Construction of a Recombinant Thermostable β-Amylase-Trehalose Synthase Bifunctional Enzyme for Facilitating the Conversion of Starch to Trehalose

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A fusion gene that encoded a polypeptide of 1495 amino acids was constructed from the β-amylase (BA) gene of *Clostridium thermosulfurogenes* and trehalose synthase (TS) gene of *Thermus thermophilus*. The fused gene was overexpressed in *Escherichia coli*, and a recombinant bifunctional fusion protein with BA at the N-terminal (BATS) or C-terminal (TSBA) of TS having both β-amylase and trehalose synthase activities with an apparent molecular mass of 164 kDa was obtained. BATS or TSBA catalyzes the sequential reaction in which maltose is formed from starch and then is converted into trehalose. The K_m values of the BATS and TSBA fusion enzymes for the reaction from starch to trehalose were smaller than those of an equimolar mixture of BA and TS (BA/TS). On the other hand, the k_{cat} value of BATS approximated that of the BA/TS mixture, but that of TSBA exceeded it. TSBA showed much higher sequential catalytic efficiency than the separately expressed BA/TS mixture. The catalytic efficiency of TSBA or BATS was 3.4 or 2.4 times higher, respectively, than that of a mixture of individual enzymes, showing the kinetic advantage of the fusion enzyme. The thermal stability readings of the recombinant fusion enzymes BATS and TSBA were better than that of the mixture of individual recombinant enzymes. These results apparently demonstrate that fusion enzymes catalyzing sequential reactions have kinetic advantages over a mixture of both enzymes.

KEYWORDS: Trehalose; *Thermus thermophilus* trehalose synthase; bifunctional enzyme; starch

INTRODUCTION

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is a nonreducing disaccharide widely distributed in various organisms, including bacteria, algae, fungi, yeasts, insects, and in some plant and mammalian tissues (*1–8*). This compound may serve as an energy reservoir, a protectant from a variety of physical and chemical stresses, and a protein stabilizer in living organisms (*1, 9–14*). It is an essential component for maintaining cell viability. The absence of reducing ends in trehalose makes it highly resistant to heat, pH, and Maillard's reaction (*15–18*). In addition, trehalose can protect proteins against denaturation because of desiccation and freezing (*19, 20*), and its water-holding capability makes its application possible in additives, stabilizers, and sweeteners that are quite useful in the food, cosmetic, and pharmaceutical industries (*21–24*). If trehalose can be produced economically, it should have even more potential commercial applications.

Recently, trehalose productions through fermentation of yeast (*12*) and *Corynebacterium* (*25*), through extraction from transformed plants (*28*), and through enzymatic processes from starch (*26, 27*) and maltose (*15, 19*) have been reported. Up to now, several trehalose-synthesizing enzyme systems have been reported in microorganisms (*25, 29–32*). Trehalose can be metabolized in several ways, which invole different enzyme systems including trehalose-6-phosphate synthetase and trehalose-6-phosphate phosphatase (*27, 33, 34*), trehalose synthase (TS) (*31, 35*), maltose phosphorylase and trehalose phosphorylase (*31*), glycosyltransferase (*36*), and maltooligosyltrehalose trehalohydrolase and maltooligosyltrehalose synthase (*25*). TS (E.C. 5.4.99.16), which converts maltose into trehalose in the absence of coenzyme, has been identified in *Thermus* strains and other microorganisms (*31, 35*).

Establishment of recombinant multifunctional fusion enzyme has great potential in the protein engineering of enzymes (*37, 38*). Fusion of structural genes encoding enzymes that catalyze sequential reactions has several advantages, such as simple expression of a single recombinant unit containing multiple genes and one-step purification of recombinant proteins. When two enzymes are present in a complex, their physical proximity catalyzing sequential reactions might increase the reaction rate

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by facilitating transfer of the reaction intermediate to the catalytic sites of the next enzymes.

In this study, we have successfully constructed a DNA fragment that contains two genes encoding TS from *Thermus thermophilus* and β-amylase (BA) (E.C.3.2.1.2) from *Clostridium thermosulphurogenes* (*39−41*), and this gene was expressed in *Escherichia coli*. The recombinant bifunctional fusion enzymes, TSBA and BATS, catalyze the sequential reactions for the production of trehalose from starch, an abundant and cheap raw material, more efficiently than a combination of individual native enzymes.

