

## Bifunctional Recombinant Fusion Enzyme Between Maltooligosyltrehalose Synthase and Maltooligosyltrehalose Trehalohydrolase of Thermophilic Microorganism *Metallosphaera hakonensis*

Seo, Ju-Seok<sup>1</sup>, Ju Hee An<sup>2</sup>, Jong-Joo Cheong<sup>1</sup>, Yang Do Choi<sup>1</sup>, and Chung Ho Kim<sup>2\*</sup>

<sup>1</sup>Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Korea

<sup>2</sup>Department of Food and Nutrition, Seowon University, Cheongju 361-742, Korea

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**MhMTS and MhMTH are trehalose ( $\alpha$ -D-glucopyranosyl-[1,1]- $\alpha$ -D-glucopyranose) biosynthesis genes of the thermophilic microorganism *Metallosphaera hakonensis*, and encode a maltooligosyltrehalose synthase (MhMTS) and a maltooligosyltrehalose trehalohydrolase (MhMTH), respectively. In this study, the two genes were fused in-frame in a recombinant DNA, and expressed in *Escherichia coli* to produce a bifunctional fusion enzyme, MhMTSH. Similar to the two-step reactions with MhMTS and MhMTH, the fusion enzyme catalyzed the sequential reactions on maltopentaose, maltotriosyltrehalose formation, and following hydrolysis, producing trehalose and maltotriose. Optimum conditions for the fusion enzyme-catalyzed trehalose synthesis were around 70°C and pH 5.0–6.0. The MhMTSH fusion enzyme exhibited a high degree of thermostability, retaining 80% of the activity when pre-incubated at 70°C for 48 h. The stability was gradually abolished by incubating the fusion enzyme at above 80°C. The MhMTSH fusion enzyme was active on various sizes of maltooligosaccharides, extending its substrate specificity to soluble starch, the most abundant natural source of trehalose production.**

**Keywords:** Trehalose, maltooligosyltrehalose synthase, maltooligosyltrehalose trehalohydrolase, bifunctional fusion enzyme, *Metallosphaera hakonensis*

Trehalose,  $\alpha$ -D-glucopyranosyl-[1,1]- $\alpha$ -D-glucopyranose, is a nonreducing diglucoside ubiquitously distributed in various living organisms [2, 3]. This disaccharide has high water-holding activity, thereby maintaining the membrane fluidity under a variety of low-water conditions [4, 17, 18, 22]. Trehalose has been utilized in various industries as a preservative material for foods, cosmetics, and medicines

[14]. During the last decades, therefore, a simple and effective (low-cost) procedure for the commercial production of trehalose has been pursued. The trehalose biosynthetic pathway was initially identified in the studies with *E. coli* and yeast. In these microorganisms, trehalose-6-phosphate (T6P) synthase converts UDP-glucose and glucose 6-phosphate into T6P [2, 19, 21], and T6P phosphatase further dephosphorylates the T6P intermediate to trehalose [5].

It has been reported that a group of thermophilic archaeobacteria possess amylolytic activity and produce trehalose from maltooligosaccharides. This trehalose biosynthesis is catalyzed by a set of thermostable enzymes; maltooligosyltrehalose synthase (MTS) [15] and maltooligosyltrehalose trehalohydrolase (MTH) [16]. MTS has glucosyltransferase activity and converts the first  $\alpha$ -1,4-glycosidic linkage at the reducing end into  $\alpha$ -1,1 linkage to produce a maltooligosyltrehalose. MTH hydrolyzes the second  $\alpha$ -1,4-glycosidic linkage of the intermediate, yielding one molecule of trehalose. The thermophilic archaeobacteria *Sulfolobus* [9–11] and *Metallosphaera* [20] produce trehalose using this enzymatic system. In addition, trehalose biosynthesis pathways of a number of mesophilic eubacteria, including *Arthrobacter* [12], *Rhizobium* [13], and *Brevibacterium* [8] have proven to be identical to this system. The thermostable enzymes, MTS and MTH, efficiently catalyze the reactions for a relatively long time at extremely high temperatures without being denatured. In addition, the thermostable system provides a way to protect the reaction system from undesirable microbial contaminations and achieve cost-curtailed in the trehalose production [1, 14]. An alternative way to lower the cost of trehalose production is to construct a bifunctional fusion enzyme. Fusion of multiple enzymes provides physical proximity between the catalytic sites of the individual enzymes, which carry out the sequential reactions more efficiently [6, 8, 19].

