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Molecular cloning and characterization of trehalose synthase from *Thermotoga maritima* DSM3109: Syntheses of trehalose disaccharide analogues and NDP-glucoses

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ABSTRACT

A gene (ORF TM0392) encoding a putative trehalose synthase (TmTreT) in *Thermotoga maritima* was cloned and expressed in *Escherichia coli*. The recombinant enzyme was purified to homogeneity by heat treatment and a glutathione-sepharose affinity column chromatography. The purified enzyme existed exclusively as a monomer in a native state. The optimum pH and temperature for this enzyme were 6.0 and 65 °C. The glutathione-S-transferase (GST)-fusion enzyme had greater thermostability than thrombin-treated free enzyme. TmTreT had diverse substrate specificities. The enzyme effectively created a free trehalose from several nucleoside diphosphate (NDP)-glucoses as a donor and glucose as an acceptor. Inversely, the enzyme was also capable of employing several NDPs such as UDP, ADP, GDP, and CDP with trehalose to produce corresponding NDP-glucoses. The enzyme was able to employ other monosaccharides, such as mannose and fructose, as acceptors to synthesize disaccharide analogues of trehalose. The mannose-containing analogue was not hydrolyzed by trehalase and the rat intestinal enzymes. Furthermore, the analogue showed a competitive inhibition to the intestinal disaccharidases with K_i values of approximately 0.8–1.6 mM. The results suggest that the enzyme is an useful trehalose synthase that can regenerate NDP-glucoses from NDPs and produce the inhibitory trehalose analogues of indigestible disaccharides.

1. Introduction

The nonreducing dissacharide trehalose is composed of two glucose molecules linked by an α,α-1.1-glycosidic linkage [1]. This sugar is known to serve as a compatible solute of some microorganisms by protecting proteins and cellular membranes from stress conditions and serve as a carbon and energy reserve [2–4]. There are at least five different pathways for the biosynthesis of trehalose. The most common pathway reported in eubacteria, archaea, fungi, insects, and plants, and it involves two enzymatic steps catalyzed by trehalose-6-phosphate synthase (TPS) and trehalose-phosphate phosphatase (TPP) [5–7]. TPS catalyzes the transfer of glucose from nucleotide sugar, typically UDP-glucose (UDP-Glc), to glucose-6-phosphate (G6P) to produce trehalose-6-phosphate (T6P) and nucleoside diphosphate (NDP). Additionally, TPP dephospholyates T6P to trehalose. Another alternative pathway for trehalose synthesis involves trehalose synthase (TreS) in *Pimelobacter* sp. [8] and *Thermus aquaticus* [9], which catalyzes the intramolecular rearrangement of maltose into trehalose, as well as the maltooligosyltrehalose synthase/hydrolase (TreY/TreZ) system in the genus *Sulfolobus*, which converts maltooligosaccharides into trehalose [10]. A trehalose phosphorylase (TreP) can catalyze trehalose synthesis from glucose-1-phosphate (G1P) and glucose in fungi and a few bacteria [11]. In addition, a recently discovered pathway involves a trehalose synthase (TreT) that catalyzes the synthesis of trehalose from NDP-Glc, like UDP-Glc, and glucose, rather than G6P in hyperthermophilic bacteria and archaea.

Until now, the TreT has been examined in the hyperthermophilic archaea *Thermococcus litoralis*, *Pyrococcus horikoshii*, *Thermoproteus tenax*, and halotolerant thermophilic bacterium *Rubrobacter xylanophilus* [12–15]. TreT from *T. litoralis* has been proposed to have a major role in the hydrolysis of trehalose rather than the formation of trehalose in the cell. On the contrary, other similar TreTs from *T. tenax* and *R. xylanophilus* have been characterized to mainly aid in the production of trehalose. *T. tenax* and *R. xylanophilus*

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contain the genes of TreT and the TPS/TPP system in their genomes and both the TreT and TPS/TPP pathways may be active *in vivo* in synthesizing trehalose. Particularly, TreT from *T. tenax* has been shown to be unidirectional and active only in the reaction of trehalose synthesis. However, other TreTs from *T. litoralis*, *P. horikoshii*, and *R. xylanophilus* have been reported to be able to reverse catalysis and to have different substrate preferences.

In the present study, the gene encoding TreT enzyme (TmTreT) from another hyperthermophilic bacterium, *Thermotoga maritima*, has been cloned and expressed in *Escherichia coli*. We have investigated the reversibility of the TmTreT catalysis, the substrate preferences in the catalysis, and the effectiveness of producing novel trehalose analogues. In addition, the effect of protein fusion with GST has been evaluated using enzyme stability and activity. We have found that the enzyme has particular specificities of the transglycosylation in the formation of disaccharide analogues of trehalose and NDP-Glcs based on the reversibility of the enzyme.