MATERIALS AND METHODS

Materials. All saccharides including maltose, trehalose, and starch were purchased from Sigma. Protein assay reagents and dyes were from Bio-Rad Lab. Columns for protein separation were obtained from Pharmacia Biotech. Other chemicals and reagents were of analytical grade.

Bacterial Strains and Cultivation. *T. thermophilus* and *C. thermosulfurogenes* were obtained from the American Type Culture Collection (ATCC). *T. thermophilus* ATCC 33923 was grown in 1260 Thermus B-P (ATCC medium), pH 7.0, at 70 °C. *C. thermosulfurogenes* ATCC 33743 was grown in 1398 modified low phosphate-buffered basal medium (ATCC medium), pH 7.3, at 60 °C under anaerobic conditions.

Construction of Recombinant Expression Plasmids. Genes encoding BA and TS were cloned from *C. thermosulfurogenes* and *T. thermophilus* genomic DNA, respectively. The ORF of BA is 1563 bp long and encodes 520 amino acids. The ORF of TS is 2898 bp long and encodes 965 amino acid residues. The estimated molecular masses of the two polypeptides are 57 and 106 kDa, respectively.

The BA gene was obtained from *C. thermosulfurogenes* genomic DNA by the *Pfu* polymerase chain reaction (PCR) with the primers P_{BA1} (5′-**TG**AGCATAGCACCAAATTTCAAAG-3′) and P_{BA2} (5′-CCGGAATTC**TTA**ATTTTGCCATGTAATGGTGAC-3′), which contain the translation initiation and termination codons (bold) of the *C. thermosulfurogene* BA gene, respectively. *Nde*I and *Eco*RI sites (underlined) were introduced into P_{BA1} and P_{BA2}, respectively. The amplified DNA was ligated into *Nde*I- and *Eco*RI-digested pET-20b-(+) (Novagen) to generate pET-BA.

The TS gene was obtained from *T. thermophilus* genomic DNA by the *Pfu* PCR with the primers P_{TS1} and P_{TS2}, which contain the translation initiation and termination codons (bold) of the *T. thermophilus* TS gene, respectively. *Nde*I and *Sal*I restriction enzyme sites (underlined) were introduced into P_{TS1} (5′-GGAATTCCATA**TG**GAC-CCCCTCTGGTACAAGG-3′) and P_{TS2} (5′-GCCGACGTCGAC**CTA**G-GCTTTTCCGGCCTTGG-3′), respectively. The amplified DNA was ligated into *Nde*I- and *Sal*I-digested pET-20b(+) to produce pET-TS.

To make an expression construct for the BATS fusion enzyme, the BA gene was amplified in the same manner with the primers P_{BA1} and P_{BA5} (5′-CCGGAATTCATTTTGCCATGTAATGGTG-3′). The primer P_{BA5} does not contain the termination codon of the *C. thermosulfurogene* BA gene. *Nde*I and *Eco*RI restriction enzyme sites (underlined) were introduced into P_{BA1} and P_{BA5}, respectively. The amplified DNA was ligated into *Nde*I- and *Eco*RI-digested pET-20b(+) to produce pET-BAd. The TS fragment was amplified with the primers P_{TS4} (5′-CCGGAATTCTTAGGATCTAGATCTGCCGAATTA**ATG**GACCC-CCTCTGGTACAAGGACG-3′) and P_{TS2} and digested with *Eco*RI and *Sal*I, then ligated into *Eco*RI- and *Sal*I-digested pET-BAd. This plasmid, named pET-BATS, was transformed into *E. coli* Tuner(DE3)-pLysS.

To make an expression construct for the TSBA fusion enzyme, the TS gene was amplified in the same manner with the primers P_{TS1} and P_{TS3} (5′-CCGGAATTCGGCTTTTCCGGCCTTGGCCTGC-3′). The primer P_{TS3} does not contain the termination codon of the *T. aquaticus* TS gene. *Nde*I and *Eco*RI restriction enzyme sites (underlined) were introduced into P_{TS1} and P_{TS3}, respectively. The amplified DNA was ligated into *Nde*I- and *Eco*RI-digested pET-20b(+) to produce pET-TSd. The BA fragment was amplified with the primers P_{BA3}

(5′-CCGGAATTCTTAGGATCTAGATCTGCCGAATTA**ATG**AG-CATAGCACCAAATTTCAAAG-3′) and P_{BA4} (5′-CCGCTCGAG**T-TA**ATTTTGCCATGTAATGGTGAC-3′), digested with *Eco*RI and *Xho*I, and then ligated into *Eco*RI- and *Sal*I-digested pET-TSd. This plasmid, named pET-TSBA, was transformed into *E. coli* Tuner(DE3)-pLysS.

