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Molecular Cloning and Characterization of Trehalose Biosynthesis Genes from Hyperthermophilic Archaebacterium *Metallosphaera hakonesis*

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Abstract The trehalose (α-D-glucopyranosyl-[1,1]-α-D-glucopyranose) biosynthesis genes *MhMTS* and *MhMTH*, encoding a maltooligosyltrehalose synthase (MhMTS) and a maltooligosyltrehalose trehalohydrolase (MhMTH), respectively, have been cloned from the hyperthermophilic archaebacterium *Metallosphaera hakonesis*. The ORF of *MhMTS* is 2,142 bp long, and encodes 713 amino acid residues constituting a 83.8 kDa protein. *MhMTH* is 1,677 bp long, and encodes 558 amino acid residues constituting a 63.7 kDa protein. The deduced amino acid sequences of MhMTS and MhMTH contain four regions highly conserved for MTSs and three for MTHs that are known to constitute substrate-binding sites of starch-hydrolyzing enzymes. Recombinant proteins obtained by expressing the *MhMTS* and *MhMTH* genes in *E. coli* catalyzed a sequential reaction converting maltooligosaccharides to produce trehalose. Optimum pH of the MhMTS/MhMTH enzyme reaction was around 5.0 and optimum temperature was around 70°C. Trehalose-producing activity of the MhMTS/MhMTH was notably stable, retaining 80% of the activity after preincubation of the enzyme mixture at 70°C for 48 h, but was gradually abolished by incubating at above 85°C. Addition of thermostable 4-α-glucanotransferase increased the yield of trehalose production from maltopentaose by 10%. The substrate specificity of the MhMTS/MhMTH-catalyzed reaction was extended to soluble starch, the most abundant maltodextrin in nature.

Keywords: Trehalose, maltooligosyltrehalose synthase, maltooligosyltrehalose trehalohydrolase, *Metallosphaera hakonesis*, hyperthermophilic archaebacterium, 4-α-glucanotransferase

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Trehalose, α-D-glucopyranosyl-[1,1]-α-D-glucopyranose, is ubiquitous in various organisms including fungi, algae, bacteria, insects, yeasts, and plants [4, 5]. The nonreducing disaccharide serves as a carbohydrate reserve and a protective agent against a variety of environmental stresses such as drought, heat, and salinity [6, 24, 25, 30]. Trehalose has various industrial applications, being utilized as a preservative material for unstable foods, cosmetics, and medicines [21].

In *E. coli* and yeast, trehalose biosynthesis occurs via an enzymatic system composed of trehalose-6-phosphate (T6P) synthase and T6P phosphatase [5, 27, 28]. T6P synthase converts UDP-glucose and glucose-6-phosphate into T6P that is further dephosphorylated to trehalose by T6P phosphatase [8].

A group of thermophilic archaebacteria produces trehalose, using a thermostable enzyme system somewhat different from that of *E. coli*. For instance, thermophilic *Sulfolobus* possesses amylolytic activity and thereby synthesizes trehalose from maltooligosaccharides [15, 16, 18]. This trehalose biosynthesis is accomplished by a sequential reaction catalyzed by maltooligosyltrehalose synthase (MTS) [22] and maltooligosyltrehalose trehalohydrolase (MTH) [23]. MTS exerts a glucosyltransferase activity at the reducing end of maltooligosaccharides to convert the first α-1,4-glycosidic linkage into α-1,1 linkage, producing a maltooligosyltrehalose. MTH then hydrolyzes the second α-1,4-glycosidic linkage of the intermediate to release one molecule of trehalose. In addition to *Sulfolobus*, several mesophilic eubacteria including *Arthrobacter* [19], *Rhizobium* [20], and *Brevibacterium* [14] also produce trehalose via the same enzymatic system.

The enzymes from thermophiles carry out the trehalose biosynthesis reaction at extremely high temperature without being denatured. Even a small amount of the enzymes, therefore, will catalyze the reaction efficiently for a relatively

long time. Furthermore, reaction at high temperature could be a way to protect the reaction system from contamination by undesirable mesophilic microorganisms. Thus, the thermostable system has proven to be valuable for cost-curtailment, which permits practical application of trehalose in the food and cosmetics industries [3, 21].

