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Articles

Construction of Bifunctional Fusion Enzyme between Maltooligosyltrehalose Synthase and Maltooligosyltrehalose Trehalohydrolase of *Sulfolobus acidocaldarius* and Overexpression in *E. coli*

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Two genes encoding maltooligosyltrehalose synthase (SaMTS) and maltooligosyltrehalose trehalohydrolase (SaMTH) were isolated from a hyperthermophilic microorganism, *Sulfolobus acidocaldarius* (ATCC 49462). ORFs of the SaMTS and SaMTH genes are 2,163 and 1,671 bp long and encode 720 and 556 amino acid residues, respectively. A bifunctional fusion enzyme (SaMTSH) was constructed through the gene fusion of SaMTS and SaMTH. Recombinant SaMTS, SaMTH, and SaMTSH fusion enzyme were overexpressed in *E. coli* BL21. SaMTS and SaMTH produced trehalose and maltotriose from maltopentaose in a sequential reaction. SaMTSH fusion enzyme catalyzed the sequential reaction in which the formation of maltotriosyltrehalose was followed by hydrolysis leading to the synthesis of trehalose and maltotriose. The SaMTSH fusion enzyme showed the highest activity at pH 5.0-5.5 and 70-75°C. SaMTS, SaMTH, and SaMTSH fusion enzyme were active in soluble starch, which resulted in the production of trehalose.

Key words: Sulfolobus acidocaldarius, *maltooligosyltrehalose synthase, maltooligosyltrehalose trehalohydrolase, trehalose, bifuntional fusion enzyme.*

Trehalose (α-D-glucopyranosyl-[1,1]-α-D-glucopyranose) is a nonreducing disaccharide of glucose found in various organisms including bacteria, algae, fungi, yeasts, and insects, and in some plants.[1] In nature, trehalose serves not only as a carbohydrate reserve but also as a protectant against a variety of physical and chemical stresses in different organisms.[2-5] It is shown to preserve the integrity of biological membranes by holding H_2O molecules,[6] thus allowing the desert plants to naturally tolerate the complete dehydration and rehydration cycle.[7] It expected to serve for various applications, for example as a sweetner, a stabilizer for dried or frozen foods, in cosmetics, and as a drug additives.[8] Recently this property is applied to food and phamaceutical industry.

Trehalose biosynthesis is well-characterized in *E. coli* and yeast. Trehalose is synthesized by two enzymes in both cells. In the first step, T6P is produced by T6P synthase from UDPG and G6P. In the second step, trehalose is produced via dephosphorylation of T6P by T6P phosphatase.[9] Recently, MTS and MTH were isolated, and their genes were cloned from several bacteria.[10-14] MTS acts on the α-1,4 linkage at the reducing end of maltodextrin, and transforms it into α-1,1 linkage to produce a maltooligosyltrehalose. MTH hydrolyzes the α-1,4-glycosidic linkage between the maltooligosyl group and trehalose of MT. Thus, trehalose is produced from maltodextrins by MTS and MTH working in a sequential manner.[10-13]

The preparation of recombinant bifunctional enzyme appears to have great potential in enzyme technology. A bifunctional enzyme can be prepared by joining the structural genes of the two enzymes; the translational stop signal at the 3'-end of the first gene is removed and ligated in-frame to the ATG start codon of the second gene. Upon fusion, the novel chimeric gene encodes a single polypeptide chain carrying both active sites. Over the past few years, a variety of artificial bifunctional enzymes have been prepared through gene fusion *in vitro*, and their physical and chemical properties analyzed.[10,15,16]

One of the hyperthermophilic microorganism, *Sulfolobus acidocaldarius*, is known to produce trehalose via enzymes similar to MTS ans MTH.[8] In this paper, the genes encoding SaMTS and SaMTH were cloned from *S. acidocaldarius* and overexpressed in *E. coli* through recombination into pRSET expression vector. To produce trehalose via maltooligosyltrehalose in a concerted fashion, an artificial bifunctional enzyme, SaMTSH, was produced through the in-frame fusion of SaMTS and SaMTH genes. All recombinant and fusion proteins were characterized for the production of trehalose from maltopentaose and starch.