We cloned the trehalose biosynthesis genes, *MhMTS* and *MhMTH*, from the thermophilic microorganism *Metallosphaera*

\*Corresponding author

Phone: 82-43-299-8745; Fax: 82-43-299-8745;

E-mail: chkim@seowon.ac.kr

*hakonensis*, as reported previously [20]. In the present study, an artificial bifunctional fusion enzyme was produced through in-frame fusion of the two genes, and enzymatic properties of the recombinant fusion enzyme were characterized in detail.

## MATERIALS AND METHODS

### Bacterial Strains and Chemicals

The strain (JCM8857) of *Metallosphaera hakonensis* was obtained from the Japan Collection of Microorganisms (JCM), and grown on JCM165 medium at 70°C. The *E. coli* strains BL21(DE3)pLysS were obtained from Promega, and grown on LB medium with or without proper antibiotics. Solid medium for bacterial growth was prepared by adding 1.5% (w/v) Bacto-agar. Trehalose and various maltooligosaccharides were purchased from Sigma Chemical Co. and used as standards for the chromatographic analyses.

### Construction of *MhMTSH* Fusion Gene and Expression in *E. coli*

The expression vector for the fusion enzyme, pRBMhMTSH, was constructed through PCR using four synthetic primers. The open reading frame (ORF) of *MhMTS* was amplified through polymerase chain reaction (PCR) using the primer 1 (5'-CGGAAAGAATTC-ATGCTAGTCGCAACCTAT-3') containing the translation initiation codon (underlined) of *MhMTS* and the primer 2 (5'-GAATTTGCCCAAACATACCTTTCATCAAACC-3'). The ORF of *MhMTH* was amplified through PCR using the primer 3 (5'-GGTTTTGATGAAAGGTATGTTGGCGCAAATTC-3'), which is complementary to the primers 2 and 4 (5'-CGGAAAGAATTCTTAGGTTTCCTCAACCTC-3') containing the termination codon (underlined) of *MhMTH*. The two PCR products were mixed and subjected to PCR using the primers 1 and 4, and the amplified *MhMTSH* fusion gene was introduced into the pRSET-B (Invitrogen) to generate pRBMhMTSH.

The DNA construct pRBMhMTSH was transformed into *E. coli* BL21(DE3)pLysS, and transcription was induced by 1 mM IPTG at 21°C for 16 h. Protein samples were analyzed by discontinuous SDS-polyacrylamide gel electrophoresis [7].

### Carbohydrate Analyses

Oligosaccharides were analyzed with thin-layer chromatography (TLC) or high pH ion chromatography (HPIC). For TLC, 5 µl of reaction mixture was loaded on a Silica gel F<sub>254</sub> plate (Merk), and developed twice with a solvent mixture (n-butanol: ethanol: water=5:3:2). For visualization, 25% sulfuric acid was sprayed on the plate and charred. The visualized spots were quantified using a GS 700 imaging densitometer (BioRad). Alternatively, the reaction mixture was analyzed with a DX500 HPIC system (Dionex) equipped with a 4×250 mm Carbo-Pak PA1 column. The oligosaccharides loaded onto the column were eluted with a continuous sodium acetate gradient, from 0 to 250 mM in 150 mM sodium hydroxide solution, over 30 min at room temperature. The eluted oligosaccharide fractions were monitored with an ED40 potential amperometric detector (Dionex).