In this study, the genes encoding trehalose-synthesizing enzymes were cloned from the hyperthermophilic archaebacterium *Metallosphaera hakonesis*. Enzymatic properties in trehalose biosynthesis of the corresponding recombinant proteins were characterized. In addition, using soluble starch as a substrate, we found that the product yield was significantly increased by addition of 4-α-glucanotransferase in the trehalose-synthesizing reaction.

MATERIALS AND METHODS

Bacterial Strains and Chemicals

Metallosphaera hakonesis (JCM 8857), *M. sedula*, *Sulfolobus solfataricus*, *S. brierleyi*, and *S. metallicus* were obtained from the Japan Collection of Microorganism (JCM). The bacteria were grown on JCM165 medium at 70°C. *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 made with 1.5% (w/v) Bacto-agar. Standard trehalose and maltooligosaccharides were purchased from Sigma Chemical Co.

Cloning of *MhMTS* and *MhMTH* Genes

DNA fragments containing the two genes (*MhMTS* and *MhMTH*) encoding MhMTS and MhMTH were cloned from *M. hakonesis*. Briefly, the bacterial genomic DNA was isolated, digested by either EcoRI or BglII, separated on a 1.0% agarose gel, and hybridized on a blot (GenScreen Plus membrane; Du Pont) with the MTS-MTH gene probe from *S. solfataricus* [15]. Under low stringency conditions, a 2.1-kb band from EcoRI digestion and a 5.5-kb band from BglII digestion were hybridized. The bands were isolated from the gel and inserted into pUC18 at the EcoRI and BglII sites, respectively. The translation initiation sites for the enzymes were determined by comparing the nucleotide sequences with those of MTS and MTH genes from other thermophilic bacteria.

Expression of Recombinant MhMTS and MhMTH Enzymes

The ORFs of *MhMTS* and *MhMTH* were amplified by the polymerase chain reaction (PCR) and introduced into a pRSET bacterial expression vector (Invitrogen). *MhMTS*-specific primers (with EcoRI restriction sites in boldface) were 5'-CGGAAA**GAATTC**ATGCTAGTCGCAACCTAT-3' and 5'-CGGAAA**GAATTC**TTAACCTTTCATCAAAAC-3' containing translation initiation and termination codons (underlined), respectively. *MhMTH*-specific primers were 5'-CGGAAA**GAATTC**ATGTTTGGCGCAAATTCA-3' and 5'-CGGAAA**GAATTC**TTAGGTTTCCTCAACCTC-3'. The PCR product was digested with EcoRI, and cloned into the pRSET-B vector to produce pRBMhMTS and pRBMhMTH.

The DNA constructs pRBMhMTS and pRBMhMTH were transformed into *E. coli* BL21(DE3)pLysS, respectively, and transcription was induced by 1 mM IPTG at 21°C for 16 h. Protein products were purified by Ni^{2+}-NTA-agarose affinity chromatography [1], following the manufacturer's instructions (Qiagen). Protein concentration was measured by the Bradford method [2], using bovine serum albumin as a standard.

Carbohydrate Analyses

Sugars were detected and quantified by performing thin-layer chromatography (TLC) or high pH ion chromatography (HPIC). For TLC, 5 μl of reaction mixtures was spotted on a Silica gel F_{254} plate (Merk), and developed twice in a solvent mixture (n-butanol:ethanol:water=5:3:2). Diluted sulfuric acid (25%) was sprayed on the plate and charred for visualization, and each spot was quantified with a GS 700 imaging densitometer (BioRad). HPIC was carried out using a 4×250 mm Carbo-Pak PA1 column on a DX500 HPIC system (Dionex) at room temperature. The sugar mixture was loaded onto the column and eluted with a continuous sodium acetate gradient, from 0 to 250 mM in 150 mM sodium hydroxide solution, over 30 min. The eluted sugar fractions were monitored with an ED40 potential amperometric detector (Dionex).