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Abbreviations: G6P, glucose-6-phosphate; HPIC, high-performance ion chromatography; MTH, maltooligosyltrehalose trehalohydrolase; MTS, maltooligosyltrehalose synthase; SaMTH, maltooligosyltrehalose trehalohydrolase of *Sulfolobus acidocaldarious*; SaMTS, maltooligosyltrehalose synthase of *Sulfolobus acidocaldarious*; SaMTSH, bifunctional fusion enzyme of SaMTH and SaMTS; T6P, trehalose-6-phosphate; UDPG, UDP-glucose.

Materials and Methods

Bacterial strains. *Sulfolobus acidocaldarius* (ATCC 49462) was used for the cloning of genes encoding SaMTS and SaMTH. *E. coli* MC1061 was used for transformation and plasmid propagation, and *E. coli* BL21 for the expression of recombinant SaMTS, SaMTH, and SaMTSH genes. *S. acidocaldarius* were grown in the sulfolobus media (ATCC 1723 media) at 75°C for 5 days, and *E. coli* were grown in LB.[17]

Enzymes and chemicals. Restriction enzymes and DNA modifying enzymes were purchased from Promega. All enzymes were used as recommended by their manufacturers. DIG-DNA detection system was purchased from Boehringer Mannheim. GenScreen plus membrane was from DuPont. Trehalose, maltooligosaccharides, and other chemicals were purchased from Sigma Chemical Co.

Construction of expression plasmids. The open reading frames of SaMTS, SaMTH, and SaMTSH were introduced into the pRSET *E. coli* expression vector through PCR amplification and overexpressed in *E. coli* BL21. The SaMTS-specific primers were 5'-GGGGTACCATGATATC AGCAACCTACAG-3' and 5'-GGGGTACCTTACATTCT AACTAGTATCC-3' containing translation initiation and termination codons (underlined), respectively. The PCR product was digested with *Kpn*I and cloned into the pRSET-B vector to produce pRSaMTS. The SaMTH-specific primers were 5'-GGGGTACCATGTTTTCGTTCGGTGGA AA-3' and 5'-GGGGTACCTCATTCTAATTGATATACCCC -3' containing translation initiation and termination codons (underlined), respectively. The PCR product was digested with *Kpn*I and cloned into the pRSET-B vector to produce pRSaMTH. Polymerase chain reaction was carried out in a volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 100 ng of template DNA, 100 pmole of each primer, and 2.5 units of Taq DNA polymerase. DNA was amplified as follows: 3 min at 94°C; followed by 30 cycles of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C; finally extension at 72°C for 5 min was allowed.

The expression vector for the fusion enzyme, pRSaMTSH, was constructed through PCR using 4 primers. The ORF of SaMTS was produced through PCR using the primer 1 (5'-GGGGTACCATGATATCAGCAACCTACAG-3') containing translation initiation codon of SaMTS and primer 2 (5'-AGGATACTAGTTAGAATGATGTTTTCGTTCGGT GG-3'). The ORF of SaMTH was produced through PCR using the primer 3 (5'-CCACCGAACGAAAACATCATT CTAACTAGTATCCT-3') which is complementary to primer 2 and primer 4 (5'-GGGGTACCTCATTCTAATTGATATA CCCC-3') containing termination codon of SaMTH. The two PCR product were subjected to PCR, using primer 1 and primer 4, and SaMTSH fusion gene was introduced to the pRSET-B to produce pRSaMTSH.

Expression and enzyme assay of SaMTS, SaMTH, and SaMTSH fusion enzyme. pRSaMTS, pRSaMTH, and pRSaMTSH were transformed into *E. coli* BL21, and induced by adding 1 mM IPTG at 37°C for 4 h. Protein samples were analyzed through discontinuous SDS-polyacrylamide gel electrophoresis.[18] Concentrations of the expressed enzymes were estimated using a densitometer with BSA as a standard protein.

