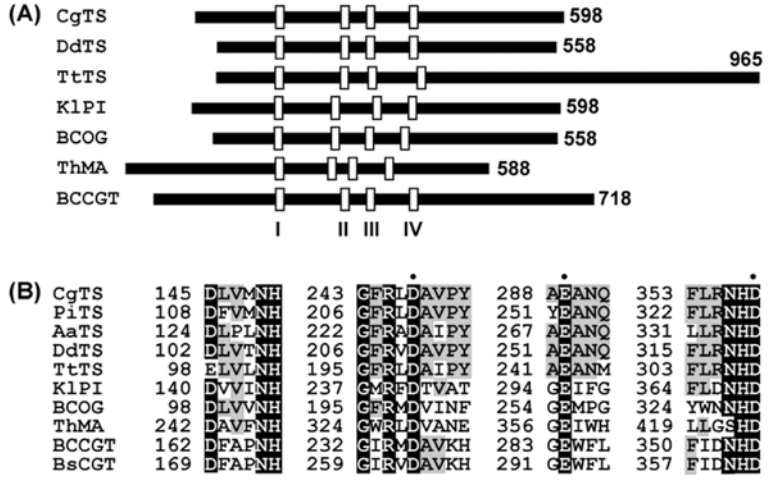

Fig. 1. Comparison of amino acid residues in the 4 conserved regions found in glycoside hydrolase family 13 enzymes. (A) 4 conserved regions (I-IV) are schematically shown on the basis of sequence alignment between CgTS and related enzymes. (B) Amino acid sequence of 4 conserved regions. Highly conserved amino acid residues in all enzymes and the conserved amino acid residues in only TreSs are indicated as white letters on black boxes and black letters on gray boxes, respectively. The catalytic residues of the family 13 enzymes are indicated with black dots. PiTS, *Pimelobacter* sp. TreS (P72235); AaTS, *Arthrobacter aurescens* TreS (ACL80570); DdTS, *Deinococcus deserti* TreS (ACO45593); TtTS, *Thermus thermophilus* TreS (Q5SL15); KlPI, *Klebsiella* sp. isomaltulose synthase (Q8KR84); BCOG, *Bacillus cereus* oligo-1,6-glucosidase (P21332); ThMA, *Thermus* sp. maltogenic amylase (O69007); BCCGT, *Bacillus circulans* cyclodextrin glucanotransferase (P43379)

Table 1. Effect of metal ions and reagents on activity of CgTS

Metal ion	Relative activity	Metal ion	Relative activity
Control	100	$CuSO_4$	45.4
$CaCl_2$	93.0	$MnCl_2$	60.9
$FeSO_4$	114	$NiSO_4$	49.4
$MgCl_2$	112	EDTA	68.5
$ZnSO_4$	71.6	SDS	0.25

80% of CgTS activity was maintained from pH 6.5 and 7.5, whereas the pH stability profile was broad (residual activity of 80% from pH 5.0 to 8.5). About 80% of activity was remained at 40°C, but CgTS was not stable at higher temperature than 40°C.

The addition of 5 mM Fe^{2+} and Mg^{2+} slightly increased the activity, showing 110-115% of the initial activity, whereas the addition of 5 mM sodium dodecyl sulfate (SDS) strongly inhibited the enzyme activity (Table 1). Interestingly, CgTS showed 45% of the initial activity in the presence of 5 mM Cu^{2+} which leaded to sharp decrease in the activity of other known TreSs (7-9).

Effect of reaction temperature on trehalose production by CgTS TreS enzymes catalyze not only transglycosylation reaction to produce trehalose but also hydrolysis reaction to split maltose to glucose. Although the hydrolysis activity of TreS is weaker than its transglycosylation activity, hydrolysis activity increases as the temperature rises, leading to decrease in the trehalose yield (7,9). Koh *et al.* (18) proposed that the structural flexibility of TreSs