### Enzyme Assay

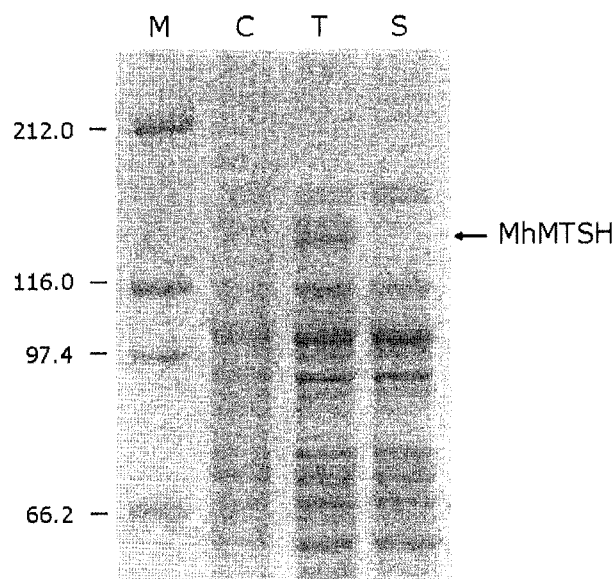
Trehalose synthesis activity was measured by incubating 5 µl of crude MhMTSH fusion enzyme preparation (soluble fraction) and

5 mM maltooligosaccharides in 100 µl of reaction mixture containing 100 mM sodium phosphate buffer (pH 5.0). In most cases, if not mentioned otherwise, the reaction was conducted at 70°C for 30 min. When soluble starch (1.0%) was used as a substrate, the reaction was carried out for 24 h. The reaction was terminated by heating the reaction mixture at 95°C for 5 min.

## RESULTS AND DISCUSSION

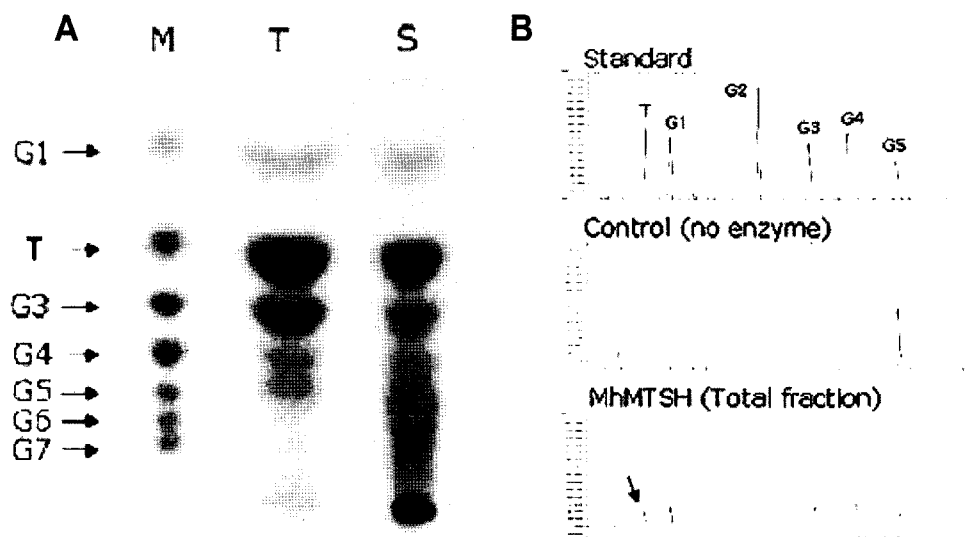
### Expression of Recombinant MhMTSH Fusion Enzyme in *E. coli*

An in-frame fusion gene between *MhMTS* and *MhMTH* was constructed through PCR using the two template genes and 4 synthetic primers containing the translation initiation codon of *MhMTS* and the termination codon of *MhMTH*, respectively. In this PCR reaction, an additional two primers complementary to each other at the 5'- and 3'-ends of the two genes, respectively, were mixed together to join the amplified DNA fragment representing the two genes. As a result, a DNA fragment containing a new ORF encoding a fusion protein MhMTSH, in which the last amino acid (Gly) of MhMTS was fused directly to the first amino acid (Met) of MhMTH without any linker, was generated. The DNA fragment was introduced into the bacterial expression vector, pRSET-B, to produce pRBMhMTSH. The DNA construct was transformed into *E. coli* and expressed by IPTG induction. As analyzed through discontinuous



**Fig. 1.** Expression of a recombinant MhMTSH fusion enzyme in *E. coli*.

The *MhMTSH* fusion gene was inserted into pRSET-B vector to produce pRBMhMTSH. Gene transcription was induced by IPTG and protein extracts were analyzed on SDS-PAGE. Lane M, molecular weight markers; Lane C, crude extract of *E. coli* harboring empty pRSET-B plasmid; Lane T, total protein extract of *E. coli* harboring pRBMhMTSH; Lane S, soluble fraction of protein extract of *E. coli* harboring pRBMhMTSH.



