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Expression, Purification, and Characterization of the Maltooligosyltrehalose Trehalohydrolase from the Thermophilic Archaeon *Sulfolobus solfataricus* ATCC 35092

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The maltooligosyltrehalose trehalohydrolase (MTHase) mainly cleaves the α-1,4-glucosidic linkage next to the α-1,1-linked terminal disaccharide of maltooligosyltrehalose to produce trehalose and the maltooligosaccharide with lower molecular mass. In this study, the *treZ* gene encoding MTHase was PCR-cloned from *Sulfolobus solfataricus* ATCC 35092 and then expressed in *Escherichia coli*. A high yield of the active wild-type MTHase, 13300 units/g of wet cells, was obtained in the absence of IPTG induction. Wild-type MTHase was purified sequentially using heat treatment, nucleic acid precipitation, and ion-exchange chromatography. The purified wild-type MTHase showed an apparent optimal pH of 5 and an optimal temperature at 85 °C. The enzyme was stable at pH values ranging from 3.5 to 11, and the activity was fully retained after a 2-h incubation at 45−85 °C. The k_{cat} values of the enzyme for hydrolysis of maltooligosyltrehaloses with degree of polymerization (DP) 4−7 were 193, 1030, 1190, and 1230 s^{-1}, respectively, whereas the k_{cat} values for glucose formation during hydrolysis of DP 4−7 maltooligosaccharides were 5.49, 17.7, 18.2, and 6.01 s^{-1}, respectively. The K_M values of the enzyme for hydrolysis of DP 4−7 maltooligosyltrehaloses and those for maltooligosaccharides are similar at the same corresponding DPs. These results suggest that this MTHase could be used to produce trehalose at high temperatures.

KEYWORDS: Maltooligosyltrehalose trehalohydrolase; trehalose; thermophilic enzyme; *Sulfolobus*; *E. coli*; starch

INTRODUCTION

Maltooligosyltrehalose trehalohydrolase (EC 3.2.1.141, MTHase, also known as glycosyltrehalose trehalohydrolase, glycosyltrehalose-hydrolyzing enzyme, and trehalose-forming enzyme) mainly cleaves the α-1,4-glucosidic linkage next to the α-1,1-linked terminal disaccharide of maltooligosyltrehalose to produce trehalose and the maltooligosaccharide with lower molecular mass. Several bacteria are capable of producing MTHase, such as *Arthrobacter* sp. Q36 (*1*), *Arthrobacter ramosus* S34 (*2*), *Corynebacterium glutamicum* (*3*), *Deinococcus radiodurans* (*4*), *Rhizobium* sp. M-11 (*5*), *Sulfolobus solfataricus* MT4 (*6*), *Sulfolobus solfataricus* KM1 (*7*), *Sulfolobus acidocaldarius* ATCC 33909 (*8*), and *Sulfolobus shibatae* DMS 5389 (*9*).

Trehalose is a nonreducing disaccharide that contains two glucose molecules linked by an α-1,1-glucosidic linkage and is widely present in insects, fungi, and bacteria. Because trehalose can protect proteins and lipid membranes from desiccation, freezing, high temperature, and osmotic stress, the applications of trehalose have been found in many different areas, such as the use as a preservative or stabilizer for cells, organs, food, cosmetics, and medicines (*10*). As the applications of trehalose are increasing, a productive process with a high yield of trehalose is gaining more attention.

In addition to MTHase, maltooligosyltrehalose synthase (EC 5.4.99.15, MTSase, also known as glycosyltrehalose synthase, glyosyltrehalose-producing enzyme, and trehalosyl dextrin-forming enzyme) is also used in the process of trehalose production from starch (*1*−*3*, *5*−*9*). MTSase mainly catalyzes an intramolecular transglycosylation reaction to produce a nonreducing maltooligosyltrehalose by converting the α-1,4-glucosidic linkage at the reducing end of maltooligosaccharide to an α-1,1-glucosidic linkage. Trehalose is currently synthesized from starch on an industrial scale by using MTSase and MTHase obtained from mesophilic bacterium *Arthrobacter* sp. Q36 or *Rhizobium* sp. M-11 (*11*).

Using the thermophilic enzymes to produce trehalose from starch has several advantages. The reaction is generally carried

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out at a high temperature, which can accelerate the reaction rate, lower the degree of retrogradation of starch, and decrease the risks of microbial contaminations in the reaction mixtures (*12*, *13*). In addition, starch is a relatively inexpensive substrate; therefore, using starch could lower the production cost. In a previous process, thermophilic MTSase and MTHase were combined with a debranching enzyme to produce trehalose from starch with a yield of around 82% (*13*, *14*). This limit in yield of trehalose was due to the side-hydrolysis reactions of maltooligosaccharides catalyzed by MTSase and MTHase (*13*, *14*). However, the substrate specificity of MTHase on this side-hydrolysis reaction has received little attention.

Recently the complete genome of thermophilic archaeon *S. solfataricus* ATCC 35092, also known as P2, has been sequenced (*15*). The MTSase from this strain has been examined in our previous study (*16*), but the MTHase, encoded by the *treZ* gene, from this strain remained uncharacterized. In the present study the *treZ* gene of MTHase was PCR-cloned from the genomic DNA of *S. solfataricus* ATCC 35092, and the cloned gene was overexpressed in *Escherichia coli*. In addition to characterizing the general properties of this recombinant MTHase, we also investigated the substrate specificity and kinetic parameters of the enzyme on both hydrolysis reactions of maltooligosyltrehaloses and maltooligosaccharides, respectively.

