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Characterization of a trehalose-degrading enzyme from the hyperthermophilic archaeon *Sulfolobus acidocaldarius*

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We purified a cytosolic trehalase (TreH) from a thermoacidophilic archaeon *Sulfolobus acidocaldarius*. Enzyme activity in cell-free extracts indicated that trehalose degradation in the cell occurred via the hydrolytic activity of TreH, and not via TreP (phosphorolytic activity) or TreT (transfer activity). TreH was purified to near-homogeneity by DEAE anion-exchange chromatography, followed by size exclusion and HiTrap Q anion-exchange chromatography, and its molecular mass was estimated as 40 kDa. Maximum activity was observed at 85°C and pH 4.5. The half-life of TreH was 53 and 41 min at 90°C and 95°C, respectively. TreH was highly specific for trehalose and was inhibited by glucose with a K_i of 0.05 mM. Compared with TreH from other trehalases, TreH from *S. acidocaldarius* is the most thermostable trehalase reported so far. Furthermore, this is the first trehalase characterized in the Archaea domain.

[**Key words:** Trehalose; Trehalase; *Sulfolobus acidocaldarius*; Substrate specificity]

In many microorganisms, cellular functions have evolutionarily adapted to sudden changes in environments. In addition to the development of structural factors for cellular protection (e.g., formation of the cell wall against turgor pressure), microorganisms accumulate disaccharides, polyols, or amino acids in the cytoplasm (1,2). Small organic molecules called compatible solutes protect macromolecules, such as enzymes or membranes, from damage. The role of compatible solutes involves osmotic adjustment for protection of cells and cell components from freezing, high temperatures, and oxygen radicals, and these solutes serve as sources of carbon, energy, and nitrogen (3). Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside), a non-reducing disaccharide, is very stable and serves as an osmolyte under water-stressed conditions imposed by high/low temperature, osmolarity, or desiccation (4,5). Physical properties of trehalose include high hydrophilicity, chemical stability (i.e., it is a nonreducing sugar), multiple polymorphs (crystalline and amorphous states), ability to destroy water networks, and absence of internal hydrogen bond formation (6,7).

Trehalose has been found in bacteria, eukaryotes, and archaea (5,8,9). Thermophilic archaea belonging to the genus *Sulfolobus* produce trehalose (10). Biochemical and genomic analyses of *Sulfolobus* spp. indicate that the biosynthesis of trehalose occurs via two reactions: maltooligosyltrehalose synthase (TreY) catalyzes the conversion of maltodextrin to maltooligosyltrehalose, and then maltooligosyltrehalose trehalohydrolase (TreZ) hydrolyzes this product to form free trehalose (11–13). Three genes in this trehalose biosynthetic pathway were cloned from *Sulfolobus*, namely *treZ*, *treX*, and *treY*. TreY and TreZ catalyze the reactions described above. TreX is a glycogen debranching enzyme that catalyzes the hydrolysis of intracellular glycogen into maltodextrin (14). However, the mechanism of utilization of the synthesized trehalose in this organism has not been studied yet. *In vivo*, trehalose catabolism proceeds via hydrolysis by trehalase (TreH), phosphorolysis by trehalose phosphorylase (TreP), or reverse reaction catalyzed by trehalose glycosyltransferring synthase (TreT). A comparative genomic analysis implied that *saci_1827* would be a *treP* or *treT* gene in the *Sulfolobus acidocaldarius* genome. Here, we report the mechanism of trehalose metabolism in *S. acidocaldarius*. Enzyme activity measurement for trehalose degradation reveals that *S. acidocaldarius* can degrade trehalose without any cofactor, unlike TreP or TreT, which require phosphate or nucleotide diphosphate-glucose, respectively, indicating that the enzyme activity is caused by TreH. As far as we know, this is the first report of an archaeal TreH. The TreH activity was further confirmed by the substrate specificity of the enzyme.

MATERIALS AND METHODS

Strain and culture condition *S. acidocaldarius* DSM639 was grown aerobically in Brock medium supplemented with 0.1% (w/v) tryptone, 0.005% (w/v) yeast extract (YT medium) at pH 3.0 and 77°C. The medium was supplemented with 0.2% (w/v) glucose as sole carbon and energy source. Growth was monitored by measurement of the optical density at 600 nm (OD_{600}) with an Ultrospec 2100 pro UV/Vis spectrophotometer.

