Saci_1816: A Trehalase that Catalyzes Trehalose Degradation in the Thermoacidophilic Crenarchaeon *Sulfolobus acidocaldarius*

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Previously, a cytosolic trehalase (TreH) from the hyperthermophilic archaeon *Sulfolobus acidocaldarius* was reported; however, the gene responsible for the trehalase activity was not identified. Two genes, *saci_1816* and *saci_1250*, that encode the glycoside hydrolase family 15 type glucoamylase-like proteins in *S. acidocaldarius* were targeted and expressed in *Escherichia coli*, and their abilities to hydrolyze trehalose were examined. Recombinant Saci_1816 hydrolyzed trehalose exclusively without any help from a cofactor. The mass spectrometric analysis of partially purified native TreH also confirmed that Saci_1816 was involved in proteins exhibiting trehalase activity. Optimal trehalose hydrolysis activity of the recombinant Saci_1816 was observed at pH 4.0 and 60°C. The pH dependence of the recombinant enzyme was similar to that of the native enzyme, but its optimal temperature was 20–25°C lower, and its thermostability was also slightly reduced. From the biochemical and structural results, Saci_1816 was identified as a trehalase responsible for trehalose degradation in *S. acidocaldarius*. Identification of the *treH* gene confirms that the degradation of trehalose in *Sulfolobus* species occurs via the TreH pathway.

Keywords: Glycoside hydrolase family 15, *Sulfolobus acidocaldarius*, trehalase, trehalose

Introduction

Extremophilic microorganisms accumulate osmolytes to sustain equilibrium during times of environmental stress. Compatible solutes are materials that are not used in metabolism and make it possible to adjust intracellular turgor pressure and stabilize macromolecules under heat and salt stress [1]. *Sulfolobus acidocaldarius* is a thermoacidophilic archaeon that grows in temperatures ranging 70–90°C. Accumulation of trehalose has been reported in *Sulfolobus* species [2, 3]. However, the physiological role of trehalose has not yet been confirmed. Trehalose has been proposed to play a role as an osmo- and thermoprotectant. A two-step TreYZ pathway (from starch to trehalose) for trehalose synthesis was reported in *S. acidocaldarius* [4]. The genes *treY* and *treZ* were identified, and their maltooligosyltrehalose synthase and maltooligosyltrehalose trehalohydrolase activities were confirmed [4–12]. Furthermore, a homolog of trehalose glycosyltransferring

synthase (TreT), which can catalyze the reversible formation of trehalose from glucose and NDP-glucose, was also found, but the biochemical properties of this enzyme were not elucidated [13]. There have been several descriptions of trehalose synthesis in *Sulfolobus* species; however, the pathway for degradation of trehalose in this organism has not been studied. It has been proposed that TreT may be involved in degrading trehalose in *Thermococcus litoralis*; however, the activity that hydrolyzes trehalose into glucose and NDP-glucose has not been confirmed [14, 15]. In our previous study, we reported that the cell-free extracts of *S. acidocaldarius* showed trehalase (TreH) activity, indicating that this species can degrade intracellular trehalose via the TreH pathway [16]. TreH hydrolyzes trehalose into two glucose molecules without the aid of a cofactor. Most of trehalases have been classified as a member of the glycoside hydrolase 37 (GH37) family and GH65 family in the Carbohydrate-Active enZYmes database (CAZy; <http://www.cazy.org/>). However, no putative genes encoding

GH37 or GH65 family trehalases were detected in the *S. acidocaldarius* genome. Recently, a new type of trehalase assigned to the GH15 family has been identified in *Thermoplasma* species [17]. Based on the primary structure, *Thermoplasma* trehalases are more similar to glucoamylases from the identical GH15 family than to trehalases classified to the GH37 and GH65 families. Nonetheless, GH15 family enzymes share a common α -helix-rich catalytic domain designated the $(\alpha/\alpha)_6$ barrel, similar to GH37 and GH65.

In this study, the gene encoding the enzyme responsible for TreH activity was identified, and TreH activity was confirmed by expressing the recombinant enzyme in *Escherichia coli*. The properties of this recombinant enzyme were examined by comparison with those of the native enzyme.