Enzyme Assay

Trehalose synthesis activity was measured by incubating 0.1 pmol of purified enzymes and 5 mM maltooligosaccharides in 100 μl of reaction mixture containing 100 mM sodium phosphate buffer (pH 5.0) at 70°C for 30 min. In the case where soluble starch (1.0%) was used as a substrate, the reaction was carried out for 24 h. The reaction was terminated by heating at 95°C for 5 min, and the reaction products were analyzed by TLC or HPIC. The thermal stability test was conducted by preincubating the enzyme preparation 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 above.

RESULTS AND DISCUSSION

Isolation of *MhMTS* and *MhMTH* Genes from *M. hakonesis*

Several thermophilic bacteria were collected, and their trehalose contents determined by HPIC. Significant amount

of trehalose was detected in *M. hakonesis* and *S. acidocaldarius*, whereas little, if any, was in *M. sedula, S. brierleyi*, and *S. metallicus* (data not shown). The genes responsible for the trehalose biosynthesis in *M. hakonesis* were isolated by hybridization of the bacterial genomic DNA with the MTS-MTH gene probe from *S. solfataricus* [15]. The two genes (*MhMTS* and *MhMTH*) encoding MhMTS and MhMTH, respectively, were located side-by-side with mutually reverse orientation in a DNA fragment (data not shown).

The ORFs of the *MhMTS* and *MhMTH* were determined by comparing the nucleotide sequences with those of MTS and MTH genes from other bacteria; *S. solfataricus* [15], *S. shibatae* [17], and *S. acidocaldarius* [22, 23]. The ORF of *MhMTS* is 2,142 bp long, and encodes 713 amino acid residues constituting a 83.8 kDa protein. *MhMTS* showed 79%, 76%, and 68% of homology with the MTS genes from *S. solfataricus*, *S. shibatae*, and *S. acidocaldarius*, respectively. *MhMTH* is 1,679 bp long, and encodes 558 amino acid residues constituting a 63.7 kDa protein. *MhMTH* showed 82%, 79%, and 72% of nucleotide sequence homology to those of *S. solfataricus*, *S. shibatae*, and *S. acidocaldarius*, respectively.

The deduced amino acid sequences of MhMTS and MhMTH from *M. hakonesis* were compared with those of homologous enzymes from *S. solfataricus*, *S. acidocaldarius*, *S. shibatae*, *Brevibacterium helvolum* [14], *Arthrobacter* sp. [19], and *Rhizobium* sp. [20]. MhMTS and MhMTH showed 58% and 64% homology in amino acid sequences with homologous enzymes from *S. solfataricus* [15], respectively. Multiple sequence alignment revealed four regions highly conserved for MTSs and three for MTHs (data not shown). It was noticed through GenBank database analysis that these domains are common to all α-amylolytic enzymes such as α-amylases, pullulanases, cyclomaltodextrin glucanotransferases, and debranching enzymes, constituting substrate-binding sites of the starch hydrolysis enzymes [9, 10, 11]. The crystal structure of SsMTH (also called SsGTHase) has been determined, illustrating a substrate-binding cleft wall that appears to be critical for the substrate selectivity [7]. Phylograms for each group of enzymes, generated using the CLUSTAL W program, yielded similar patterns for MTSs (*treY*s) and MTHs (*treZ*s) (data not shown), suggesting that these two enzymes have evolved through a common history. Nucleotide sequences of the genes have been deposited to the GenBank database under accession numbers AY444508 for *MhMTS* and AY444509 for *MhMTH*.

Trehalose Synthesis by MhMTS and MhMTH

The *MhMTS* and *MhMTH* were introduced into *E. coli* expression vector, and expressed into proteins (Fig. 1). The estimated molecular weights of the recombinant MhMTS and MhMTH were approximately 90 kDa and 65 kDa, respectively, including the hexahistidine domain from the pRSET-B vector. These values are in agreement with the values predicted from their deduced amino acid sequences.