Preparation of cell-free extracts Cells were grown in 100 mL of YT medium containing 0.2% (w/v) glucose at 77°C. Cells were harvested by centrifugation (8000 ×*g* for 30 min at 4°C) during the exponential phase of growth and resuspended in 20 mM sodium phosphate (pH 7.4). Cells were then lysed by sonication (Ultrasonic generator, Nihonseiki Kaisha Ltd.; 200 μA, 7–9 min) on ice and centrifuged at 2000 ×*g* for 10 min to remove the cell debris. Insoluble proteins were removed by centrifugation at 16,000 ×*g* for 40 min at 4°C. The resulting cell-free extracts were used as crude enzymes. The protein concentration of crude

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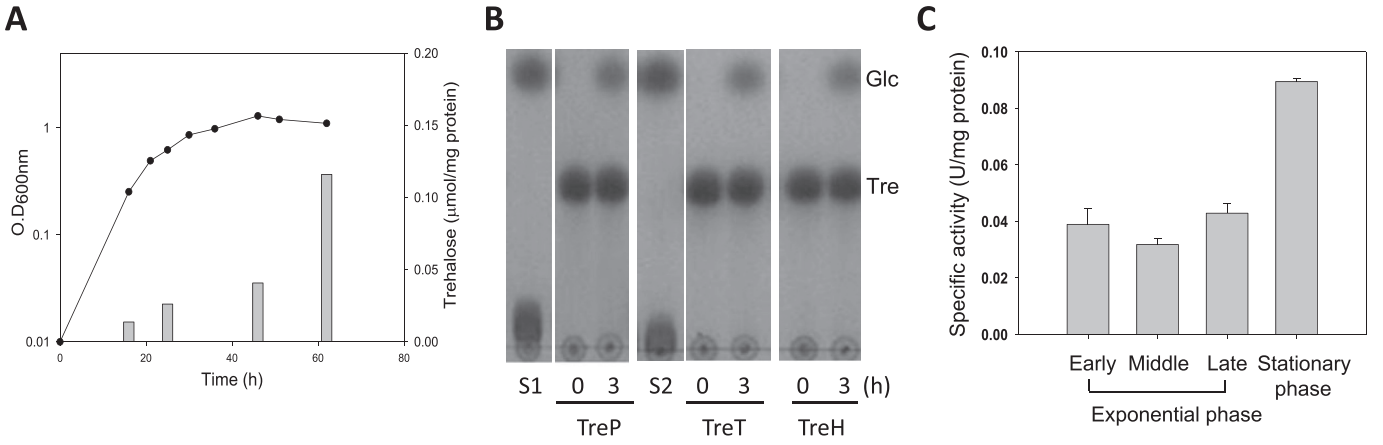

FIG. 1. Trehalose accumulation and trehalase activity in *Sulfolobus acidocaldarius*. (A) Cell growth and accumulation of trehalose. *S. acidocaldarius* DSM639 cells were grown in Brock's medium supplemented with 0.2% glucose, and trehalose content in cell-free extracts was measured by HPLC. (B) TLC analysis of degradation products of three different pathways. Cell-free extracts were incubated at 80°C for 3 h with trehalose alone (TreH), trehalose and phosphate (TreP), and trehalose and UDP/ADP (TreT). S1, glucose and glucose-1-phosphate standards; S2, glucose and UDP-glucose standards. Glc, glucose; Tre, trehalose. (C) Trehalase activity at different growth phases. Cells were harvested at different growth phases and the enzyme activity in cell-free extracts was measured in 20 mM sodium acetate (pH 4.5) with 10 mM trehalose at 80°C for 30 min. Amount of released glucose was quantified by the GOP assay.

enzymes was estimated by the Bradford method using bovine serum albumin as the standard.

Activity assays For the examination of trehalose degradation pathway (TreH, TreP, and TreT), the reaction was carried out at 80°C for 3 h. The cell-free extracts (30 μg) were used in total volume of 100 μL. TreH activity assay was carried out with 20 mM sodium acetate (pH 4.5) and 10 mM trehalose as a substrate and TreP activity was determined in the presence of 10 mM trehalose and 10 mM potassium phosphate as a substrate. The reverse TreT activity was determined in the presence of 10 mM trehalose, 2.5 mM UDP or ADP, and 20 mM $MgCl_2$. Degradation products of trehalose were examined by thin-layer chromatography (TLC). For the standard TreH assay, a reaction mixture (100 μL) containing 10 mM trehalose and the enzyme solution (1 μg) in 20 mM sodium acetate (pH 4.5) was incubated for 30 min at 80°C, and then, stopped on ice. Amount of glucose generated by enzyme reaction was measured by a glucose oxidase–peroxidase (GOP) assay at 37°C for 30 min. Glucose in the reaction mixture (50 μL) was quantified by coupling the production of gluconic acid and hydrogen peroxide to oxidation of *o*-dianisidine by using glucose oxidase and peroxidase in the GOP assay kit (100 μL) to form a colored product. The increase in absorption at 540 nm is proportional to the glucose concentration. One unit of enzyme activity is defined as 1 μmol of glucose formed per min under standard assay conditions.