Materials and Methods

Strains and Growth Conditions

E. coli DH5 α and C43(DE3) cells harboring pRIL were used for cloning and overexpression, respectively. They were aerobically grown at 37°C in Luria-Bertani (LB) broth or LB agar plates. The medium for the C43(DE3)pRIL strain was supplemented with kanamycin (30 μ g/ml) and chloramphenicol (30 μ g/ml). *S. acidocaldarius* strains were aerobically grown in a shaking incubator (77°C, 180 rpm) in YT medium with 0.005% yeast extract (w/v) and 0.1% tryptone (w/v), supplemented with Brock's mineral salts. The composition of Brock's mineral salts was the following: (NH₄)₂SO₄, 1.3 g; MgSO₄·7H₂O, 0.25 g; KH₂PO₄, 0.28 g; CaCl₂·2H₂O, 0.07 g; FeCl₃·6H₂O, 0.02 g; MnCl₂·4H₂O, 1.8 mg; CuCl₂·2H₂O, 0.05 mg; ZnSO₄·7H₂O, 0.22 mg; NaMoO₄·2H₂O, 0.03 mg; VOSO₄·H₂O, 0.03 mg; NiSO₄, 5 μ g; CoSO₄·7H₂O, 0.01 mg; and Na₂B₄O₇·10H₂O, 4.5 μ g. The pH was adjusted to 3.3 with 6 M H₂SO₄ before sterilization.

Cloning of Putative *treH* Genes from *S. acidocaldarius*

The primer sequences for *Saci_1816* were the following: forward, 5'-TACATATGTTAGGTATGAAACCTCTAGGAT-3'; reverse, 5'-TACTCGAGTGTACTTAGACCTTTTACT-3'. The primer sequences for *Saci_1250* were as follows: forward, 5'-TTCATATGAATTACG CCTTACTTTCAAACG-3'; reverse, 5'-TCCTCGAGTCTTACTTCA TCAAACCTC-3'. All forward and reverse primers contained NdeI and XhoI recognition sites (underlined). Genomic DNA of *S. acidocaldarius* DSM639 was used as a template. To amplify the *saci_1816* and *saci_1250* genes, PCR was performed as follows: 1 cycle at 94°C for 5 min, followed by 30 cycles of 98°C for 10 sec, 55°C for 5 sec, and 72°C for 110 sec. The reaction mixture contained 10 pmol of the forward and reverse primers and 1.25 U of PrimeSTAR HS DNA Polymerase (TaKaRa Bio Inc., Korea). The PCR products were purified using a PCR Purification Kit (Elpis Biotech Inc., Korea) and digested with NdeI and XhoI. The

digested DNA fragments were inserted into the pET29b(+) plasmid, and the recombinant vectors were transformed into the *E. coli* C43(DE3)pRIL strain. The transformants were selected with kanamycin and chloramphenicol.

Overexpression and Purification of Recombinant TreH

The recombinant *E. coli* cells were grown overnight at 37°C in LB medium containing kanamycin (30 μ g/ml) and chloramphenicol (30 μ g/ml), and then an inoculum was added to fresh LB medium to a final concentration of 1% (v/v), also with kanamycin and chloramphenicol at 1% of the volume of the culture medium (v/v). Cells were grown at 37°C to an optical density at 600 nm of 0.4. The expression of each protein was induced by the addition of 0.1 M isopropyl-1-thio- β -D-galactopyranoside (IPTG), and then the cells were grown for an additional 5 h at 30°C. Cells were harvested by centrifugation (6,000 $\times$ g for 50 min at 4°C) and resuspended in 20 mM sodium phosphate (pH 7.4) containing 0.5 M NaCl and 20 mM imidazole. Cells were then lysed by sonication at 200 μ A for 7–9 min (Ultrasonic homogenizer US-300T; Tender Scientific Co., Ltd., Taiwan) on ice and centrifuged at 3,000 $\times$ g for 10 min to remove the cell debris. Insoluble proteins were removed by centrifugation at 16,000 $\times$ g for 40 min at 4°C. The supernatants were filtered using a 0.45- μ m syringe filter and heated to 60°C for 30 min to remove heat-labile *E. coli* proteins by centrifugation at 3,000 $\times$ g for 50 min. The soluble proteins were then subjected to Ni Sepharose High Performance (GE Healthcare, UK) affinity chromatography. The fractions containing the recombinant proteins were analyzed by SDS-PAGE.