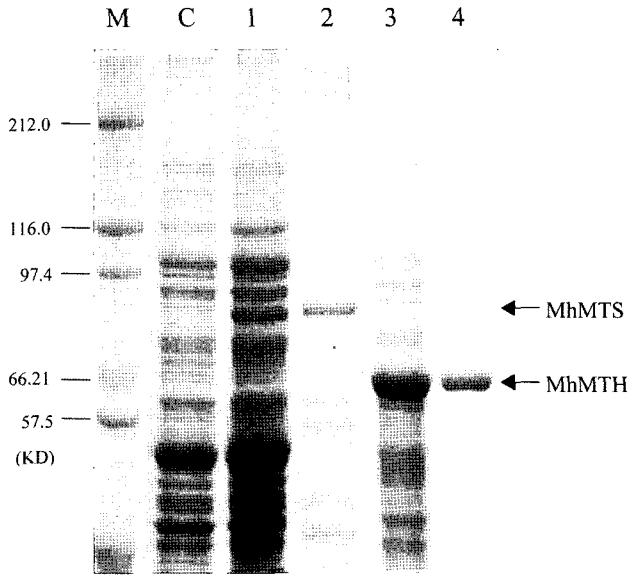

Fig. 1. Purification of recombinant MhMTS and MhMTH.
The *MhMTS* and *MhMTH* genes were inserted into the pRSET-B vector to produce pRBMhMTS and pRBMhMTH, respectively. Gene transcription was induced by IPTG and protein extracts or column fractions were analyzed on SDS-PAGE. Lane M; molecular weight markers. Lane C, crude extract of *E. coli* harboring empty pRSET plasmid; lane 1, crude extract of *E. coli* harboring pRBMhMTS; lane 2, MhMTS purified by Ni^{2+}-NTA chromatography; lane 3, crude extract of *E. coli* harboring pRBMhMTH; lane 4, MhMTH purified by Ni^{2+}-NTA chromatography.

A mixture of the purified recombinant MhMTS and MhMTH was incubated with maltopentaose, and trehalose production was examined by TLC and HPIC (Fig. 2). Maltopentaose (G_5) was converted into trehalose (T) and maltotriose (G_3) in the presence of both enzymes. In the reaction with MhMTS, maltopentaose (G_5) might be converted to an intermediate maltotriosyltrehalose (G_3T), as determined previously [12, 16–18], and then MhMTH might hydrolyze the G_3T to trehalose (T) and maltotriose (G3). In the reaction with *B. helvolum* enzymes BvMTS and BvMTH [12], G_3 was not reacted by any of those enzymes and thus not cleaved to T (or G2) and glucose (G1). In addition, the *B. helvolum* enzymes did not cleave G5 into G1 and maltotetraose (G4). In contrast, the MhMTS and MhMTH mixture generated a significant amount of G1, and G4 in the reaction with G5 (Fig. 2). Related to this observation, it was reported that MTS from *S. acidocaldarius* exhibits slight hydrolytic activity [22, 23]. Generation of G4 might not hamper the trehalose synthesis, since most of G4 might be converted to trehalose and maltose (G2), as determined in the reaction with *B. helvolum* enzymes [14].