Trehalose synthesis activity was measured by incubating 1 pmole of each enzyme in 100 μl of 20 mM McIlvaine buffer (pH 5.5) containing 5 mM maltopentaose or 1% soluble starch. The reaction was carried out at 70°C for 30 min and terminated by heating at 100°C for 5 min. The reaction product was analyzed through TLC and HPIC.

HPIC was carried out using a Carbo-Pak PA1 column on a DX500 HPIC system (Dionex) at room temperature. Saccharides were eluted through a continuous sodium acetate gradient from 0 to 250 mM in 150 mM sodium hydroxide solution over 30 min. The eluted saccharides were monitored with an ED40 potential amperometric detector.

Results

Isolation of genes for SaMTS and SaMTH from *S. acidocaldarius*. To isolate genes encoding SaMTS and SaMTH of *S. acidocaldarius*, PCR was carried out using SaMTS- and SaMTH-specific primers, which were designed according to the method of Maruta *et al.*[19] Each PCR product was digested with *Kpn*I and cloned into the pRSET-B vector to produce pRSaMTS and pRSaMTH. The ORF of SaMTS is 2,163 bp long and encodes 720 amino acid residues with a molecular weight of 84,802 (Fig. 1). The ORF of SaMTH is 1,671 bp long and encodes 557 amino acid residues with a molecular weight of 64,370 (Fig. 2). The nucleotide sequences of SaMTS and SaMTH showed 99.8 and 99.9% homology with those of *S. acidocaldarius* ATCC 33909 (GenBank accession D83245), and 64.3 and 66.4% homology with those of *S. solfatarius* KM1 (GenBank accession D64128, D64130), respectively (data not shown).

Expression of SaMTS, SaMTH, and their fusion enzyme SaMTSH. To overexpress the SaMTS and SaMTH, the ORFs of SaMTS and SaMTH were introduced into the *E. coli* expression vector, pRSET-B, respectively. To test the enzyme activity of bifunctional fusion enzyme catalyzing two consecutive reactions, genes encoding SaMTS and SaMTH were fused together in frame and expressed in *E. coli* as well. To construct the in-frame fusion gene between them, polymerase chain reaction was carried out using 4 primer as described in Materials and Methods. As a result, gene encoding fusion protein SaMTSH, in which the last amino acid (Met) of SaMTS was fused directly to the first amino acid (Met) of SaMTH without the linker, was constructed and introduced into *E. coli* expression vector, pRSET-B, to produce pRSaMTSH. The construction strategy of SaMTSH fusion gene and junction sequence between genes for SaMTS and SaMTH is shown in Fig. 3.