Activity Assays

For the TreH activity assay, a reaction mixture (500 μ l) containing 5 mM trehalose and the enzyme solution (1.5 μ g) in 20 mM sodium citrate buffer (pH 4.0) was incubated for 30 min at 60°C, and then the reaction was stopped by boiling for 10 min. The amount of glucose generated by the enzyme reaction was measured in a glucose oxidase-peroxidase (GOP) assay at 37°C for 30 min. Glucose in the reaction mixture (50 μ l) was quantified by coupling the production of gluconic acid and hydrogen peroxide to the oxidation of *o*-dianisidine, using glucose oxidase and peroxidase in the GOP assay kit (100 μ l) to form a colored product. The amount of increase in absorption at 540 nm is proportional to the glucose concentration. One unit of enzyme activity is defined as 1 μ mol of glucose formed per minute under standard assay conditions. The pH dependence of the purified enzyme for TreH activity was determined at 60°C and at a pH of 3.0–8.5 using the following buffer systems: 20 mM sodium citrate (pH 3.0–4.5), sodium acetate (pH 4.5–5.5), sodium phosphate (pH 5.5–7.5), and Tris-HCl (pH 7.5–8.5). The temperature dependence was determined in 20 mM sodium citrate (pH 4.0) buffer and at 40–90°C for 30 min. The thermostability of the enzyme was determined by incubating the enzyme solution (1.5 μ g) in 20 mM sodium citrate (pH 4.0) buffer at temperatures ranging from 60°C to 90°C. To examine the hydrolytic patterns of the putative TreH, the enzyme

solution (1.5 µg) in 20 mM sodium citrate (pH 4.0) buffer was incubated with 5 mM trehalose at 60°C.

Thin-Layer Chromatography (TLC) Analysis

The reaction products and their respective standard solutions were applied to silica plates (type 60 F₂₅₄; Merck, Germany), which were subsequently developed with 1-butanol:acetic acid:water (7:4:1 (v/v/v)). The developed TLC plates were completely dried and then visualized by dipping them in a solution containing 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) H₂SO₄ in methanol, followed by heating at 120°C for 5 min.

Partial Purification of Native TreH from *Sulfolobus acidocaldarius*

The purification of native TreH was slightly modified as described previously [16]. *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 (50 ml of 20 mM sodium phosphate, pH 7.4), and resuspended in 100 ml of the same buffer. After sonication, cell lysates were centrifuged at 3,000 ×g for 10 min to remove the cell debris. This cell crude extract was loaded onto a HiTrap DEAE fast flow 5 ml column (GE Healthcare, USA). The column was washed with the same buffer used in cell resuspension, and the bound proteins were eluted at a linear gradient of 0–0.5 M NaCl by AKTA prime FPLC (GE Healthcare, USA). All proteins were separated in each 5 ml fraction and trehalose hydrolyzing activity was measured. The fractions showing trehalose hydrolyzing activity were collected and further applied to HiLoad 16/600 Superdex 200 pg (GE Healthcare, USA) size-exclusion chromatography. The active fractions were collected and desalted on a HiPrep 26/10 desalting column (GE Healthcare, USA) with cell resuspension buffer (20 mM sodium phosphate, pH 7.4). The desalted proteins were applied to a HiTrap Q HP 1 ml column (GE Healthcare, USA) equilibrated with cell resuspension buffer. After washing, the bound proteins were eluted with a linear gradient of 0–0.5 M NaCl. All proteins were separated in 1 ml fractions and the fractions exhibiting trehalose hydrolyzing activity were used as partially purified trehalase, which was used for mass spectrometry analysis for the identification of the gene for TreH.