MATERIALS AND METHODS

Materials. The genomic DNA of *S. solfataricus* ATCC 35092 was obtained from the American Type Culture Collection (Manassas, VA). *E. coli* BL21-CodonPlus (DE3)-RIL was obtained from Stratagene (La Jolla, CA). Plasmid pET-15b was from Novagen (Madison, WI). Vent DNA polymerase and mung bean nuclease were purchased from New England BioLabs (Beverly, MA). T4 DNA ligase and restriction enzymes were supplied by Promega (Madison, WI). Glucose (G_1), maltose (G_2), maltotriose (G_3), maltotetraose (G_4), maltopentaose (G_5), maltohexaose (G_6), maltoheptaose (G_7), glucoamylase, 3,5-dinitrosalicylic acid (DNS), bovine serum albumin (BSA), isopropyl-β-D-thiogalactoside (IPTG), and phenylmethanesulfonyl fluoride (PMSF) were from Sigma (St. Louis, MO). Ultrafree-15 and Microcon centrifugal filter units were obtained from Millipore (Bedford, MA). Sephacryl S-200 HR and protein low molecular mass standards were from Amersham Pharmacia Biotech (Piscataway, NJ).

Amplification of the *treZ* Gene. The *treZ* gene was amplified by using the Polymerase Chain Reaction (PCR). Two primers were designed on the basis of the *treZ* sequence of *S. solfataricus* (GenBank accession no. AE006815 Region: 7425 .. 9110). To clone the *treZ* gene into the pET-15b vector, the *Xho*I and *Bam*HI restriction sites were included in the forward and reverse primers, respectively. The primer sequences are as follows: 5XtreZ (forward primer), 5′-CCC GGG TCG A**CT CGA G**AT GAC GTT TGG TTA TAA ATT AGA TGA-3′; 3BtreZ (reverse primer), 5′-TTA GCA GCC **GGA TCC** CTA AAG TTT ATA TAA AGC AAA TCC CT-3′; the *Xho*I and *Bam*HI restriction sites are in boldface. The reaction was carried out in 100 μL of reaction mixture containing the genomic DNA of *S. solfataricus* ATCC 35092, two primers, dNTP, Vent DNA polymerase, and Vent DNA polymerase buffer, and was performed by using a GeneAmp PCR system 2400 (Perkin-Elmer, Wellesley, MA) according to the following conditions in sequence: 95 °C for 5 min, an amplification, and a final extension at 72 °C for 10 min. The amplification profile was 1 min at 95 °C, 1 min at 60 °C, and 2 min at 72 °C.

Construction of Expression Vector for MTHase. The 1.7 kbp PCR-amplified fragment was purified and then digested with *Xho*I and *Bam*HI. The digested fragment was inserted into the pET-15b vector, resulting in a recombinant vector designated pET-15b-*treZ*. The amplified *treZ* gene was fused in frame with the His-tag coding sequence on pET-15b; therefore, the expressed recombinant MTHase possessed a His-tag on its N-terminal region. The sequences of the entire *treZ* gene and the fused His-tag coding region on pET-15b-*treZ* were confirmed by DNA sequencing. To express a wild-type MTHase—which contains no extra His-tag coding sequence derived from the cloning steps—the pET-15b-*treZ* was digested with *Nco*I and *Xho*I to remove the His-tag coding sequence, made blunt by mung bean nuclease and ligated, resulting in a recombinant clone designated pET-15b-ΔH-*treZ*. DNA sequencing was also performed to ascertain the removal of the His-tag coding sequence. All DNA automatic sequencings were carried out by Mission Biotech Corp. (Taipei, Taiwan).

Expression of MTHase by *E. coli*. The pET-15b-ΔH-*treZ* vector was transformed into *E. coli* BL21-CodonPlus (DE3)-RIL to express MTHase. One single colony from a newly transformed culture plate was inoculated into 10 mL of terrific broth (TB) medium supplemented with 100 μg/mL ampicillin plus 34 μg/mL chloramphenicol and grown at 37 °C until the OD_{600} reached around 0.6. Cells were collected by centrifugation and resuspended in 4 mL of fresh medium. A volume of 3 mL of the resuspended culture was then added to 600 mL of fresh medium containing 100 μg/mL of ampicillin plus 34 μg/mL chloramphenicol and grown until the OD_{600} reached around 0.6. The culture was then induced by an addition of IPTG to a final concentration of 0.5 mM. After a further culture at 20 °C for 16 h, the cells were then harvested by centrifugation and stored at −70 °C before further processing.

Preparation of Cellfree Extract. Frozen cells (6 g) expressing wild-type and His-tagged MTHases were suspended in 18 mL of lysis buffers A and B, respectively. Lysis buffer A contained 20 mM Tris-HCl (pH 8.0) and 1 mM benzamidine. Lysis buffer B contained 20 mM Tris-HCl (pH 7.9), 5 mM imidazole, 0.5 M NaCl, and 1 mM benzamidine. The suspended cells were disrupted using a French Press disruptor (Sim-Aminco, Rochester, NY) at 20000 psi (1360 atm). The cellfree extract was then prepared by removing the insoluble fractions from the supernatant of the above mixture by centrifugation at 10000*g* for 2 h.

Purification of Wild-Type Enzyme. Heat treatment was first used to precipitate most of the undesired proteins by incubating the cell-free extract in an 80 °C water bath for 1 h followed by centrifugation to remove the heat-labile proteins. A 10% (w/v) streptomycin sulfate stock solution was then added to a final concentration of 1% (w/v) to precipitate the nucleic acids. After centrifugation at 10000*g* for 1 h, the supernatant was dialyzed against 20 mM Tris-HCl buffer (pH 8.5) and was subsequently loaded onto a Q-Sepharose column (1.6 × 10 cm), which was pre-equilibrated with the same dialysis buffer. The column was first washed thoroughly with the same buffer until the absorbance of 260 nm reached baseline. Finally, a linear gradient of 0−0.4 M NaCl in the above buffer was used to elute the bound proteins. The eluted fractions containing enzyme activity were collected and dialyzed against a buffer containing 20 mM Tris-HCl (pH 8.0).