**Fig. 2.** Trehalose-producing activity of a MhMTSH fusion enzyme.

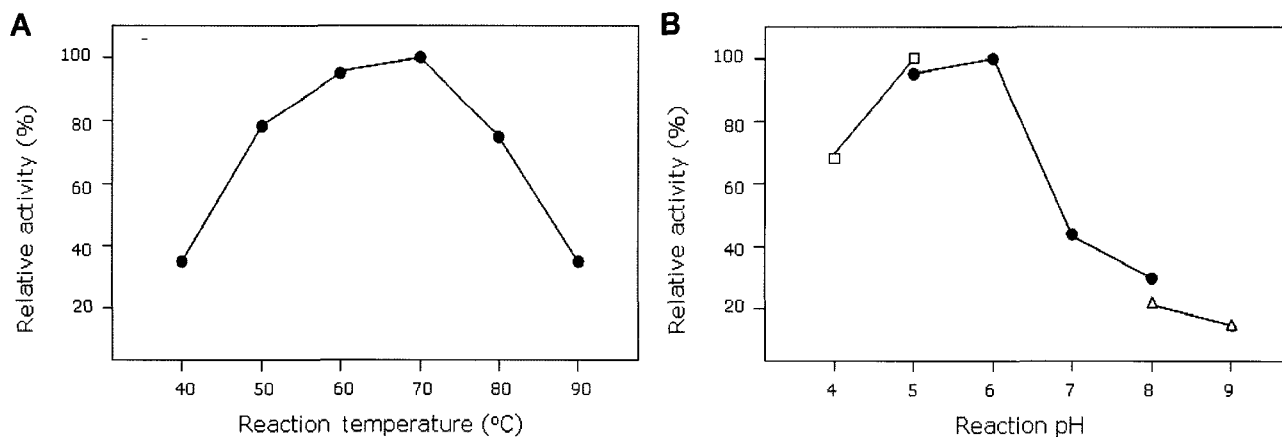
Recombinant MhMTSH fusion enzyme was incubated with 5 mM maltopentaose at 70°C for 30 min and analyzed by TLC or HPIC. **A.** TLC analysis of the reaction products. Lane M: mixture of standard maltooligosaccharides. G1, G3, G4, G5, G6, G7, and T denote glucose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, and trehalose, respectively; Lane T: reaction product obtained after incubation with the total protein extract of *E. coli* harboring pRBMhMTSH; Lane S: reaction product obtained after incubation with the soluble fraction of protein extract of *E. coli* harboring pRBMhMTSH. **B.** HPIC profile of the reaction products produced by the MhMTSH fusion enzyme. The arrow indicates the trehalose peak.

SDS-polyacrylamide gel electrophoresis, the estimated molecular mass of the recombinant fusion enzyme MhMTSH was approximately 150 kDa, including hexahistidine domain from the pRSET-B vector (Fig. 1). This value is in agreement with that predicted from the deduced amino acid sequence [20].

### Trehalose Synthesis by MhMTSH Fusion Enzyme

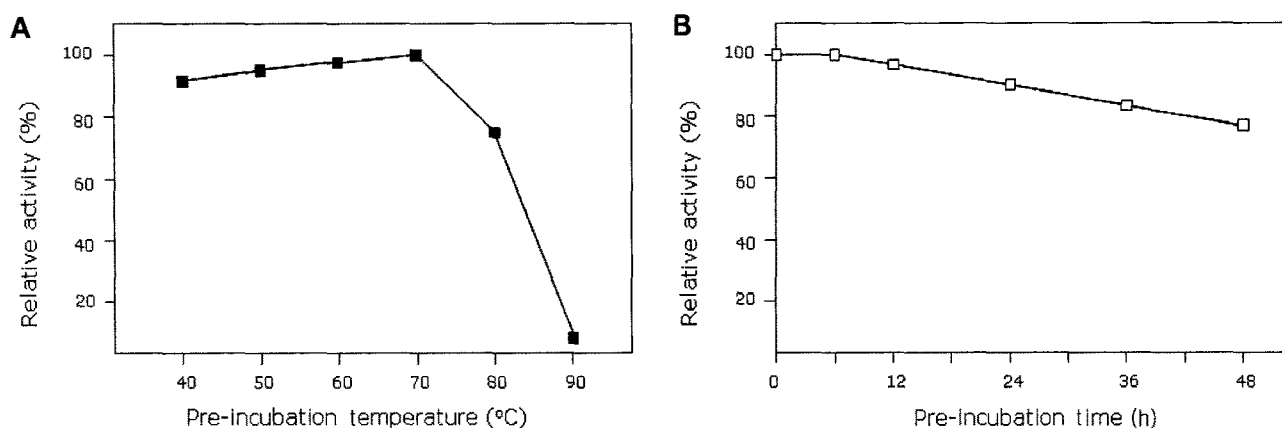
The enzymatic activity and substrate specificity of the fusion enzyme were examined. Crude MhMTSH fusion enzyme was incubated with maltopentaose ( $G_5$ ), and trehalose production was examined by TLC and HPIC. The crude