Molecular Mass Determination of Native TreH

To estimate the molecular mass of native TreH and recombinant TreH, size-exclusion chromatography was performed. The AKTA prime FPLC and HiLoad 16/600 Superdex 200 pg column were used. Gel filtration marker kit MWGF1000 (Sigma-Aldrich, USA) was used as the molecular weight standard according to the manufacturer's instruction. All running conditions were the same as follows: flow rate, 1 ml/min; fraction size, 5 ml.

Mass Spectrometry (MS) Analysis

In-gel pieces were decolorized by reacting in 50% (v/v) acetonitrile (ACN) solvent for 15 min. The chymotrypsin and trypsin solutions (1 µg) were added to the decolorized in-gel piece samples and

incubated at 37°C for 16 h. Then, 2 µl of 1 M dithiothreitol was added and the pieces were stored at room temperature for 1 h, and then 4 µl of 1 M iodoacetamide was added and the samples were further incubated at room temperature for 1 h. Finally, 200 µl of 50% (v/v) ACN solvent was added to recover the hydrolyzed peptide through gel dehydration. The samples were dried using a SpeedVac (Thermo Fisher Scientific, USA). Solid-phase extraction was performed using Sep-Pak (Waters, USA) and then drying again using SpeedVac. Then, 15 µl of water containing 0.1% (v/v) formic acid was added and the sample was kept refrigerated until analysis.

For LC-MS/MS, a Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, USA) was used. The MS was coupled with an UHPLC Ultimate 3000 (Dionex, Germany) system and peptide separation was proceeded on an Acclaim PepMap RSLC C18 column (2 µm, 100 Å, 75 µm × 15 cm, nanoViper; Dionex, Germany). The samples were eluted in gradient mode at a flow rate of 0.3 µl/min as follows: 5% B at 0–5 min; 5–30% B at 5–33 min; 30–50% B at 33–38 min; 50–80% B at 38–40 min; 80% B at 40–43 min; 80–5% B at 43–45 min; and 5% B at 45–59 min. The mobile phase was water containing 0.1% (v/v) formic acid (solvent A) and ACN containing 0.1% (v/v) formic acid (solvent B) and the injection volume was 1 µl. The MS conditions were set as follows: spray voltage, +2,500 V; capillary temperature, 300°C. Peptides were scanned at *m/z* 150 to 1,800. LC-MS/MS data were evaluated using Thermo Proteome Discoverer 2.1.0.81 (Thermo Fisher Scientific, USA).

Results

Comparison of the Amino Acid Sequences of Saci_1816 and Saci_1250 with Those of Other Archaeal TreHs

Two putative TreHs, Saci_1250 and Saci_1816, were selected as enzyme candidates responsible for trehalose degradation in *S. acidocaldarius*. Saci_1816 showed 43% sequence identity with Saci_1250. Sequence alignments with two TreHs, Ta0286 (*Thermoplasma acidophilum* TreH) and MSMEG_4535 (*Mycobacterium smegmatis* trehalase), revealed five conserved regions found in GH15 family TreHs [17, 18]. Two glutamic acid residues of Saci_1816, Glu374 and Glu524, in regions 3 and 5, which are known as catalytic residues in this family, were also conserved. This suggested that both candidates may possess TreH activity (Fig. 1).

Saci_1816 Showed TreH Activity

To determine which protein possessed TreH activity, two candidate genes were cloned into the pET29b vector, and the resulting plasmids were introduced into *E. coli*. Both proteins were expressed as a soluble protein when the transformed cells were induced by IPTG, and each protein