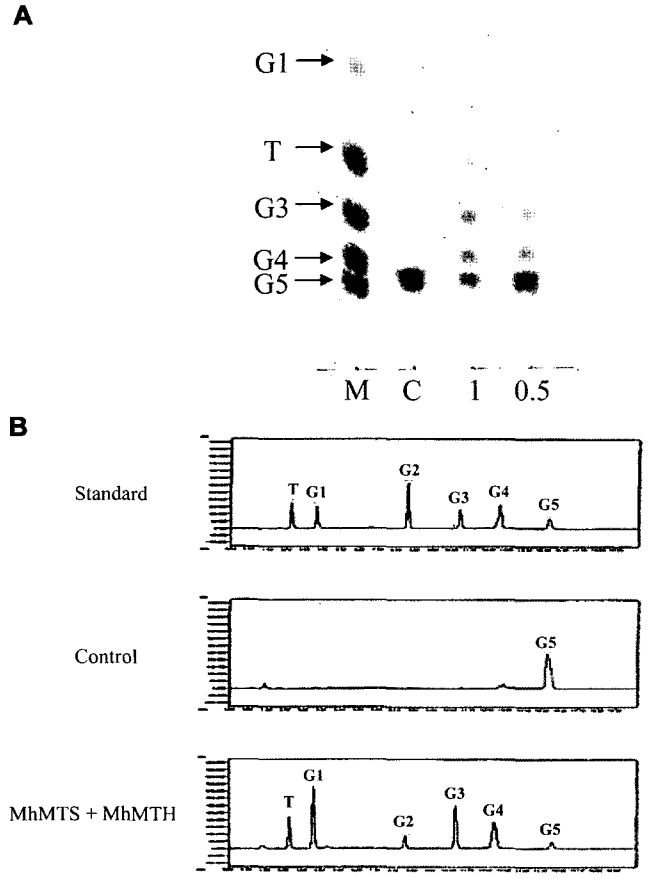

Fig. 2. Trehalose-producing activity of a MhMTS/MhMTH mixture. A mixture of recombinant MhMTS and MhMTH was incubated with 5 mM maltopentaose at 70°C. **A.** TLC analysis of the reaction products. Lane M; mixture of standard maltooligosaccharides. G1, G3, G4, G5, and T denote glucose, maltotriose, maltotetraose, maltopentaose, and trehalose, respectively. Lane C, reaction product obtained after incubated with the heat-denatured enzyme mixture. Lanes 1 and 0.5 denote reaction times 1 and 0.5 h, respectively, with the MhMTS/MhMTH mixture. **B.** HPIC profile of the reaction products produced by the MhMTS/MhMTH mixture (reaction for 0.5 h).

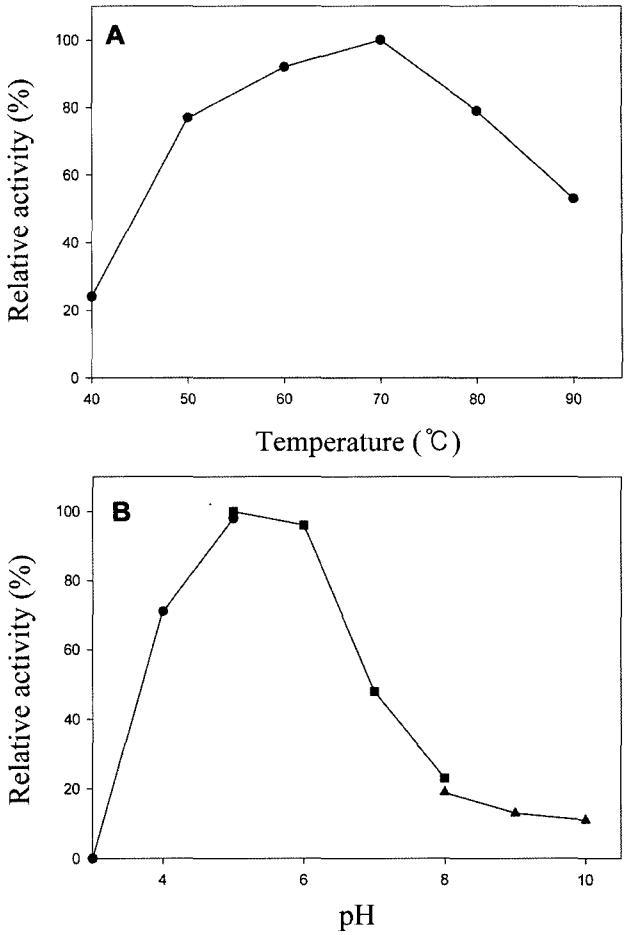

Fig. 3. Optimum conditions for the activity of MhMTS/MhMTH mixture.
A. Effect of temperature on the trehalose production. Purified MhMTS/MhMTH mixture (0.1 pmol each) was incubated with 5 mM of maltoheptose at various temperatures for 30 min with each. **B.** Effect of pH on the trehalose production. For the pH test, 0.1 M McIlvaine buffer solutions (pH 3–5), 0.1 M sodium phosphate buffer solutions (pH 5–8), and 0.1 M sodium bicarbonate buffer solutions (pH 8–10) were used. Reactions were conducted at 70°C for 30 min.