MhMTSH fusion enzyme converted maltopentaose ( $G_5$ ) to trehalose (T) and maltotriose ( $G_3$ ) (Fig. 2), as did the mixture of MhMTS and MhMTH [20]. This result demonstrated that the MhMTSH fusion enzyme is functional and catalyzes two sequential reactions; conversion of  $G_5$  to intermediate maltotriosyltrehalose ( $G_3T$ ) and then hydrolysis of the intermediate into trehalose and  $G_3$ . There are some amounts of G1 and G4 remaining in the reaction with  $G_5$  (Fig. 2). Related to this observation, it was reported that MTS from *S. acidocaldarius* and *M. hakonensis* exhibits some degree of hydrolytic activity [15, 16, 20]. Generation of G4 might not hamper the trehalose synthesis, since most of the G4



**Fig. 3.** Optimum conditions for the activity of MhMTSH fusion enzyme.

**A.** Effect of temperature on the trehalose production. MhMTSH fusion enzyme (5  $\mu$ l) was incubated with 5 mM of maltopentaose at various temperatures for 30 min. **B.** Effect of pH on the trehalose production. For the pH test, 0.1 M McIlvaine buffer solutions (pH 4–5), 0.1 M sodium phosphate buffer solutions (pH 5–8), and 0.1 M sodium bicarbonate buffer solutions (pH 8–9) were used. Reactions were conducted at 70°C for 30 min.



**Fig. 4.** Thermostability of MhMTSH fusion enzyme.

Thermal stability test was conducted by pre-incubating the MhMTSH fusion enzyme at various temperatures (40 through 90°C) followed by rapid cooling down on ice for 5 min, and determining the residual enzyme activity as described in Materials and Methods. **A.** Effect of pre-incubation on the trehalose production. **B.** Half-life of the enzyme mixture at 70°C.

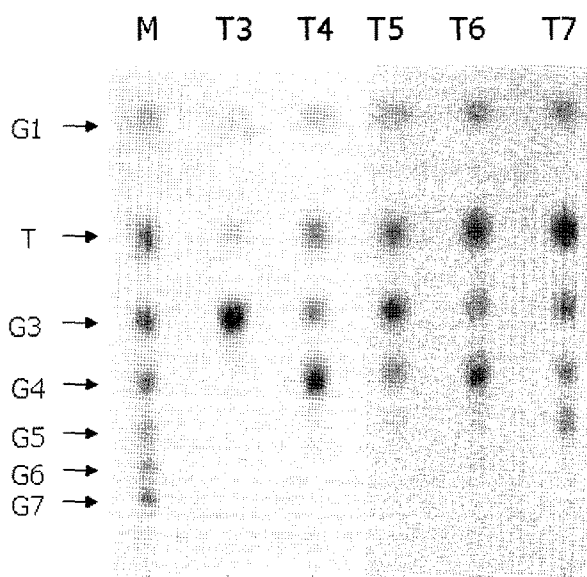
would eventually be converted to trehalose and maltose (G<sub>2</sub>), as demonstrated in the reaction with MhMTS and MhMTH of *M. hakonensis* [20].