Ta0286	-----MLVAMN	6
MSMEG_4535	MVLQQTETPDGADRKASDGPLTVTAFVPPYAAGPTLRNPFPPPIADYGFSLDCETTCLISSA	60
Saci_1816	-----MLGMKPLGFIGNGLTSAL-VDN	21
Saci_1250	-----MNYALLSNGITTAL-EKE	17
	*	
Ta0286	GFIDWGCLENFNSPAVFSSILDKKKGGYFAIYPTDTHDLYVDQYKELTNILVTEF-IKN	65
MSMEG_4535	GSVEWLCVPRPDSPSVFGAILDRGAGHFR-LGPGYGVSPVPAARYLPG-SLILETTWQHT	118
Saci_1816	GSIVWLTFFPRDPSVFGKLLDDNAGEFSIRPVEDKEK-VSQSYLV--PNVLSTTFKSSN	78
Saci_1250	GSIEWFPVPKFDSPSVFTKILDEDKGGYFLITPEKFNK-VKQQYVE--YSLI-LRTEFDD	73
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Ta0286	GKVILRTIDFMPD-----S---EYKISFPE---VHRFVETFSDFVRITIDFKPAFNY	112
MSMEG_4535	GWLIVRDALVMGFWDHIDIRSRTHRTPMOWDAEHILLRTVRCVSGIVELVMSCEPAFDY	178
Saci_1816	GKAEIVDLMPIGE-----K-----AIIRKVRTEIPLSFKIIPMFNY	114
Saci_1250	GNLILIDFLPLSL-----P-----AIIRLYEAKVPFNVEVKPLFNY	109
	* : : : : : : : : : : *	
Ta0286	ATERPLIQRVQHGFI--IFSTEK-----ENMGISTEFOLKKNADHVADVDMEPRSSSWV	164
MSMEG_4535	HRVSATWEYSGPAYGEAIAARASRNPDSPHTLRLITNLRIEGREARARTRLTEDGNVTV	238
Saci_1816	GLYRPIIRKDDGIQ-----FLNPVSRECLSLSDVPTDEIK---P-----PGTTL	157
Saci_1250	GLVNAGTETRKDGII-----YKNPESKEGLELLINGDYKIIS---PYRITVNSGKGYL	159
	. : . : . : . : . : . : . : .	
Ta0286	IALYGIHLYRP-----TDYK-SYLRLQETTDYWRKWASNSTYSG-MYHSMVMRSALAK	217
MSMEG_4535	ALSWSKH--PAP-----QTYEEAADKMWKTSEAWROWINVGDFPDHPWRYLQRSALT	291
Saci_1816	YLVYSSDCAYGPLDKGKQLENDLENSFNLTIDYWKDKI-----R-SNDEVWRTSVGVLL	210
Saci_1250	YLLYSRDLRYGLFSQKGFVYSEPYEAYSKLLYSRKELERARKPS-IYENAFYRSLSVIL	218
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Ta0286	VLFEPTGMVAAPTASLPEAIGGERNWDYRYTWIRDTAYVIEALATIGYKREAVSFLYD	277
MSMEG_4535	GLTYSPTGALLAAPTITSLPETQGERNWDYRYSWIRDSTFALWGLYTLGLDREADDFSF	351
Saci_1816	GLIYSPSGSSIAAATTSLPEAVGDSRNWDYRFVWVRDSSMISEALLYSGYVVEARRILNF	270
Saci_1250	GLIYKPSGGIIASPTTISPEIVGDERNWDYRYVWVRDSSYAIEALVKANLLTHARRALDF	278
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Ta0286	MMDVISREN----KIRTIYSIDNSDDFVEREL-DFEGYGRSRPVRIGNKAVDQLQIDQYG	332
MSMEG_4535	IADVSGANNGERHPLOVMYGVGGERSLVEEELHHLGSDYNSRPFVRIGNGAYNQROHDI	411
Saci_1816	MLALVNFT--AKPLLHPLYAVDGSPPPEIEIPWLSGYMNSRPFVRVGNAAASQIQLDIEG	328
Saci_1250	LTNLLDPS--SKSFDHPFYSVDGTPPPAEENLDWLSGFMNSKPFVRIGNAAYLQIQMDIEG	336
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Ta0286	SIVRAIHAMEKA--GGVNSYLWDFVEEMAKIEYLWKYPDSSIWEFRTPEPKQVYYSKVM	390
MSMEG_4535	TMLDSVYLHAKS--REQIPDALWPVLKNQVEEAIKHWEKPDRTGIWEVRGEPOHFTSSKIM	469
Saci_1816	FLVDAIYKYKYTSRDRVFEENWDKIKYIGDWVSKNMLKADAGWEDRGDPKHYSKVM	388
Saci_1250	AYMNALEYKRTILDKDYISSIFWAVEAISDWSSSVRGESTDIWEERGISRHYTHTKLM	396
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Ta0286	SWAAFDSAITMARDL-GLTAPIKKWKGIDQEIWNVDMTKGFDPQTNSEFVQYYSKKNVDAS	449
MSMEG_4535	CWVALDRGSKLAELQ-GEKSYAQQWRAIAEEIKADVLRAGVDKR-GVLTQRYGDDALDAS	527
Saci_1816	MWVALDRIEKIMNVKIEKDEIKEWV-----MRNCV-KD-GSFVRSSDSNDVDAN	436
Saci_1250	SWVALDRASKLAKDL-GYNKLFEEWKSRAEIKIDILNNGVKDN-HHFVRYGGDEIDAA	454
	* . * : * : . : : * : : : : : : : : : : : : * : *	
Ta0286	LLRLPILGFIPANDERFIGTMKRIEDELMD-VDGYLFR--RYREDGLKGDGSLFMLTFW	506
MSMEG_4535	LLLAVLTRFLPADDPRIRATVLAIADELTDGLVLRVRYEETDDGLAGEEGFTFICSF	586
Saci_1816	LLTLPLYDFIDVKDPIFLKTLKRIEDELMD-VDGFVKRYSQ---DFMGEAKHPFALATI	491
Saci_1250	LLTLPIYDFIPATDILFMNTLKKIDEELRVADGLYLRYK---DFMGLAKNPFTLVTIT	510
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Ta0286	YIEDLILMKRLKAARALESIIKANHLGLYSEIDEKTDGDFLGNFPOALSHLGIIRVAP	566
MSMEG_4535	LVSAIVEIGETSRKHLCEKLLSFASPLHLIYEEIEPRTRHGLGNFPOAFTHLALINAV	646
Saci_1816	LARVYIRLGRKERAMELLDKVLKPSGTLYLIGEHIDVENMEYTGNYPOAFVHAQLLALK	551
Saci_1250	MARVYIRLKEFDRARWLLLETILKCNQDLGLIGEHVIDPETCEARGNYPHLFPHSGMVL	570
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Ta0286	KLEEAFLKRVK--K---INE--	581
MSMEG_4535	HVIRAEEDSSGVFVPANAPM	668
Saci_1816	EVKGLST-----	558
Saci_1250	EFDEVR-----	576
	. .	