The pH dependence of the purified enzyme for TreH activity was determined at 80°C with a pH range of 3.0–8.0 by using the following buffer systems: 20 mM sodium citrate (pH 3.0–4.5), sodium acetate (pH 4.5–6.5), and HEPES (pH 6.5–8.0). The temperature dependence was determined in 20 mM sodium acetate (pH 4.5) in a range of 65–100°C for 30 min. Thermostability of the enzyme was determined by incubating the enzyme solution (1 μg) in 20 mM sodium acetate (pH 4.5) at different temperatures ranging from 80 to 100°C. To examine the hydrolytic patterns of TreH, the enzyme solution (1 μg) in 20 mM sodium acetate (pH 4.5) was incubated with 1 mM each substrate at 80°C for 30 min.

Extraction of intracellular trehalose The cells grown in YT medium with glucose were harvested every 6 h by centrifugation at 2000 ×*g* for 30 min. The cell pellets were washed with distilled water twice, centrifuged at 16,000 ×*g* for 10 min at 4°C, dissolved in 1 mL of distilled water, and sonicated for 2 min. After further centrifugation at 16,000 ×*g* for 30 min at 4°C, the supernatant was obtained. To remove soluble proteins from the supernatant, a final concentration of 80% (v/v) ethanol was added to the supernatant, which was then incubated at 100°C for 30 min to aggregate the denatured proteins. The residual fractions were then dried using a rotary vacuum evaporator prior to analysis (15).

Purification of TreH *S. acidocaldarius* DSM639 was grown at 77°C in YT medium supplemented with 0.2% (w/v) glucose. Cells were harvested in the late exponential growth stage, washed twice with buffer A (50 mL of 20 mM Tris–HCl, pH 8.0), and resuspended in 20 mL of the same buffer. After sonication, the cell debris was removed by centrifugation at 16,000 ×*g* for 40 min. Approximately 60 mg of soluble protein was loaded to a PD-10 column (GE Healthcare, UK) filled with DEAE Sepharose Fast Flow (Amersham Biosciences, Sweden). The column was washed with buffer A, and the bound proteins were eluted at a step gradient of 0–0.5 M NaCl. The fractions exhibiting trehalose-degrading activity were pooled and concentrated. The concentrated enzyme solution was then applied to a HiPrep 16/60 Sephacryl S-200 HR column (16 × 600 mm, GE Healthcare) and eluted with 0.2 M NaCl. The active fractions were pooled and dialyzed against buffer A. The enzyme solution was finally applied to a column of HiTrap Q HP

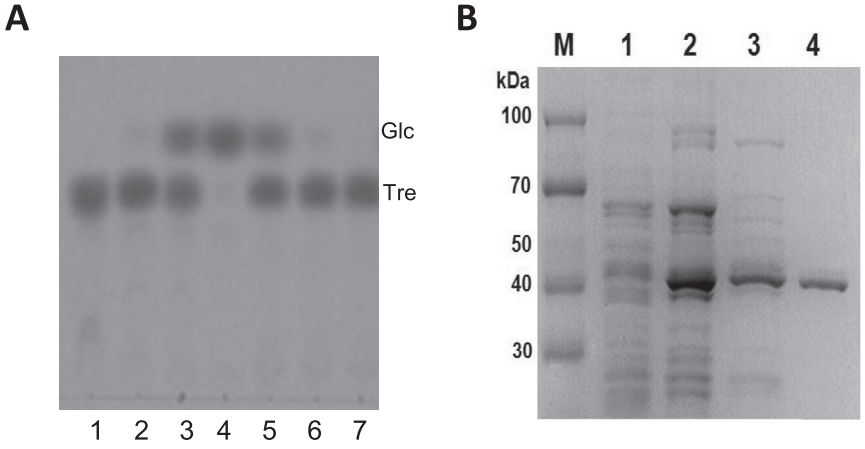

FIG. 2. Purification of TreH. (A) TLC chromatogram of TreH activity during HiTrap Q HP chromatography. Eluted fractions were assayed for TreH activity as described in the Materials and methods section. Lanes 1–7, eluted fraction numbers 8–14. (B) SDS-PAGE of TreH. Lane M, molecular mass markers; lane 1, cell-free extracts; lane 2, DEAE Sepharose Fast Flow purification; lane 3, Sephacryl S-200 HR gel filtration; lane 4, HiTrap Q HP purification. Glc, glucose; Tre, trehalose.