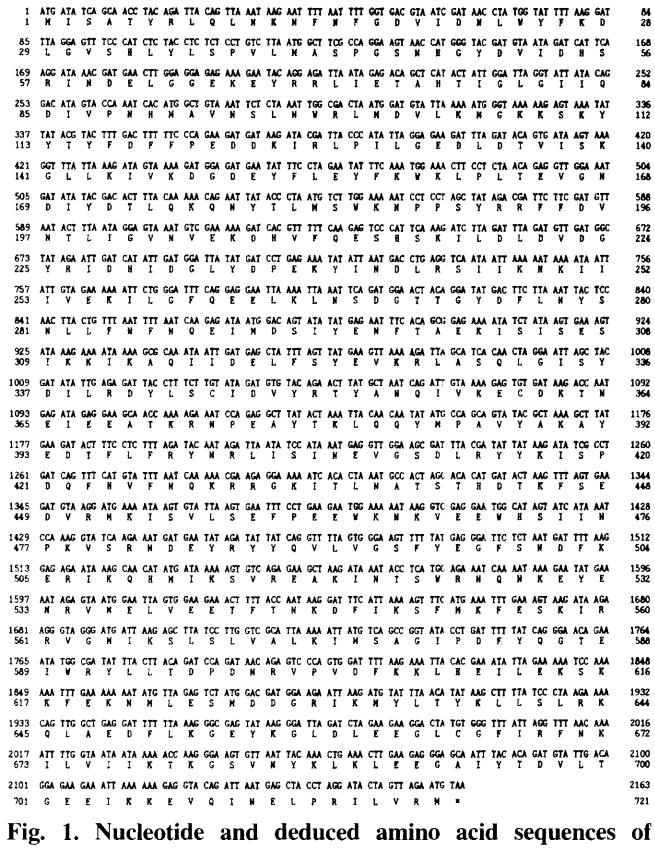

Fig. 1. Nucleotide and deduced amino acid sequences of maltooligosyltrehalose synthase (SaMTS) from hyperthermophilic microorganism, *Sulfolobus acidocaldarius* (ATCC 49462). The nucleotide sequence of SaMTS shows 99.8% homology with that of *S. acidocaldarius* ATCC 33909 (GenBank accession D83245), and 64.3% with that of *S. solfatarius* KM1 (GenBank accession D64128).

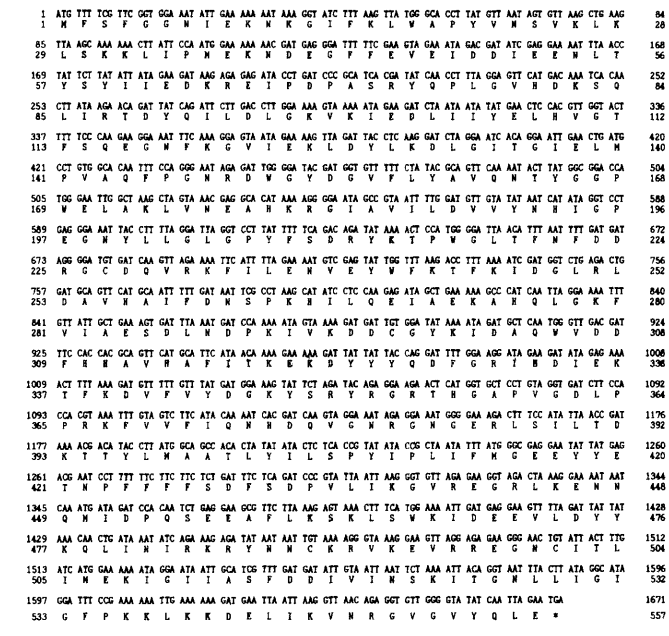

Fig. 2. Nucleotide and deduced amino acid sequences of maltooligosyltrehalose trehalohydrolase (SaMTH) from hyperthermophilic microorganism, *Sulfolobus acidocaldarius* (ATCC 49462). The nucleotide sequence of SaMTH shows 99.9% homology with that of *S. acidocaldarius* ATCC 33909 (GenBank accession D83245), and 66.4% homology with that of *S. solfatarius* KM1 (GenBank accession D64130).

To analyze the enzymatic activities and the substrate specificities of SaMTS, SaMTH, and SaMTSH, they were overexpressed in *E. coli* and analyzed through discontinuous SDS-polyacrylamide gel electrophoresis (Fig. 4). The estimated molecular weight of recombinant SaMTS, SaMTH, and SaMTSH containing hexahistidine domain derived from pRSET vector were approximately 85,000, 65,000, and 150,000, respectively. These values are in accord with the values calculated from their deduced amino acids. The concentration of expressed enzymes were estimated using a densitometer with BSA as a standard protein.

Recombinant fusion enzyme SaMTSH produces trehalose from maltopentaose. To analyze the enzymatic activities and the substrate specificities, 1 pmole each of crude enzymes, SaMTS and SaMTH, were incubated with maltopentaose (G_5) (G_n, where n is the number of glucose unit linked by α-1,4 linkage), respectively, and the reaction products were analyzed through HPIC (Fig. 5). Figure 5A shows the HPIC profile of standard carbohydrates. The total protein extract of *E. coli* BL21 could not react on G_5 (Fig. 5B). On the other hand, G5 was converted into trehalose (T) and maltotriose (G_3) in the presence of SaMTS and SaMTH mixture (Fig. 5C). The intermediate peak shown in Fig. 5C,D is maltotriosyltrehalose (G_3T) as determined by Maruta *et al.* (1995) and Kato et al. (1996). These results showed that SaMTS produces G3T from G5 and SaMTH subsequently hydrolyzes G_3T to trehalose and G_3 through the sequential reaction.