Purification of His-Tagged Enzyme. The cell-free extract was diluted with an equal volume of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) prior to being loaded onto a Novagen His·Bind column (1.6 × 5 cm), which was previously equilibrated with the same binding buffer. The column was first washed with the binding buffer, then washed with a wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), and finally eluted with an elute buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) according to the protocols provided in the Novagen His·Bind Kits. The eluted fractions containing enzyme activity were collected and dialyzed against a buffer containing 20 mM Tris-HCl (pH 8.0).

HPLC Analysis of the Molecular Masses of Wild-Type and His-Tagged Enzymes. The molecular masses of both wild-type and His-tagged MTHases were measured using a BioSep-SEC-S4000 column (300 × 7.8 mm), which is a silica-based gel filtration column. The mobile phase contained 50 mM sodium phosphate buffer (pH 6.8), and the flow rate was controlled at 0.5 mL/min. The analysis was carried out under a Hitachi HPLC L-7000 (Tokyo, Japan) equipped with an UV detector. The molecular masses of wild-type and His-tagged MTHases were determined by comparison with the calibration curve derived from the molecular mass standards.

Preparation of Maltooligosyltrehaloses. The preparation of maltosyltrehalose (G_2T), maltotriosyltrehalose (G_3T), maltotetraosyltrehalose (G_4T), and maltopentaosyltrehalose (G_5T) was according to the procedures described by Kato et al. (*17*). The MTSase reactions were

carried out at 75 °C for 2−4 h in 0.05 M citrate−phosphate buffer (pH 5) by using 100 mM G_4−G_7 as substrates. At the end of each MTSase reaction, a 0.1 N NaOH treatment was used to decompose the remaining reducing sugars, and then the reaction mixture was deionized by Amberlite IR-120 A and IRA-900 (Fluka, Buchs, Switzerland). The purified MTSase was prepared according to the method given in our previous study (*16*).

Enzyme Activity Assay. The MTHase activity was assayed at 60 °C for 10 min by using 2.8 mM G_4T as substrate in 0.05 M citrate−phosphate buffer (pH 5). One unit of MTHase activity was defined as the amount of enzyme required to produce 1 μmol of trehalose in 1 min. MTHase cleaves G_4T into equimolar amounts of trehalose and G_4, and because the latter oligosaccharide is the only reducing sugar in the mixture, the concentration of G_4, and hence the activity of the enzyme, can be determined by the DNS assay (*18*). In determining the effects of pH and temperature on the activity and stability of enzyme, the following buffers with a final concentration of 50 mM were used in different pH ranges: citrate−phosphate buffer (pH 3.5−7), Tris-HCl buffer (pH 7.5−8.5), and $NaHCO_3$−Na_2CO_3 buffer (pH 9.5−11). For determination of the optimal pH, the enzyme (0.311 μg/mL) was assayed at different pH values under the standard conditions. For determination of pH stability, the enzyme (3.11 μg/mL) was incubated at various pH values at 4 °C for 24 h, and the remaining activities were assayed under the standard conditions. For determination of the optimal temperature, the enzyme (0.311 μg/mL) was assayed at different temperatures under the standard conditions. For determination of thermostability, the enzyme (3.11 μg/mL) was incubated at various temperatures at pH 5 for 2 h, and the remaining activities were assayed under the standard conditions. For determination of the effects of metal ions on the activity, the enzyme (0.311 μg/mL) was assayed at 60 °C and pH 5 as in the standard conditions except that the buffers contained EDTA or a metal ion at a final concentration of either 0.4 or 4 mM.

HPLC Analysis of the Hydrolysis Products of MTHase Using Maltooligosaccharides as Substrates. The hydrolysis products of MTHase using maltooligosaccharides as substrates were measured by a Hitachi HPLC L-7000 equipped with a refractive index detector. The enzyme reactions were carried out at 60 °C for 1.5−36 h in 0.05 M citrate−phosphate buffer (pH 5) by using 14 mM G_2−G_7 as substrates. The reactions were stopped by incubating the mixture in boiling water for 10 min, and then the mixtures were filtered through the Microcon centrifugal filter unit with a YM-3 membrane (MWCO 3000) to remove the enzyme. The filtrates were analyzed by a Vercopak Nucleosil 5 μm NH_2 column (4.6 × 250 mm) with a mobile phase consisting of 30% (v/v) H_2O and 70% (v/v) acetonitrile.

Mass Spectrometric Analysis. The mass spectrometric analysis was performed on a MicroMass (Manchester, U.K.) Quattro-BIOQ mass spectrometer as previously described (*19*).

Protein Concentration Measurement. Protein concentration was determined according to Bradford's method (*20*) with BSA as standard.

Measurement of Glucose Formation from the Hydrolysis of Maltooligosaccharides. The substrates, G_4−G_7 (14 mM), were incubated with MTHase in 50 mM citrate−phosphate buffer at pH 5 and 60 °C for 10 min. The reactions were stopped by adding 0.4 volume of 4 M Tris-HCl buffer at pH 8.0 followed by incubation of the mixtures in boiling water for 10 min. The glucose that was released from the hydrolysis of the substrates was measured according to the glucose oxidase method (*21*).

Enzyme Kinetics. The initial rates of hydrolysis of G_2T−G_5T and G_4−G_7 were determined at 60 °C in 50 mM citrate−phosphate buffer at pH 5 by using 8−10 substrate concentrations ranging from 2 to 50 mM. Samples taken at five different time intervals were stopped by adding DNS or 4 M Tris-HCl buffer (pH 8.0). In the hydrolysis of G_2T−G_5T, the molar concentration of the released trehalose was equal to that of the released G_2−G_5 measured by the DNS method (*18*) as described in the method of enzyme activity assay. In the hydrolysis of G_4−G_7, the released glucose was measured by the glucose oxidase method (*21*). Values of k_{cat} and K_M were calculated by fitting the initial rates as a function of substrate concentration to the Michaelis−Menten equation using Enzfitter software (Elsevier-Biosoft).