Fig. 1. Alignment of Saci_1816 and Saci_1250 sequences with those of other bacterial and archaeal trehalases.

Ta0286, *Thermoplasma acidophilum* trehalase; MSMEG_4535, *Mycobacterium smegmatis* trehalase. Blue and black boxes represent the five highly conserved regions of GH15 trehalases and two catalytic residues (nucleophile and acid/base) in the active site, respectively. Multiple sequences were aligned using the UniProt alignment program (<http://www.uniprot.org/align/>).

was purified by Ni-NTA affinity chromatography. SDS-PAGE analysis of each purified recombinant protein showed a single band with an apparent molecular mass of 63.6 kDa (Fig. 2A), which agreed with the expected size

based on the primary structure. To examine TreH activity, the purified proteins were incubated with trehalose at 60°C, and the reaction products were examined by TLC. As shown in Fig. 2B, glucose was released from trehalose by

Fig. 2. Purification of recombinant Saci_1816 and Saci_1250.

(A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified proteins. Lane 1, molecular mass marker; lane 2, Saci_1816; lane 3, Saci_1250. (B) Thin-layer chromatogram of the products of the enzyme reaction in 20 mM sodium citrate (pH 4.0) with 5 mM trehalose at 60°C.

the enzyme reaction. Saci_1816 alone showed TreH activity; Saci_1250 did not. This indicated that *saci_1816* encodes a TreH responsible for trehalose degradation in *S. acidocaldarius*.