2. Materials and methods

2.1. Bacterial strains, plasmids, and chemicals

E. coli strain MC1061 [F⁻ *araD139 recA13 Δ(araABC-leu)7696 galU galK ΔlacX74 rpsL thi hsdR2 mcrB*] and BL21(DH3) were used as hosts for gene manipulation and expression of the recombinant protein. Plasmid p6xHis119 was used as a cloning vector [13]. Plasmid pGEX4T1 was used as an expression vector, which contained the tac promoter, the glutathione-S-transferase (GST) tag, and the ampicillin resistance gene. The *E. coli* transformants were cultured at 37 °C in Luria–Bertani (LB) medium supplemented with ampicillin (100 μg/ml). Uridine diphosphate (UDP), adenosine diphosphate (ADP), guanosine diphosphate (GDP), UDP-glucose (UDP-Glc), ADP-glucose (ADP-Glc), and GDP-glucose (GDP-Glc) were purchased from Sigma Chemical Co (St. Louis, MO, USA). ADP was also obtained from Amresco, Inc. (Solon, Ohio, USA) and Genechem, Inc. (Daejeon, Korea). Cytidine diphosphate (CDP) and CDP-glucose (CDP-Glc) were purchased from Genechem, Inc. The α,α-trehalose, porcine kidney trehalase, as well as an acetone powder of rat intestinal enzymes (trehalase, sucrase, maltase, and isomaltase) were also obtained from Sigma. All other chemicals used were of reagent grade.

2.2. PCR cloning and expression of ORF TM0392

The genomic DNA of *T. maritima* ATCC 43589 was obtained from the American Type Culture Collection. The nucleotide sequence of the ORF TM0392 in the bacterium was originally retrieved from GenBank with the BLAST program [16], which was annotated to encode a hypothetical protein. The gene was amplified with PCR using *Tag* DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) and the genomic DNA of *T. maritima* as a template. The oligonucleotide primers for the 5′- and 3′-flanking ends of the gene were designed as 5′-GGGAGGTCTAGACGTGGATGTTGTGTT-3′ (forward) and 5′-AAGGCAAGCTTTCACCTCAACAGATCT-3′ (reverse), which contained an XbaI and a HindIII restriction sites, respectively (underlined). PCR amplification consisted of an initial denaturation step at 98 °C for 2 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 5 s, and extension at 72 °C for 2 min in the presence of 1.5 mM $MgCl_2$. The 1.22 kb amplified DNA fragment was subsequently digested with XbaI and HindIII, and thereafter ligated into the expression vector p6xHis119 to construct p6xHis119-TmTreT and transformed into the competent *E. coli* MC1061. The target gene was then subcloned into the expression vector pGEX4T1 with a BamHI and an EcoRI to construct pGEX4T1-TmTreT and transformed into the competent *E. coli* BL21(DE3). The *E. coli* BL21(DE3) transformants harboring pGEX4T1-TmTreT were able to produce the recombinant enzyme with a 1 mM lactose analog isopropyl-β-D-thiogalactopyranoside (IPTG) induction. The determination of the nucleotide sequence for the target gene was performed using the BigDye terminator cycle sequencing kit for the ABI 377 Prism (Perkin-Elmer, Norwalk, USA). The other genetic manipulations were conducted using methods from Sambrook et al. [17]. The sequence analysis and homology search in the GenBank and UniProt databases were performed using the BLAST program.

2.3. Purification of the recombinant enzyme

After the *E. coli* BL21(DE3) harboring the expression vector pGEX4T1-TmTreT was grown in ampicillin-supplemented LB broth overnight, the cells were cultured further for 12 h with IPTG induction and harvested by centrifugation (7000 × *g*, 10 min, 4 °C). The pellet was resuspended in phosphate buffered saline (PBS) buffer (pH 7.3) containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4. The cell suspension was disrupted by sonication (4 × 5 min, output control 4, 50% duty cycle; VC-600, Sonics & Materials, Newtown, Conn., USA), followed by centrifugation (10,000 × *g*, 10 min at 4 °C). The resulting supernatant was heated at 70 °C for 20 min and centrifuged. The recombinant GST-fusion protein in the supernatant was purified by Glutathione Sepharose 4 Fast Flow (FF) affinity column chromatography (GE Healthcare Bio-Sciences, Sweden), in which the GST-fusion recombinant protein was eluted with 50 mM Tris–HCl (pH 8.0) containing 40 mM reduced glutathione. The eluted fractions containing the fusion protein were pooled and treated with thrombin protease (40 U/mg protein) for 24 h at 25 °C. After dialysis with the PBS buffer, the GST portion of the fusion protein was removed through the Glutathione Sepharose 4 FF column, and the eluted solution was applied to a Q-Sepharose FF column (Hi-Load 16/10) equilibrated with 20 mM Tris–HCl (pH 7.5). This solution was then eluted with a linear NaCl gradient (0.0–1.0 M) in the same buffer to separate the GST and the thrombin protease from free native TmTreT. The purified TmTreT was concentrated by ultrafiltration (10,000 MWCO, Millipore Co., Bedford, Mass., USA) after dialysis against 50 mM sodium acetate buffer (pH 6.0) and used for further investigation. The purity and molecular mass of the recombinant GST-fusion and free TmTreT protein was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with a 10% (w/v) acrylamide gel.