Table 1. Comparison of the Deduced Amino Acid Sequences of MTHase from *S. solfataricus* ATCC 35092 with Those of Other MTHases from the *Sulfolobus* Genus

source	accession no.[a]	no. of residues	identity[b] (%)
S. solfataricus ATCC 35092	P95867	561	100
S. solfataricus MT4	none[c]	561	99.4
S. solfataricus KM1	Q55088	559	79.9
S. shibatae B12	Q9UWN9	559	78.5
S. acidocaldarius ATCC 33909	Q53641	556	60.0

[a] The deduced amino acid sequences were obtained from the TrEMBL protein sequence database. [b] The identity values were analyzed by SIM-Local similarity program on the website http://tw.expasy.org/tools/sim-prot.htmL (*22*). [c] The deduced amino acid sequence of MTHase from *S. solfataricus* MT4 was from de Pascale et al. (*23*).

RESULTS AND DISCUSSION

Gene Analysis and Comparison of Amino Acid Sequences of Several MTHases from the *Sulfolobus* Genus. MTHase from *S. solfataricus* ATCC 35092 possesses 561 amino acids and a calculated molecular massof 64371 Da, which was deduced from the nucleotide sequence of the *treZ* gene and analyzed by using the Compute pI/Mw tool at http://tw.expasy.ch/tools/pi_tool.htmL (*22*), respectively. MTHase from *S. solfataricus* ATCC 35092 shares a >75% identity with other MTHases from the *Sulfolobus* genus except *S. acidocaldarius* (**Table 1**). In addition, a 99.4% identity of amino sequences existed between MTHase from *S. solfataricus* ATCC 35092 and that from *S. solfataricus* MT4 (*23*) with the following residues indicating the only changes: [G4A; L7I; C461Y].

By the information from CAZy, an Internet resource on glycosyl hydrolases at http://afmb.cnrs-mrs.fr/CAZY/, these MTHases have been previously classified in family 13 of glycosyl hydrolases on the basis of their amino acid sequence similarities according to the classification developed by B. Henrissat (*24*−*26*). Families 13, 70, and 77 of glycosyl hydrolases constitute Clan GH-H, which contains functionally and structurally related enzymes (*27*). Many enzymes from Clan GH-H acting on starch, such as α-amylase, pullulanase, isoamylase, and cyclodextrin glucanotransferase, are known as the α-amylase family of enzymes (*27*). There are four highly conserved regions of amino acid sequences in the α-amylase family of enzymes (*27*). Except for one residue, [Ser257] of MTHase from *S. shibatae* B12, and three residues, [Leu250; Phe280; Phe371] of MTHase from *S. acidocaldarius*, the amino acid sequences of the four highly conserved regions in these MTHases are identical. The four conserved regions also contain three essential amino acid residues, Asp252, Glu283, and Asp377 (numberings according to the enzyme from *S. solfataricus* KM1), which play a catalytic role in the active site of the α-amylase family (*27*, *28*). The three-dimensional structures of MTHases from *S. solfataricus* KM1 and *Deinococcus radiodurans* also suggested that residues Asp252, Glu283, and Asp377 are catalytic residues (*4*, *29*). Asp252 acts as the catalytic nucleophile/base, and Glu283 is the catalytic proton donor (*4*, *29*). Asp377 is not directly involved in the catalysis but has been proposed to stabilize the transition state and to maintain Glu283 in the correct protonation state (*4*).

Expression and Purification of MTHase. The protein expression levels of both wild-type and His-tagged MTHases were higher in the absence of IPTG than in the presence of 0.5 mM IPTG induction, and these expression levels of wild-type were higher than those of His-tagged MTHases under the same induction conditions. In the absence of IPTG induction, the

Table 2. Summary of Purification Steps of Wild-Type and His-Tagged MTHases Expressed in *E. coli* BL21-CodonPlus (DE3)-RIL

enzyme	purification step	total protein (mg)	total activity (units)	activity recovery (%)	specific activity (units/mg)	purification fold
wild-type	crude extract	1180	79600	100	67.5	1
	heat treatment[a]	145	54100	67.9	373	5.53
	Q Sepharose	25.0	20300	25.4	812	12.0
His-tagged	crude extract	924	19500	100	21.1	1
	His•Bind	41.0	12700	65.3	310	14.7

[a] The crude extract of wild-type enzyme was heated at 80 °C for 1 h and then centrifuged to remove the precipitates.

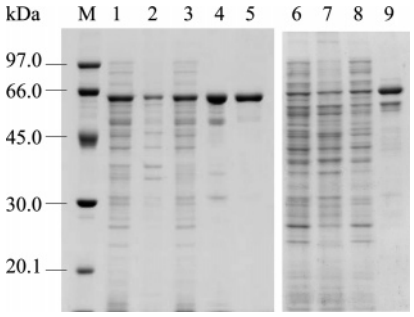

Figure 1. SDS-PAGE analysis of purifications of wild-type and His-tagged MTHases in *E. coli* BL21-CodonPlus (DE3)-RIL carrying the pET-15b-ΔH-*treZ* and pET-15b-*treZ*, respectively: (lane M) molecular mass standards; (lanes 1 and 6) total cellular proteins of the *E. coli* cells carrying the pET-15b-ΔH-*treZ* and pET-15b-*treZ,* respectively; (lanes 2 and 7) insoluble proteins for the *E. coli* cells carrying the pET-15b-ΔH-*treZ* and pET-15b-*treZ,* respectively, after cell lysis; (lanes 3 and 8) crude cell-free extracts of wild-type and His-tagged MTHases; (lane 4) partially purified fraction of wild-type MTHase after heat treatment; (lane 5) purified fraction of wild-type MTHase after ion-exchange chromatography; (lane 9) partially purified fraction of His-tagged MTHase after metal-affinity chromatography.