SaMTSH fusion enzyme converted G_5 to G_3 and trehalose as did the mixture of SaMTS and SaMTH (Fig. 5D). This result demonstrated that the SaMTSH fusion enzyme is functional and catalyzes two sequencial reactions from G_5 to G_3T and then to trehalose and G_3.

Biochemical properties of SaMTSH fusion enzyme. The effects of temperature and pH on the activity of SaMTSH fusion enzyme are shown in Fig. 6. A 5 mM maltopentaose was incubated for 30 min at 70°C with 1 pmole of crude recombinant SaMTSH fusion enzyme at various temperatures and pHs. The SaMTSH fusion enzyme showed the highest activity at 70-75°C (Fig. 6A), and the fusion enzyme was stable at temperatures below 80°C (data not shown). For the pH test, McIlvaine buffer (pH 3.0-7.0), 0.1 M Tris-HCl buffer (pH 8.0), and 0.1 M $NaHCO_3$-Na_2CO_3 buffer (pH 9.0-10.0) were used. The optimum pH of SaMTSH fusion enzyme was 5.0-5.5 (Fig. 6B), and was stable between pH 4.5 to 7.0 (data not shown).

Bifunctional fusion enzyme produces trehalose from soluble starch. To examine the reactivity of enzymes on soluble starch, mixture of crude enzymes, SaMTS/SaMTH, or fusion enzyme, SaMTSH, were incubated with 1%

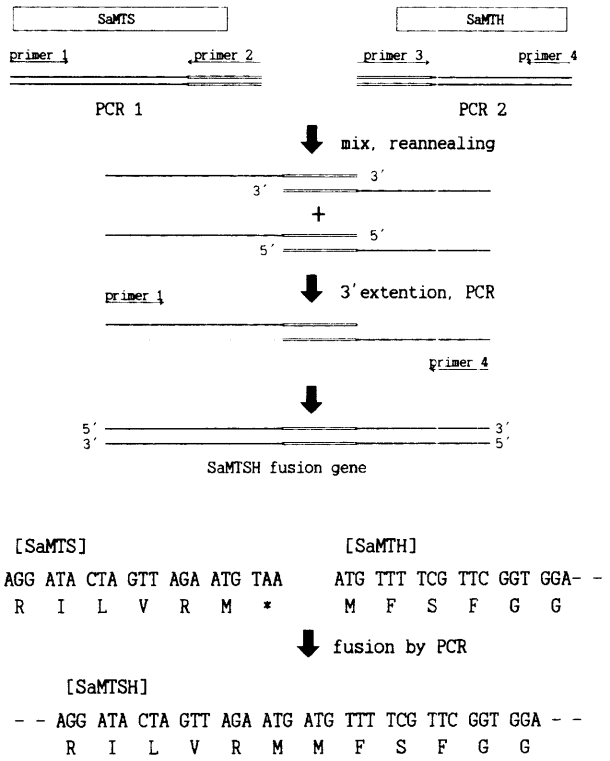

Fig. 3. Strategy for the cloning of a SaMTSH fusion gene through two step PCR and DNA sequence of the fused region of SaMTS and SaMTH.