2.4. Molecular weight determination

The native molecular weight of TmTreT was estimated by gel filtration chromatography with a Shodex KW-804 column (8 mm × 300 mm, Showa Denko, Japan) connected to a Waters Model 626 system and Dionex AD20 detector at 224 nm. Protein samples (20 μl of 1.5–2.5 mg/ml) were applied to the column system equilibrated in a 50 mM sodium phosphate buffer (pH 7.0) with 0.1 or 1.0 M NaCl and eluted with the same buffers at a flow rate of 0.4–0.5 ml/min. Tryroglobulin (Mw 669,000), ferritin (443,000), aldolase (158,000), conalbumin (75,000), and ovalbumin (43,000) (GE Healthcare Bio-Sciences, Sweden) were used as standard proteins for the calibration of apparent molecular weight (Da) of the enzyme in native state.

2.5. Enzyme assay

The enzyme activity was assayed by trehalose-forming activity using substrates of UDP-Glc (16.4–32.8 mM) and glucose (110–220 mM) in 50 mM sodium acetate buffer (pH 6.0) at 40 and 60 °C as a standard condition [13]. The substrate solution was reacted in the standard buffer (pH 6.0) with the purified enzyme (0.1–0.85 mg/ml) to analyze whole reaction progress for 24 h. The reaction was stopped by the addition of 1N HCl and the amount of trehalose produced was quantitatively analyzed by high performance anion exchange chromatography (HPAEC), using a Dionex CarboPac PA100 column (0.4 cm × 25 cm, Dionex Co., Sunnyvale, CA, USA) with an isocratic elution of 150 mM sodium hydroxide at a flow rate of 1.0 ml/min [18]. The protein concentration was determined according to the Bradford method with bovine serum albumin used as a standard [19].

2.6. Effects of reaction conditions

For the determination of optimal pH, the enzyme activity for the trehalose formation was compared in pH range from 3.0 to 12.0 with various 50 mM buffers as follows: sodium citrate (pH 3.0–4.0), sodium acetate (pH 4.5–6.0), sodium phosphate (pH 6.5–7.0), Tris–HCl (pH 7.0–9.0), and glycine–NaOH (pH 10.0–12.0). To examine the pH stability, the enzyme was first incubated in various pH buffers for 1 h at 40 °C and the remaining activity was measured under the standard condition described above. The optimal temperature of the enzyme was investigated in a range of 37–90 °C in the standard buffer (pH 6.0). To determine the thermal stability, the enzyme was pre-incubated up to 2 h in the standard buffer at temperatures ranging from 60 to 90 °C. After aliquots of the sample were taken and placed immediately on ice, the residual activities were assayed under the standard conditions. The effects of metal ions and organic solvents on the enzyme activity of the trehalose formation were also investigated using $CaCl_2$, $MgCl_2$, $MnCl_2$, plus methanol, ethanol, dimethylsulfoxide (DMSO), and dimethylformamide (DMF), respectively. The enzyme was incubated in the absence and presence of 5 mM divalent cations or a 10% (v/v) organic solvent in the standard buffer for 1 h at 40 °C. The enzyme was also incubated with 5 mM EDTA in the same buffer. Immediately after the pre-incubation, an appropriate aliquot was taken and the enzyme activity was measured.

2.7. Transglycosylation reaction

To determine the substrate specificities of TmTreT, the enzyme reaction was performed in the presence of NDP-Glc as the glucosyl donor and monosaccharide as the acceptor for synthesis of trehalose or disaccharide analogue, or inversely with NDP as the acceptor and trehalose as the donor for synthesis of NDP-Glc. For the forward reaction of the trehalose synthesis, the enzyme (0.1–0.85 mg/ml) was incubated with various NDP-Glc (25 mM), including UDP-Glc, ADP-Glc, GDP-Glc, and CDP-Glc and glucose (110 mM), in the standard buffer (pH 6.0) at 60 °C. For the synthesis of trehalose analogue, several monosaccharides (56 mM) including galactose, fructose, and mannose were employed as acceptors with the UDP-Glc donor [20]. The reaction was carried out to monitor whole reaction progress for 24 h and stopped by the addition of 1N HCl. The reaction mixture was centrifuged at 12,000 × *g* for 10 min and filtered using 0.45-μm membrane filters for further experiments. For the reverse synthesis of NDP-Glc, the enzyme was incubated with trehalose (260 mM) and several NDPs (11.3–12.4 mM) including UDP, ADP, GDP, and CDP in the same buffer at 40 °C for 3–48 h. The reaction was also performed with