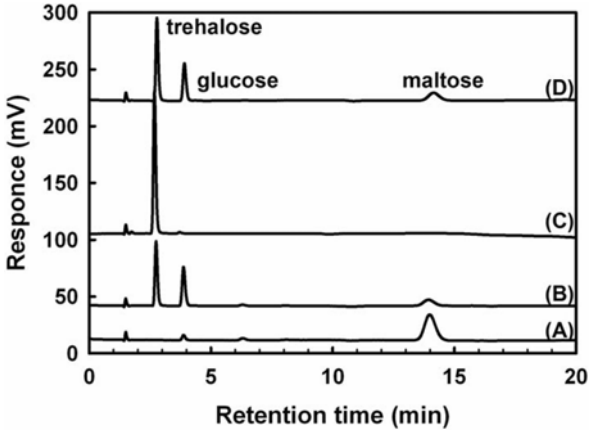

Fig. 2. Analysis of the products of CgTS-catalyzed reaction by HPAEC. Purified recombinant enzymes were incubated for 15 hr. (A) 0.5% maltose, (B) reaction mixture using maltose as the substrate, (C) 0.5% trehalose, and (D) reaction mixture using trehalose as the substrate

increases at a higher temperature, allowing water molecules to attack the covalent glycosyl-enzyme intermediate for hydrolysis before the formation of the α,α-1,1-glycosidic bond through transglycosylation reaction (18). In order to enhance the conversion yield, the reaction temperatures were reduced by 20-35°C compared to their optimal temperatures to get the best conversion yield (7,9,20). However, such lowering reaction temperature requires longer reaction time due to accompanying the decrease in transglycosylation activity.

To check the trehalose production by CgTS, the

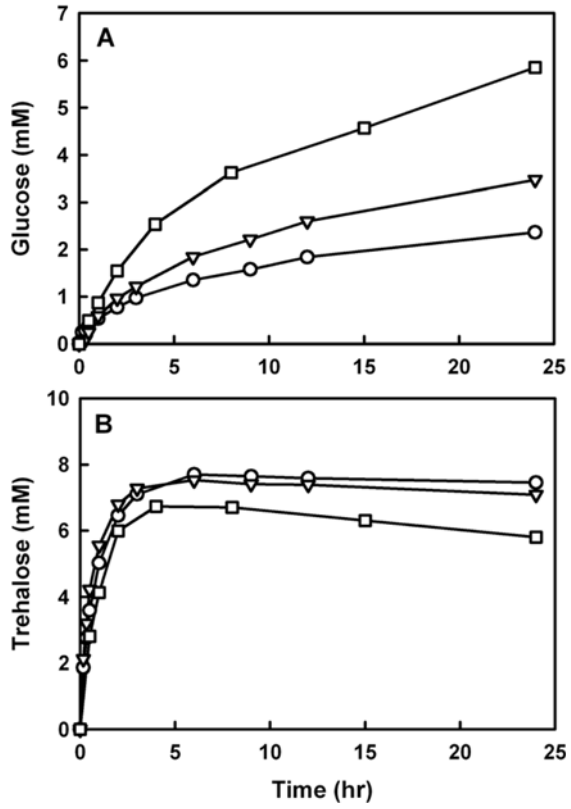

Fig. 3. Time course analysis of production of glucose (A) and trehalose (B) from maltose by CgTS. ○, △, and □ represent the reactions at 25, 30, and 35°C, respectively.

reactions with 0.5% maltose as the substrate were carried out at different temperatures (25, 30, and 35°C), and the products in the reaction mixtures were analyzed using HPAEC. The best conversion yields and the required reaction times at 25, 30, and 35 were 67% at 9 hr, 69% at 9 hr, and 59% at 4 hr, respectively (Fig 3). The reactions produced more glucose and less trehalose when the reaction was performed under a higher temperature (Fig. 3), which is consistent with other TreSs cases (7, 9). The amount of trehalose was rapidly reduced at late stage of the reaction at its optimum temperature (35°C), whereas the hydrolysis activity of CgTS decreased at a temperature lower than 35°C, leading to enhancing the conversion yields. There was no further significant increase in the yield from 30 to 25°C (Fig. 3B). Consequently, lowering temperature by 5°C was enough to get the maximum conversion yield, which is significantly less reduction of temperature than those for other TreSs. As an example, the reaction time required for the best conversion yield by a TreS from *Picrophilus torridus* increased from 6 hr at its optimum temperature (45°C) to 48 hr at 20°C (9).

In conclusion, the gene for CgTS was cloned, and its enzymatic properties were investigated. The hydrolysis activity of CgTS markedly decreased at lower temperatures by only 5°C than its optimum temperature compared to its transglycosylation activity, leading to an improved conversion yield of trehalose from maltose.