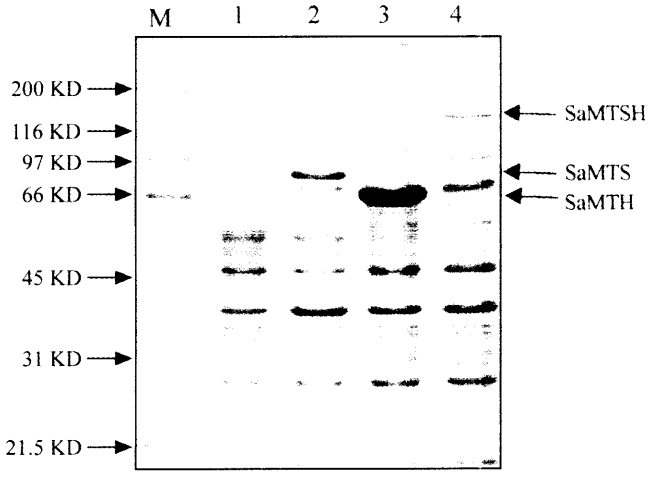

Fig. 4. Expression of recombinant SaMTS, SaMTH, and SaMTSH in *E. coli*. The individual ORFs for SaMTS and SaMTH were introduced into the pRSET expression vector and expressed in *E. coli*. Protein extracts prepared from induced *E. coli* were analyzed through 12.5% SDS-PAGE and stained with Coomassie Blue. Lane M: Molecular weight marker, Lane 1: crude extract of *E. coli* harboring pRSET plasmid only, Lane 2: crude extract of *E. coli* harboring pRSaMTS, Lane 3: crude extract of *E. coli* harboring pRSaMTH, Lane 4: crude extract of *E. coli* harboring pRSaMTSH

soluble starch, and the reaction was carried out at 70°C for 24 h. When the soluble starch was incubated with the mixture of SaMTS and SaMTH, 18.1% of soluble starch

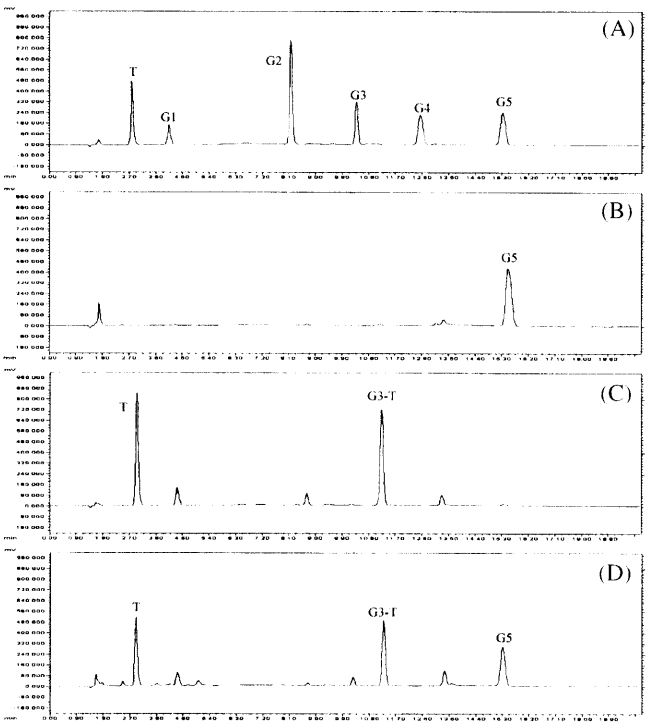

Fig. 5. HPIC analysis of reaction products obtained from maltopentaose through the enzyme activity of recombinant SaMTS, SaMTH, and SaMTSH. A 5 mM of maltopentaose was incubated at 70°C for 30 min with 1 pmole of each crude recombinant enzymes, SaMTS and SaMTH (B), SaMTSH fusion enzyme (C). Reaction products were analyzed through HPIC. G5, G3, G3T, G2, G1, and T denote maltopentaose, maltotriose, maltotriosyltrehalose, maltose, glucose, and trehalose, respectively.

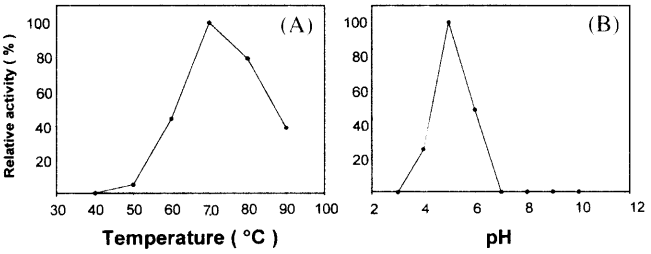

Fig. 6. Effects of temperature and pH on the activity of SaMTSH fusion enzyme. A: Effect of temperature. A 5 mM of maltopentaose was incubated at various temperatures for 30 min with 1 pmole of crude recombinant SaMTSH fusion enzyme. B: Effect of pH. For the pH test, McIlvaine buffer (pH 3.0-7.0), 0.1 M Tris-HCl buffer (pH 8.0), and 0.1 M $NaHCO_3$-Na_2CO_3 buffer (pH 9.0-10.0) were used. A 5 mM of maltopentaose was incubated at 70°C for 30 min with 1 pmole of crude recombinant SaMTSH fusion enzyme at various pHs.

were converted into trehalose (Fig. 7A), and 15.9% of soluble starch were converted into trehalose through the reaction of SaMTSH fusion enzyme (Fig. 7B). These results demonstrated that trehalose is produced from soluble starch through the sequential reaction of SaMTS and SaMTH or by SaMTSH fusion enzyme.