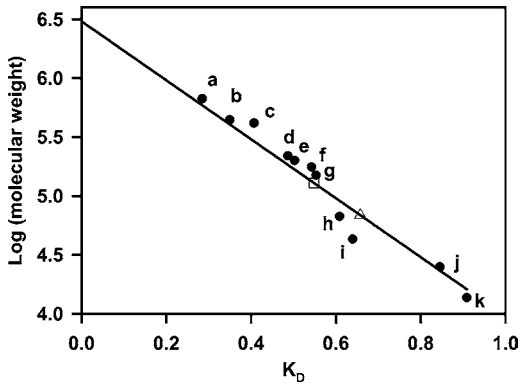

Figure 2. Determination of the molecular masses of wild-type and His-tagged MTHases by HPLC analysis. The protein standards (●) included a, thyroglobin (669 kDa); b, apoferritin (443 kDa); c, ferritin (416 kDa); d, catalase (219 kDa); e, β-amylase (200 kDa); f, adolase (176 kDa); g, alcohol dehydrogenase (150 kDa); h, albumin (67 kDa); i, ovalumin (43 kDa); j, chymotrypsinogen A (25 kDa); and k, ribonuclease A (13.7 kDa). Wild-type (□) and His-tagged (△) MTHases were plotted with the protein standards. The K_D values were calculated using the equation $K_D = (V_e - V_0)/(V_T - V_0)$, where V_e is the elution volume and V_T and V_0 represent the total liquid volume and the void volume of the column, respectively.

yields of the active wild-type and His-tagged MTHases expressed in *E. coli* BL21-CodonPlus (DE3)-RIL were 13300 and 3250 units/g of wet cells, respectively. The yield of 13300 units/g of wet cells was much higher than the previously reported yields of 2822 and 726 units/g of wet cells for the *E. coli* JM109 and Rb-791 expressing *S. solfataricus* KM1 and MT4 enzymes, respectively (*11*). However, in the presence of IPTG induction, the yields of the active wild-type and His-tagged MTHases expressed in *E. coli* were only 3230 and 2280 units/g of wet cells, respectively. The expression level of target protein can be affected by many factors, such as the gene toxicity, proteolytic degradation of target protein, secondary structure in the mRNA transcript, and excessive rare codon usage (*30*, *31*). In general, IPTG is used to inactivate lac repressor and to induce the overexpression of target gene. In the absence of IPTG, the expression of the target gene is not induced and under its basal expression level due to the gene leakage. The expression of MTHase from *Sulfolobus solfataricus* MT4 studied by de Pascale et al. also had shown that the highest level of protein expression was obtained in the absence of IPTG induction (*23*). Because the gene of MTHase contains many rare codons, such as AGG and AGA, we suspect that the overexpression of MTHase gene induced by IPTG may conversely inhibit the machinery of protein synthesis.

The expressed wild-type and His-tagged MTHases were mostly soluble and constituted about 80 and 60%, respectively, of the total expressed MTHases that appeared in the cell-free extract (**Figure 1**, lanes 1−3 and 6−8). Wild-type MTHase was purified from the cell-free extract of *E. coli* sequentially by heat treatment, nucleic acid precipitation, and ion-exchange chromatography, whereas His-tagged MTHase was purified from the cell-free extract directly by metal chelating chromatography (**Table 2**). The purified wild-type MTHase showed a single band on SDS-PAGE, indicating that a high purity of protein was obtained (**Figure 1**, lane 5). However, the His-tagged enzyme was only partially purified after metal chelating chromatography (**Figure 1**, lane 9). The apparent molecular masses of wild-type and His-tagged MTHases estimated under denaturing conditions by SDS-PAGE were around 64 and 66 kDa, respectively (**Figure 1**), which are in good agreement with the calculated molecular masses. Because there are 23 amino acid residues inherited from the His-tag fragment, the molecular mass of the His-tagged enzyme is about 2.5 kDa larger than that of wild-type enzyme. The molecular masses of the purified wild-type and the partially purified His-tagged MTHases under non-denaturing conditions were then analyzed by HPLC using the BioSep-SEC-S4000 gel filtration column. The purified wild-type MTHase had a peak at 18.99 min, whereas His-tagged MTHase appeared as a peak at 20.33 min (data not shown). By comparison with the calibration curve derived from the molecular mass standards, the molecular mass of wild-type MTHase under non-denaturing conditions was around 128.8 kDa and that of His-tagged MTHase around 68.7 kDa (**Figure 2**). Because the theoretical molecular masses of one subunit for wild-type and His-tagged MTHases are around 64.4 and 66.9 kDa, the estimated 128.8 and 68.7 kDa corresponded to 2 and 1.03 subunits, respectively. These results suggest that the purified wild-type MTHase is a homodimer, whereas the purified His-tagged MTHase is a monomer. The three-dimensional structure

Table 3. Substrate Specificity of Wild-Type MTHase from *S. solfataricus* ATCC 35092[a]

DP of substrate	maltooligosyl-trehalose	trehalose formation rate [mol·(mol of MTHase^{-1})·s^{-1}]	maltooligo-saccharide	glucose formation rate [mol·(mol of MTHase^{-1})·s^{-1}]	ratio of G/T[b] (%)
4	G_2T	165 ± 10[c]	G_4	5.46 ± 0.47	3.31
5	G_3T	996 ± 55	G_5	12.3 ± 0.5	1.23
6	G_4T	1090 ± 80	G_6	12.9 ± 0.3	1.18
7	G_5T	1250 ± 30	G_7	6.49 ± 0.95	0.52

[a] G_2T–G_5T and G_4–G_7 were used as substrates to determine the trehalose and glucose formation rates, respectively. The substrate (14 mM) was incubated with the enzyme (0.311 μg/mL for G_2T–G_5T and 3.11 μg/mL for G_4-G_7) at 60 °C, pH 5. [b] Ratio of glucose to trehalose formation. [c] Mean ± SD from triplicate experiments.

of MTHase from *S. solfataricus* KM1 had also revealed that the enzyme is a homodimer covalently linked by an intermolecular disulfide bond at residue C298 (*29*), which corresponds to C301 of MTHase from *S. solfataricus* ATCC 35092. Several previous studies suggested that a His-tag at the N or C terminus of protein affected the oligomerization state of protein (*32*−*34*). Because the His-tagged MTHase contains 23 additional amino acid residues in its N terminus, this extra fragment probably does not contain any secondary element (*32*) and hangs around residue C324, which corresponds to C301 of wild-type MTHase; it may, therefore, interfere with the formation of the intermolecular disulfide bond at residue C324.