Characterization of the Recombinant Saci_1816

The recombinant TreH (Saci_1816) hydrolyzed trehalose specifically; it did not hydrolyze any other trehalose analogs such as maltose, nigerose, and turanose. The enzyme showed typical TreH properties. The pH and temperature dependence of the recombinant TreH were examined. The optimal pH of the recombinant TreH for trehalose was 4.0, and the activity profiles at various pHs were very similar to those of native TreH from *S. acidocaldarius* (Fig. 3A) [16]. However, the optimal temperature (60°C) and thermostability (half-life of 41 min at 90°C) were decreased compared with those of native TreH (Figs. 3B and 3C). Previously, the optimal activity of native TreH was observed at approximately 80–85°C, with a half-life of 53 min at 90°C. To investigate the differences in the thermal properties of recombinant and native TreH, size-exclusion chromatography of both proteins was performed. The trehalose hydrolyzing activity of the partially purified native TreH was detected in the proteins near 190 kDa molecular size (Fig. 4A). Unexpectedly, the purified recombinant TreH showed two different forms despite one band being seen in SDS-PAGE. One form was close to 200 kDa, which is similar to native TreH, and the other was nearly 480 kDa in size (Fig. 4B). Based on this

result, the native form of TreH was expected to be a trimer. The two different forms observed in the recombinant TreH are presumably due to the mixture with incompletely folded proteins.

Saci_1816 Is Indeed TreH

In our previous study, the molecular mass of native TreH was estimated to be 40 kDa [16]. If the molecular mass of the previous protein was correct, the molecular mass of the recombinant TreH observed in this study is too large. To confirm this discrepancy, MALDI-TOF analysis of the protein previously assessed with TreH was performed and the protein was found to be a glutamate dehydrogenase (Saci_0155, 46.2 kDa) (data not shown). Attempts to purify native TreH to homogeneity failed. Instead, the enzyme partially purified by three consecutive chromatographic steps was examined by SDS-PAGE, and the bands close to 65 kDa, similar to the predicted molecular mass of recombinant TreH, were subjected to LC-MS/MS analysis to identify the correct trehalase-encoding gene. MS/MS analysis of the three different protein bands identified the ~65 kDa protein as the predicted trehalase Saci_1816 (Fig. 5 band 1 and Table 1). The ~60 and ~53 kDa proteins were also detected and corresponded to acetolactate synthase (Saci_2281) (Fig. 5 band 2 and Table 1) and phosphoenolpyruvate carboxylase (Saci_0059) or phosphoribosylamine-glycine ligase (Saci_1613) (Fig. 5 band 3, Table 1).

Fig. 3. Effects of pH (A) and temperature (B) on the activity and thermostability (C) of the recombinant TreH.

(A) To determine the optimal pH, the following buffers were used: pH 3.0–4.5, sodium citrate (closed circles); pH 4.5–5.5, sodium acetate (open circles); pH 5.5–7.5, sodium phosphate (open triangles); and pH 7.5–8.5, Tris-HCl (closed triangles). Activity was assayed at 60°C for 30 min in the various buffers (20 mM). (B) To determine the optimal temperature, activity was measured at the indicated temperatures under standard assay conditions. (C) To determine thermostability, the enzyme was incubated at 60°C (closed circles), 75°C (open circles), 80°C (closed triangles), and 90°C (open triangles) in 20 mM sodium citrate buffer (pH 4.0) without the substrate, and the residual activity was measured as above. Error bars represent the standard deviation from three separate experiments. A_0 and A are the initial and residual activities, respectively, of the enzyme after incubation for the indicated time periods.

Fig. 4. Molecular mass determination of native (A) and recombinant TreH (B) by size-exclusion chromatography.

The trehalose hydrolyzing activity (gray bar) was measured using trehalose as a substrate and the maximum activity and TreH peak are marked (red dot). TreH and six molecular mass standard proteins were each subjected to size-exclusion chromatography. The molecular mass of TreH was estimated by comparing the retention time of TreH with the standard curve obtained for the molecular mass standard proteins. 1, Tyroglobulin (669 kDa); 2, apoferritin (443 kDa); 3, β -amylase (200 kDa); 4, alcohol dehydrogenase (150 kDa); 5, bovine serum albumin (66 kDa); 6, carbonic anhydrase (29 kDa).