The optimum pH of the MhMTS/MhMTH enzyme reaction for the trehalose production was around 5.0 (Fig. 3A) and optimum temperature was around 70°C at pH 5.0 (Fig. 3B). Thermal stability of the enzymes was determined by measuring residual enzyme activities after preincubating at various temperatures between 40°C and 90°C for 30 min. The trehalose-producing activity of the MhMTS/MhMTH 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 enzyme activity for the trehalose production was retained to be about 80% (Fig. 4B). The optimum temperature of the MhMTS/MhMTH enzyme reaction for the trehalose production was around 70°C at pH 5.0, which shows lower optimum temperature than other thermophilic MTS and MTH [15, 16, 18, 22, 23]. This property could have the advantage to reduce the cost to produce trehalose for industrial application by reducing the costs to keep a high temperature during the trehalose production reaction.

Effect of 4-α-Glucanotransferase

4-α-Glucanotransferase (4-α-GT) transfers a segment of the 1,4-α-D-glucan molecule to a new C-4 position in an acceptor that could be a glucose or a 1,4-α-D-glucan [30]. When maltooligosaccharides longer than maltose are used as substrates for 4-α-GT, various maltooligosaccharides with higher degrees of polymerization are synthesized [13].

We tested if addition of 4-α-GT could increase the yield of trehalose production from maltooligosaccharide substrates in the sequential reactions of MhMTS and MhMTH. Thus,

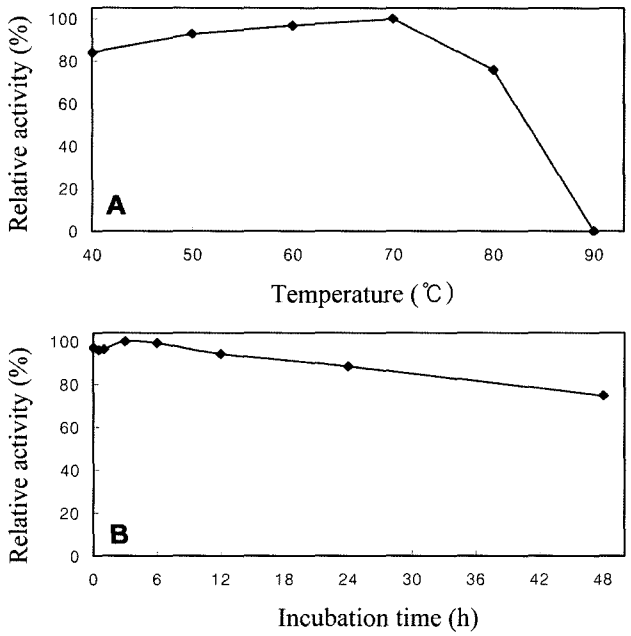

Fig. 4. Thermostability of the MhMTS/MhMTH enzyme mixture.
A. Effect of preincubation on the trehalose production. **B**. Half-life of the enzyme mixture at high temperature.

50 pmol of thermostable 4-α-GT from *Thermotoga maritima* [13] was added to the reaction mixture. In this experiment, trehalose production was increased in the presence of 4-α-GT, converting 70% of maltopentaose into trehalose after 6 h, while 60% was converted without 4-α-GT (Fig. 5). This result suggests that 4-α-GT