Originally, the His-tag enzyme was designed for purification directly from metal chelating chromatography to obtain apparent homogeneity by taking advantage of the His-tag. However, the His-tagged enzyme could be only partially purified by metal chelating chromatography. Because the protein expression level and active enzyme yield of His-tagged MTHase were less than those of wild-type MTHase and because the His-tagged enzyme was in a monomer form, which is different from the homodimer form of wild-type enzyme, we did not conduct further purification steps to obtain apparent homogeneity after metal chelating chromatography, and further studies on only the wild-type MTHase were carried out.

The molecular mass of the purified wild-type MTHase was also analyzed by electrospray ionization mass spectrometry and, after treatment with 50 mM DTT, it showed a molecular mass of 64.24 kDa. These data are consistent with the theoretical molecular mass of one subunit of the MTHase after the cleavage of the N-terminal methionine. These observations suggested that the MTHase from *S. solfataricus* ATCC 35092 might be also a homodimer covalently linked by an intermolecular disulfide bond similar to the MTHase from *S. solfataricus* KM1.

Effects of pH and Temperature on the Activity and Stability of Wild-Type MTHase. The recombinant enzyme showed an optimal activity at pH 5.0−5.5 and remained stable in the pH range from 3.5 to 11 (**Figure 3a**). The enzyme had an optimal activity at 85 °C and retained virtually all of its activity at that temperature for 2 h (**Figure 3b**). The good thermostability of this enzyme indicated that the purified recombinant MTHase was well folded and should have the same structure as that produced directly from *S. solfataricus* ATCC 35092.

Effects of Metal Ions on the Activity of Wild-Type MTHase. The addition of EDTA, Na^+, K^+, Mg^{2+}, Ca^{2+}, and Zn^{2+} had no activation or inhibition effect on MTHase activity. Adding 0.4 and 4 mM Hg^{2+} strongly inhibited MTHase, whereas Cu^{2+} inhibited it only at 4 mM. These results indicated that these metal ions were not required for MTHase activity. Although the catalytic domain of the α-amylase family of enzymes generally contains one conserved calcium ion to stabilize the interface of domains A and B, there is no metal ion bound to the domain interface in the three-dimensional structure of MTHase from *S. solfataricus* KM1 (*29*).

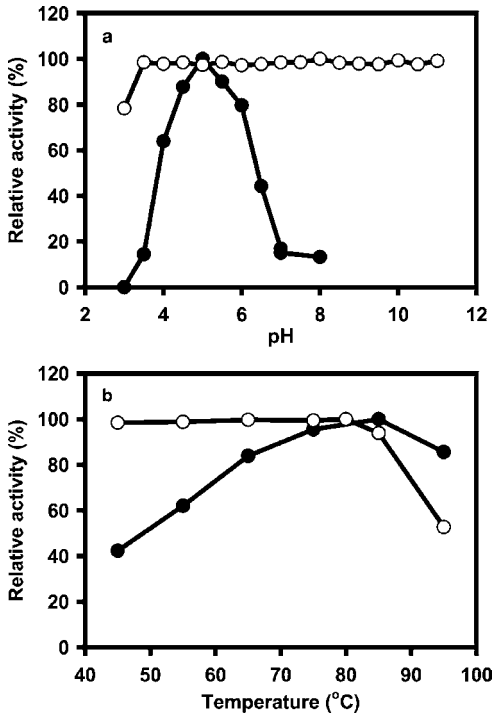

Figure 3. Effects of pH and temperature on the activity (●) and stability (○) of wild-type MTHase: (**a**) effects of pH; (**b**) effects of temperature.

Substrate Specificity. Maltooligosyltrehaloses and maltooligosaccharides of various DPs were used as substrates to study the substrate specificity of MTHase because both types of oligosaccharides could occupy the active site of MTHase for enzymatic reactions. MTHase converts maltooligosyltrehaloses to trehalose and a maltooligosaccharide with lower DP; for example, G_5T is converted to trehalose and G_5. The side reaction of this maltooligosaccharide (G_n) with MTHase would then produce glucose and a maltooligosaccharide (G_{n-1}). As shown (**Table 3**) the trehalose formation rate increased as the DP value of the substrate increased, and G_5T was the most preferred substrate tested in this study for trehalose formation. However, for glucose formation, G_6 exhibited the highest formation rate. The ratios of glucose formation to trehalose formation, which represented the ratios of the side reaction to the major reaction of MTHase, were 3.31, 1.23, 1.18, and 0.52% for DP 4−7 substrates, respectively. These results suggested that the hydrolysis reaction toward the low DP maltooligosaccharides might be one of the major reasons that caused the decreased yield of trehalose production from starch.