Discussion

The aim of this study was to identify the gene encoding the TreH that is responsible for trehalose degradation in *S. acidocaldarius*. *S. acidocaldarius* produces trehalose as a compatible solute during salinity stress conditions. Previously, we reported that degradation of accumulated trehalose was performed by intracellular TreH that hydrolyzed trehalose into two glucose molecules [16]. However, the search for a gene encoding TreH was not

Fig. 5. SDS-PAGE analysis of partially purified native TreH. The proteins numbered were selected on the basis of trehalose hydrolyzing activity and the expected TreH molecular weight and analyzed by mass spectrometry. Lane 1, size marker for protein; lane 2, partially purified native TreH.

successful because GH35 or GH65 family-type TreHs were not detected in a whole-genome analysis. GH15 family-type TreHs have recently been reported in the archaea *Thermoplasma volcanium* and *T. acidophilum* [17]. Three GH15-type genes (*saci_1198*, *saci_1250*, and *saci_1816*) were found in the *S. acidocaldarius* genome. The *saci_1198* gene product showed 56% and 52% amino acid sequence identities with glucoamylases of *Picrophilus torridus* DSM9790 and *T. volcanium* GSS1, respectively, indicating that *Saci_1198* is a GH15 glucoamylase (unpublished data). The high similarities of the deduced amino acid sequences of *Saci_1250* and *Saci_1816* and those of known thermophilic TreHs implied that one of the two proteins could be a TreH in this organism.

Both proteins were heterologously expressed and purified

in *E. coli*, and their trehalose hydrolyzing activities were examined using trehalose as a substrate. *Saci_1816* exhibited TreH activity but *Saci_1250* did not. The pH profile of recombinant *Saci_1816* showed a narrow acidic pH range and high thermostability, consistent with the properties of native TreH in *S. acidocaldarius*. The low optimal temperature and relatively low thermal stability seen in recombinant TreH are considered to be due to incomplete folding of the recombinant protein, as shown by the results of size-exclusion chromatography. A decrease in the optimal temperature and reduced thermostability have been observed in other exogenous proteins expressed in *E. coli*. Recombinant MalA expressed in *E. coli* was previously reported to be highly unstable and exhibited optimal activity at 20°C below that of the native enzyme expressed in *S. acidocaldarius* [19]. MS analysis of partially purified native TreH confirmed that *Saci_1816* is the only trehalase in *S. acidocaldarius*. As a result, *Saci_1816* is considered to be the TreH that is responsible for trehalose degradation in this organism.

In hyperthermophilic archaea, the degradation pathway for intracellularly accumulated trehalose during cell growth has not been well characterized. Based on sequence homology data, the TreP, TreT, or TreH pathways are the expected routes for trehalose degradation. TreT has been reported to degrade trehalose in *Thermococcus litoralis* and *Pyrococcus horikoshii* [14, 20]. Recently, the TreH pathway was also identified in thermophilic *Thermoplasma* species [17]. Although a putative TreT (*Saci_1827*) was found in *S. acidocaldarius*, trehalose degradation activity was not observed [16]. The identification of TreH in this organism, as well as in *Thermoplasma* species, implies that the TreH pathway may be the only route to trehalose degradation in thermoacidophilic archaea. To date, GH15-type TreHs have only been found in thermoacidophilic archaea, and their crystal structures have not yet been solved. Elucidation of these structures will be important to understand the detailed reaction mechanism of trehalose degradation in these archaeal groups.

Table 1. Mass spectrometry analysis of the native TreH with SDS gel^a.

Band	ORF no.	Annotation	MW (kDa)	Peptide count	Protein coverage (%)
1	<i>saci_1816</i>	Glycosyl hydrolase	63.6	65	65.8
2	<i>saci_2281</i>	Acetolactate synthase	59.3	18	34.7
3	<i>saci_0059</i>	Phosphoenolpyruvate carboxylase	58.4	39	65.9
	<i>saci_1613</i>	Phosphoribosylamine-glycine ligase	52.9	28	58.8

^aProtein bands were excised from the SDS gel of the fraction with trehalose degradation activity after partial purification as described in Materials and Methods. Protein bands 1 to 3, as depicted in Fig. 5, were isolated and identified by LC-MS/MS.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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