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References

1. Elbein AD, Pan YT, Pastuszak I, Carroll D. New insights on trehalose: A multifunctional molecule. Glycobiology 13: 17R-27R (2003)
2. Kaushik JK, Bhat R. Why is trehalose an exceptional protein stabilizer? An analysis of the thermal stability of proteins in the presence of the compatible osmolyte trehalose. J. Biol. Chem. 278: 26458-26465 (2003)
3. Kim BK, Ryu SI, Lee SB. Cryo- and thermo-protective effects of enzymatically synthesized β-galactosyl trehalose trisaccharide. Food Sci. Biotechnol. 17: 199-202 (2007)
4. Paiva CL, Panek AD. Biotechnological applications of the disaccharide trehalose. Biotechnol. Annu. Rev. 2: 293-314 (1996)
5. Padilla L, Krämer R, Stephanopoulos G, Agosin E. Overproduction of trehalose: Heterologous expression of *Escherichia coli* trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in *Corynebacterium glutamicum*. Appl. Environ. Microb. 70: 370-376 (2004)
6. Kim YH, Kwon TK, Park S, Seo HS, Cheong JJ, Kim CH, Kim JK, Lee JS, Choi YD. Trehalose synthesis by sequential reactions of recombinant maltooligosyltrehalose synthase and maltooligosyl-trehalose trehalohydrolase from *Brevibacterium helvolum*. Appl. Environ. Microb. 66: 4620-4624 (2000)
7. Wang JH, Tsai MY, Chen JJ, Lee GC, Shaw JF. Role of the C-terminal domain of *Thermus thermophilus* trehalose synthase in the thermophilicity, thermostability, and efficient production of trehalose. J. Agr. Food Chem. 55: 3435-3443 (2007)
8. Xiuli W, Hongbiao D, Ming Y, Yu Q. Gene cloning, expression, and characterization of a novel trehalose synthase from *Arthrobacter aurescens*. Appl. Microbiol. Biot. 83: 477-482 (2009)
9. Chen YS, Lee GC, Shaw JF. Gene cloning, expression, and biochemical characterization of a recombinant trehalose synthase from *Picrophilus torridus* in *Escherichia coli*. J. Agr. Food Chem. 54: 7098-7104 (2006)
10. Ryu SI, Park CS, Cha J, Woo EJ, Lee SB. A novel trehalose-synthesizing glycosyltransferase from *Pyrococcus horikoshii*: Molecular cloning and characterization. Biochem. Bioph. Res. Co. 329: 429-436 (2005)
11. Avonce N, Mendoza-Vargas A, Morett E, Iturriaga G. Insights on the evolution of trehalose biosynthesis. BMC Evol. Biol. 6: 109-123 (2006)
12. Vallino JJ, Stephanopoulos G. Metabolic flux distributions in *Corynebacterium glutamicum* during growth and lysine overproduction. Biotechnol. Bioeng. 41: 633-646 (2000)
13. Ikeda M, Nakagawa S. The *Corynebacterium glutamicum* genome: Features and impacts on biotechnological processes. Appl. Microbiol. Biot. 62: 99-109 (2006)
14. Wolf A, Kramer R, Morbach S. Three pathways for trehalose metabolism in *Corynebacterium glutamicum* ATCC 13032 and their significance in response to osmotic stress. Mol. Microbiol. 49: 1119-1134 (2003)
15. Carpinelli J, Krämer R, Agosin E. Metabolic engineering of *Corynebacterium glutamicum* for trehalose overproduction: Role of the TreYZ trehalose biosynthetic pathway. Appl. Environ. Microb.

72: 1946-1955 (2006)
16. Kim YW, Lee SS, Warren RAJ, Withers SG. Directed evolution of a glycosynthase from *Agrobacterium* sp. increases its catalytic activity dramatically and expands its substrate repertoire. J. Biol. Chem. 279: 42787-42793 (2004)
17. Miller GL. Use of dinitrosalicylic acid reagent for determination reducing sugar. Anal. Chem. 31: 426-428 (1959)
18. Koh S, Kim J, Shin HJ, Lee D, Bae J, Kim D, Lee DS. Mechanistic study of the intramolecular conversion of maltose to trehalose by *Thermus caldophilus* GK24 trehalose synthase. Carbohyd. Res. 338: 1339-1349 (2003)
19. Lovering AA, Lee SS, Kim YW, Withers SG, Strynadka NCJ. Mechanistic and structural analysis of a family 31 α-glycosidase and its glycosyl-enzyme intermediate. J. Biol. Chem. 280: 2102-2125 (2005)
20. Lee JH, Lee KH, Kim CG, Lee SY, Kim GJ, Park YH, Chung SO. Cloning and expression of a trehalose synthase from *Pseudomonas stutzeri* CJ38 in *Escherichia coli* for the production of trehalose. Appl. Microbiol. Biot. 68: 213-219 (2005)