HPLC Analysis of the Hydrolysis Products of Wild-Type MTHase Using Maltooligosaccharides as Substrates. To further examine the product profiles of the side reaction of MTHase, the enzyme reactions were carried out at 60 °C for 0−36 h by using G_2−G_7 as substrates. **Figure 4** shows the ln(residual concentration of substrate) versus time. The residual

Table 4. Comparison of Kinetic Parameters of MTHases from *S. solfataricus* ATCC 35092, *S. solfataricus* KM1, and *S. acidocaldarius* ATCC 33909 for Hydrolysis of G_2T–G_5T

	recombinant			natural					
	S. solfataricus ATCC 35092[a]			*S. solfataricus* KM1[b]			*S. acidocaldarius* ATCC 33909[c]		
substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1}•mM^{-1})	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1}•mM^{-1})	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1}•mM^{-1})
G_2T	193 ± 7[d]	11.1 ± 0.1	17.4 ± 0.5	339	22.4	15.1	NR[e]	16.7	NR
G_3T	1030 ± 30	7.22 ± 0.48	143 ± 6	747	5.8	129	NR	2.7	NR
G_4T	1190 ± 30	5.66 ± 0.37	210 ± 8	866	5.6	155	NR	3.7	NR
G_5T	1230 ± 40	5.89 ± 0.45	198 ± 9	756	4.7	161	NR	4.9	NR

[a] This study. Experiments were carried out at 60 °C and pH 5. The enzyme concentration was 0.311 μg/mL. [b] Kato et al. (*30*). Experiments were carried out at 60 °C and pH 5.5. [c] Nakada et al. (*31*). Experiments were carried out at 60 °C and pH 6. [d] Standard error from the curve fitting. [e] Not recorded.

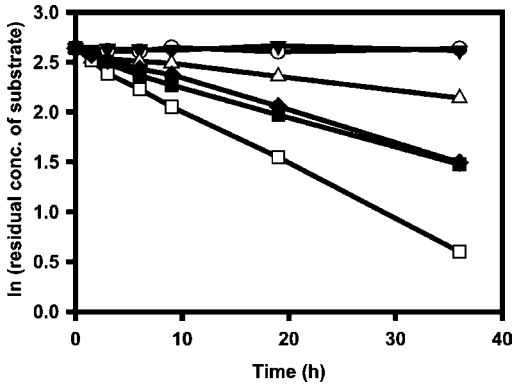

Figure 4. Residual concentrations of substrates after hydrolysis by wild-type MTHase. After hydrolysis of 14 mM DP 2–7 maltooligosaccharides by wild-type MTHase (0.311 μg/mL) at 60 °C, pH 5, the residual concentrations of substrates were determined from the HPLC analysis: (○) G_2; (▼) G_3; (△) G_4; (■) G_5; (□) G_6; (◆) G_7.

Table 5. Kinetic Parameters of Wild-Type MTHase from *S. solfataricus* ATCC 35092 for Glucose Formation in Hydrolysis of G_4–G_7 at 60 °C and pH 5[a]

substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1}•mM^{-1})
G_4	5.49 ± 0.20	10.9 ± 0.9	0.50 ± 0.20
G_5	17.7 ± 0.5	5.86 ± 0.40	3.02 ± 0.13
G_6	18.2 ± 0.7	5.63 ± 0.51	3.24 ± 0.22
G_7	6.01 ± 0.13	2.63 ± 0.20	2.29 ± 0.13

[a] The enzyme concentration was 3.11 μg/mL. [b] Standard error from the curve fitting.

concentrations of G_2 and G_3 stayed almost unchanged, suggesting that G_2 and G_3 were poorly hydrolyzed. On the other hand, G_6 was hydrolyzed most quickly among the substrates because the ln(residual concentration) of G_6 had the steepest decline with the reaction time. These data were consistent with the previous observations of maltooligosaccharide hydrolysis that was indicated by the glucose formation rate as shown in **Table 3**. **Figure 5** shows the formations of hydrolysis products of MTHase using G_4−G_7 as substrates. When G_4 was hydrolyzed, the same concentrations of G_1 and G_3 were produced, whereas no G_2 was detected, indicating that G_4 was not hydrolyzed to maltose. When G_5 was hydrolyzed, the concentrations of G_1 and G_2 were nearly the same as those of G_4 and G_3, respectively, and the concentrations of G_1 and G_4 were higher than those of G_2 and G_3 at each sampling time. These data suggest that G_5 was hydrolyzed more likely to glucose than to maltose. The G_1 formation rate was the highest, whereas the G_2 formation rate was approaching the level of G_1 formation rate as the DP of substrate increased, indicating that the substrate of higher DP tended to be hydrolyzed to maltose. In addition to the hydrolysis

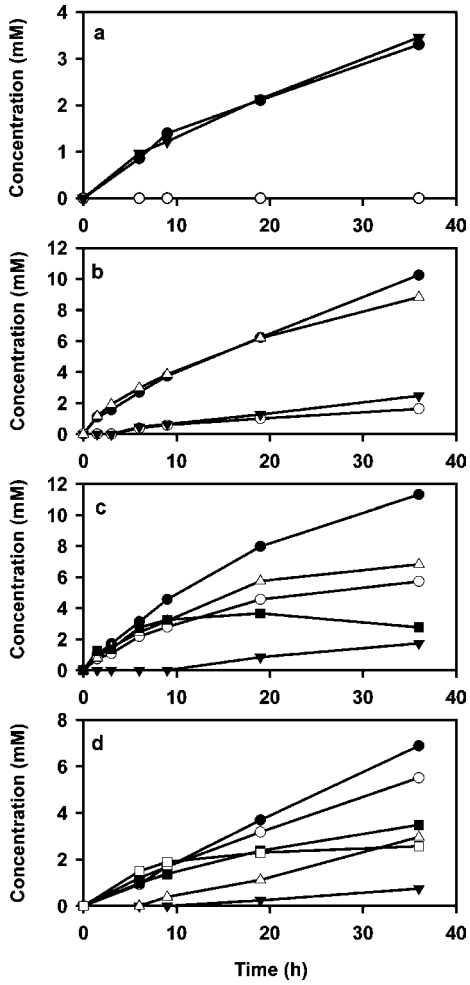

Figure 5. Hydrolysis products of wild-type MTHase using DP 4–7 maltooligosaccharides as substrates: (**a**–**d**) hydrolysis products of wild-type MTHase (0.311 μg/mL) using 14 mM G_4, G_5, G_6, or G_7, respectively, as substrate at 60 °C, pH 5. The concentrations of hydrolysis products were determined from the HPLC analysis: (●) G_1; (○) G_2; (▼) G_3; (△) G_4; (■) G_5; (□) G_6.

activity toward maltooligosyltrehaloses, an earlier study indicated that MTHase from *S. solfataricus* KM1 could also hydrolyze maltooligosaccharides from the reducing end to release either glucose or maltose (*35*). The above observations suggest that MTHase from *S. solfataricus* ATCC 35092 could also hydrolyze maltooligosaccharides to release glucose or maltose, similar to the properties of MTHase from *S. solfataricus* KM1.