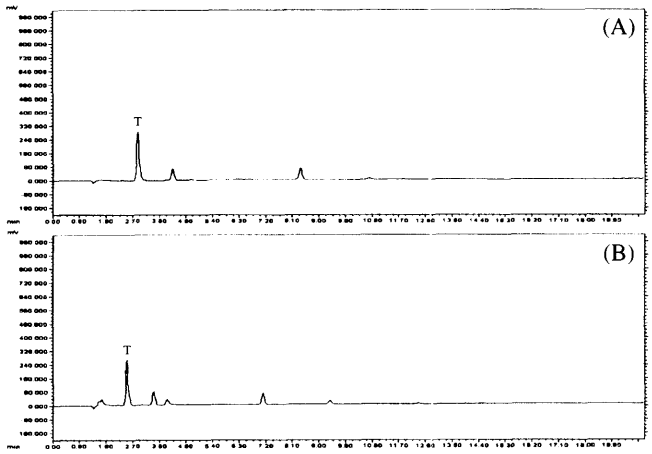

Fig. 7. Trehalose production from starch by SaMTS, SaMTH, and SaMTSH. Soluble starch (1% solution) was incubated with 1 pmole of each crude SaMTS and SaMTH mixture (A), and SaMTSH fusion enzyme (B) at 70°C for 24 h. The reaction products were analyzed through HPIC.

Discussion

Two genes encoding SaMTS and SaMTH were isolated from *S. acidocaldarius* (ATCC 49426), and enzymatic activities of each encoded protein were demonstrated. Several bacteria, such as *B. helvolum*,[10] *Arthrobacter* sp. Q36,[11] *Rhizobium* sp. M-11,[12] *S. solfataricus* KM1,[13] and *M. tuberculosis*[14] have been reported to produce trehalose via enzymatic system of MTS and MTH.

The use of several enzymes catalyzing sequential reactions is a feature of many biological metabolic processes. The simplest way to carry out an enzymatic reaction consisting of several consecutive steps is addition of all the enzymes directly to the reaction mixture. They may be added simultaneously or sequentially if they require entirely different conditions. Generating physical proximity between enzymes often provides the system with highly attractive properties. Proximity between two or more enzymes may also be achieved by making an in-frame gene fusion of the corresponding structural genes. The fusion gene encodes a polypeptide carrying two or more active centers. The preparation of artificial bifunctional enzymes appears to have great potential in enzyme technology. In this study, thermostable bifunctional fusion enzyme, SaMTSH, was constructed through the in-frame fusion of structural genes for SaMTS and SaMTH. The fusion enzyme is able to catalyze the formation of maltooligosyltrahalose, and the subsequent hydrolysis of trehalose and maltooligosyltrahalose shortens two glucose units, thus suggesting that the polypeptide chain can fold sufficiently well to yield autonomous domains.

Soluble starch was converted into trehalose through the combination reaction of SaMTS and SaMTH, or SaMTSH fusion enzyme only. The yield, however, was lower than expected even after 24 h incubation. It might be that soluble starch did not present many reducing ends to SaMTS, and if it did, SaMTH could not work efficiently because there must be a small amount of maltooligosyltrehalose structure to produce trehalose. Addition of thermostable α-amylase or starch-debranching enzyme might increase the trehalose productivity from soluble starch.[10]

Studies on other basic properties of the fusion enzyme such as their stabilities and optimal reaction conditions are still in progress. The bifunctional fusion enzyme, SaMTSH, reported in this paper may lead to the mass production of trehalose from inexpensive raw materials.

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