#### Biochemical Properties of MhMTSH Fusion Enzyme

The effects of temperature and pH on the activity of the MhMTSH fusion enzyme were examined. When 5 mM of maltopentaose was incubated for 30 min with 5  $\mu$ l of crude MhMTSH fusion enzyme, maximal trehalose production was achieved at around 70°C (Fig. 3A) and pH 5.0–6.0 (Fig. 3B). Therefore, the MhMTSH fusion enzyme shows

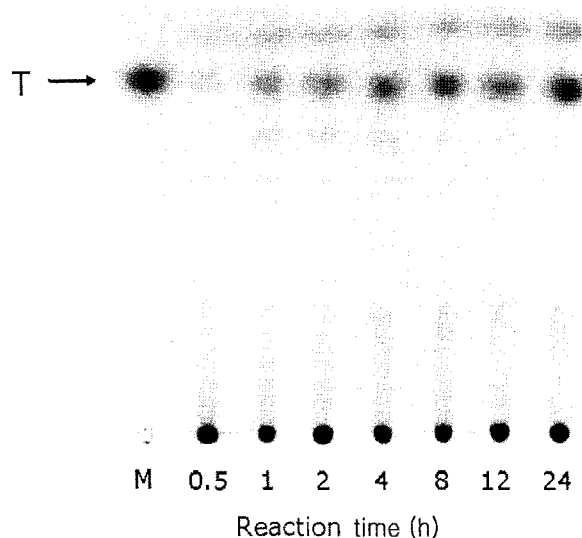
a lower optimum temperature than other thermophilic MTS and MTH [9, 15, 16]. This property could provide an advantage to reduce the cost of trehalose production for industrial application by reduction of the cost to keep a high temperature during the trehalose production reaction.

Thermal stability of the enzyme was determined by measuring residual enzyme activities after pre-incubating for 30 min at various temperatures between 40°C and 90°C. Trehalose-producing activity of the MhMTSH fusion enzyme was gradually abolished by incubating the mixture at above 80°C for 30 min (Fig. 4A). When the enzyme mixture was incubated at 70°C for up to 48 h, the residual



**Fig. 5.** Reaction activities of the MhMTSH fusion enzyme with various sizes of maltooligosaccharides.

MhMTSH fusion enzyme was reacted with 5 mM of various sizes of maltooligosaccharides and the reaction products were analyzed by TLC. Lane M, mixture of standard maltooligosaccharides. Lanes T3–T7, reaction product obtained after incubation with crude MhMTSH fusion enzyme and various sizes of maltooligosaccharides (G<sub>3</sub> to G<sub>7</sub>).



**Fig. 6.** Trehalose production from soluble starch.

MhMTSH fusion enzyme was reacted with 1% (w/v) soluble starch and the reaction products were analyzed by TLC. Lane M: maltooligosaccharide standard mixture. Lanes 0.5–24: reaction products obtained after incubation of the MhMTSH fusion enzyme with 1% (w/v) soluble starch for 0.5 to 24 h.

enzyme activity for the trehalose production was retained to be about 80% (Fig. 4B). Thus, the fusion enzyme catalyzed the trehalose-producing reaction for a long time without being denatured. In addition, undesirable contamination could be avoided by conducting the reaction at 70°C, which is an extremely high temperature for the survival of mesophilic microorganisms.

### Trehalose Production from Maltooligosaccharides and Starch

The enzyme activity of the MhMTSH fusion enzyme on diverse substrates was further examined by detecting the reaction products on a TLC plate (Fig. 5). The MhMTSH fusion enzyme was active on various sizes of maltooligosaccharides. Maltooligosaccharides were shortened by a unit of two glucose molecules per cycle of the reactions by releasing one trehalose molecule. Maltooligosaccharides having an odd number of glucose units were converted to trehalose, leaving residual maltotriose. In contrast, from maltooligosaccharides with an even number of glucose units, residual maltose remained. There was no difference in substrate specificity between the MhMTS/MhMTH mixture [20] and the MhMTSH fusion enzyme.

The crude MhMTSH fusion enzyme was incubated with 1.0% soluble starch, and the reaction was carried out for 24 h. Gradual conversion of soluble starch to disaccharides was observed as the reaction time increased up to 24 h (Fig. 6). Thus, the substrate specificity was extended to soluble starch, the most abundant maltodextrin in nature.

### Acknowledgments

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