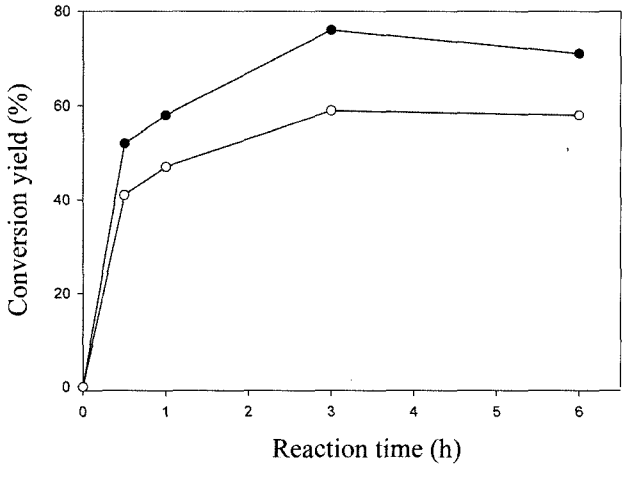

Fig. 5. Effects of 4-α-glucanotransferase on trehalose synthesis from maltopentaose.
Ten mM maltopentaose was incubated with the MhMTS/MhMTH mixture (0.1 pmol each) and 50 pmol of 4-α-glucanotransferase in a 100-μl reaction mixture. The conversion yield was determined by calculating the amount of trehalose produced in the reaction, using a densitometer. Closed (●) and open (○) circles denote the results from the reactions with and without 4-α-glucanotransferase, respectively.

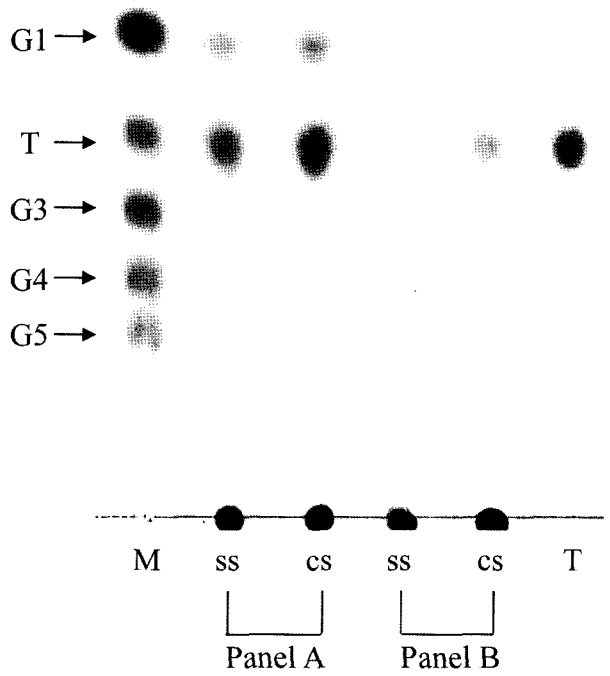

Fig. 6. Trehalose production from starch.
MhMTS/MhMTH (Panel A) and SaMTS/SaMTH (Panel B) were reacted with 1% (w/v) soluble starch or 1% liquefied corn starch, and the reaction products were analyzed by TLC. Lane M, maltooligosaccharide standard mixture. Lane ss, 1% soluble starch; lane cs, 1% liquefied corn starch; T, trehalose standard.

supplied longer maltooligosaccharides that might be better substrates for the sequential reaction catalyzed by MhMTS/MhMTH.

Trehalose Production from Starch

The substrate specificity was extended to soluble starch, the most abundant maltodextrin in nature. A mixture of MhMTS/MhMTH was incubated with 1.0% soluble starch and liquefied corn starch, respectively. A 38% solution of soluble starch and 44.3% of liquefied corn starch were converted into trehalose after 24 h incubation in the reaction with the MhMTS/MhMTH mixture (Fig. 6), demonstrating higher efficiency than SaMTS and SaMTH (almost nothing and 21.7%, respectively) from *S. acidocaldarius* [12]. The yield of trehalose production from starch was relatively low, however, which might be because starch could not provide many reducing ends available for MhMTS and MhMTH reaction. It was reported that addition of α-amylase in the mesophile BvMTS/BvMTH enzyme reaction increased the trehalose productivity (22.1% to 68.3%) [14]. α-Amylase might hydrolyze the starch into maltooligosaccharides, generating more reducing ends at which the enzymes can work on. To raise the yield of trehalose production from starch using the MhMTS/MhMTH reaction system, the reaction with α-amylase is under progress.

Acknowledgments

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