Enzyme Kinetics. Hydrolysis of G_2T−G_5T by wild-type MTHase from *S. solfataricus* ATCC 35092 was carried out at 60 °C and pH 5. A comparison of kinetic parameters (k_{cat} and

Table 6. Comparison of Some Enzymatic Properties of MTHases from *Sulfolobus* Genus (Strains *S. solfataricus* ATCC 35092, *S. solfataricus* MT4, *S. solfataricus* KM1, *S. acidocaldariu*s ATCC 33909, and *S. shibata*e DSM 5389)

	recombinant		natural		
	ATCC 35092[a]	MT4[b]	KM1[c]	ATCC 33909[d]	DSM 5389[e]
optimum temperature (°C)	85	85	70–85	75	85
optimum pH	5	5	4.5–5.5	5.5–6	4.5
thermostability[f]	80 °C, 2 h (100%) 85 °C, 2 h (∼94%)	75 °C, 2 h (100%) 85 °C, 2 h (∼80%)	85 °C, 6 h (100%)	80 °C, 1 h (100%) 85 °C, 1 h (∼93%)	75 °C, 2 h (100%) 85 °C, 2 h (∼78%)
pH stability	3.5–11	4–10.5	3.5–10	5.5–9.5	4.5–9.5
metal ion inhibition	Cu^{2+}, Hg^{2+}	NR[g]	Cu^{2+}	Cu^{2+}, Hg^{2+}	NR
Ca^{2+} activation	no	NR	no	no	NR
side-hydrolysis reaction[h]	yes	NR	yes	yes	NR

[a] This study. [b] de Pascale et al. (*23*). [c] Kato et al. (*30*). [d] Nakada et al. (*31*). [e] Di Lernia et al. (*9*). [f] The residual activities are shown in parentheses. [g] Not recorded in the published literature. [h] The hydrolysis reaction toward maltooligosaccharides.

K_M) of MTHases from different strains of *S. solfataricus* is given in **Table 4**. In general, the k_{cat} values of these MTHases increased, whereas the K_M values decreased, as the DP of substrates increased—a phenomenon observed for most amylolytic enzymes. Nevertheless, kinetic parameters of these three MTHases are very different. The MTHase from *S. solfataricus* ATCC 35092 has higher k_{cat} values for hydrolysis of G_3T-G_5T compared to those of the enzyme from *S. solfataricus* KM1 and marginally higher K_M values for the hydrolysis of G_3T-G_5T by those enzymes from *S. solfataricus* KM1 and *S. acidocaldarius* ATCC 33909. The catalytic efficiencies (k_{cat}/K_M) of MTHase from *S. solfataricus* ATCC 35092 are 111−135% of those of the enzyme from *S. solfataricus* KM1. These higher catalytic efficiencies suggest the MTHase from *S. solfataricus* ATCC 35092 can be a more efficient enzyme that could be used in trehalose production from starch.

Kinetic parameters for glucose formation in hydrolysis of G_4-G_7 by wild-type MTHase from *S. solfataricus* ATCC 35092 at 60 °C and pH 5 are given in **Table 5**. In the hydrolysis of G_4-G_7, the K_M values of wild-type MTHase decrease as the DP of substrates increase, whereas the k_{cat} values of MTHase increase as the DP of substrates increase except for hydrolysis of G_7. The K_M values for hydrolysis of maltooligosaccharides are similar to those for hydrolysis of maltooligosyltrehaloses at the same DP except for G_7. However, the k_{cat} and k_{cat}/K_M values for hydrolysis of maltooligosaccharides are only 0.5−2.8 and 1.2−2.9% of those of maltooligosyltrehaloses at the same DP. These results suggest that both maltooligosyltrehaloses and maltooligosaccharides can access the binding site of MTHase, but the latter has comparably lower hydrolysis rates. Previous studies reported similar observations of the hydrolysis of maltooligosaccharides by the MTHases from *S. solfataricus* KM1 and *S. acidocaldarius* ATCC33909 (*35*, *36*); however, the kinetic parameters were not determined in those studies.

A further comparison of some enzymatic properties of MTHases from the *Sulfolobus* genus is given in **Table 6**. Although the kinetic parameters of these MTHase were different from those shown (**Table 4**), these MTHases from different sources possess similar apparent properties, such as optimal temperature, optimal pH, and thermostability.

Conclusion. In this study the MTHase from *S. solfataricus* ATCC 35092 was cloned and expressed in *E. coli* with a high yield of active enzyme. We characterized the enzymatic properties of this recombinant enzyme and compared these properties with those of other MTHases from the *Sulfolobus* genus. Except kinetic parameters, the wild-type MTHase that we cloned and expressed has properties very similar to those of other natural or recombinant MTHases from the *Sulfolobus* genus. The results from this study also suggest that this MTHase can be used to produce trehalose at high temperatures.

ABBREVIATIONS USED

MTSase, maltooligosyltrehalose synthase; MTHase, maltooligosyltrehalose trehalohydrolase; G_1, glucose; G_2, maltose; G_3, maltotriose; G_4, maltotetraose; G_5, maltopentaose; G_6, maltohexaose; G_7, maltoheptaose; DNS, 3,5-dinitrosalicylic acid; BSA, bovine serum albumin; IPTG, isopropyl-β-D-thiogalactoside; DP, degree of polymerization; G_2T, maltosyltrehalose; G_3T, maltotriosyltrehalose; G_4T, maltotetraosyltrehalose; G_5T, maltopentaosyltrehalose.

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