Expression of Recombinant Enzymes. The *E. coli* strains Tuner-(DE3)pLysS (Novagen) were used for expression studies. To express the recombinant enzymes, transformed Tuner(DE3)pLysS was inoculated into 6 L of Luria−Bertani (LB) medium supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol in combination and then grown at 37 °C. When the A_{590} reached 0.8, IPTG was added to a final concentration of 1.0 mM. Incubation was continued for another 12 h at 20 °C, and the cells were harvested. The cell pellet was resuspended in 100 mL of lysis buffer (50 mM sodium phosphate buffer, pH 7.0, 1 mM PMSF, and 2 mM EDTA), and cells were disrupted with a French pressure cell press (Thermo Spectronic) for two cycles. The soluble fractions of the lysed cells were collected using 30 min of centrifugation at 15000*g* and 4 °C. The soluble protein fractions were concentrated by ultrafiltration using a 30000 molecular weight cutoff membrane (Millipore). The crude extracts of the recombinant enzymes were harvested for purification.

Purification of Recombinant Enzymes. The crude extracts of the recombinant enzymes were purified by the following steps. To purify the recombinant TSase, solid $(NH_4)_2SO_4$ was added to the cell-free extract to 70% saturation. The resulting precipitate was collected and dissolved in 50 mM sodium phosphate buffer (pH 7.0) and then dialyzed against the same buffer. The dialyzed solution was put on a DEAE-Sepharose column (160mm × 10 mm, Pharmacia Biotech) equilibrated with 50 mM sodium phosphate buffer (pH 7.0), and the adsorbed enzyme was eluted with a linear gradient of 0−1 M NaCl in the same buffer. The active fractions were pooled. Solid $(NH_4)_2SO_4$ was added into the enzyme solution to reach a final concentration of 1 M. The mixture was put on a Butyl-Sepharose column (160 mm × 10 mm) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 1 M $(NH_4)_2SO_4$. The adsorbed enzyme was eluted with a linear gradient of 1 to 0 M $(NH_4)_2SO_4$ in the same buffer. The active fractions were pooled and then dialyzed against 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl. The dialyzed solution was put on a Mono Q-Sepharose column (160 mm × 10 mm) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl. The adsorbed enzyme was eluted with a linear gradient of 0.1−0.5 M NaCl in the same buffer. The active fractions were pooled. The enzyme solution was concentrated with ultrafiltration using a 50000 molecular weight cutoff membrane (Amicon Ultra, Millipore) and then pooled as a purified enzyme preparation.

The recombinant BA, BATS, and TSBA were purified by the method of raw starch adsorption and desorption (*42, 43*). Solid $(NH_4)_2SO_4$ was added to the cell-free extract to 70% saturation. The resulting precipitate was collected and dissolved in 50 mM sodium phosphate buffer (pH 7.0) and then dialyzed against the same buffer. The dialyzed solution was added to the raw starch, which was washed with 50 mM acetate buffer (pH 6.0) at 4 °C, and the mixture was kept for 20 min with occasional shaking. After centrifugation (5 min, 10000*g*), the residue was washed twice with the same buffer. It was then suspended in the elution buffer containing boiled soluble starch in 50 mM acetate buffer (pH 6.0) with 5 mM CaCl₂, shaken occasionally for 30 min, and centrifuged. The supernatant solution containing recombinant enzyme was pooled and dialyzed against 50 mM sodium phosphate buffer (pH 7.0). The dialyzed solution was put on a DEAE-Sepharose column (160 mm × 16 mm) equilibrated with 50 mM sodium phosphate buffer (pH 7.0), and the adsorbed enzyme was eluted with a linear gradient of 0−1 M NaCl in the same buffer. The active fractions were pooled. Solid $(NH_4)_2SO_4$ was added into the enzyme solution to a final concentration of 1 M. The mixture was put on a Butyl-Sepharose column (160 mm × 10 mm) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 1 M $(NH_4)_2SO_4$. The adsorbed enzyme was eluted with a linear gradient of 1 to 0 M $(NH_4)_2SO_4$ in the same buffer. The active fractions were pooled and then dialyzed against 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl. The dialyzed solution was concentrated with ultrafiltration using a 30000 or 50000

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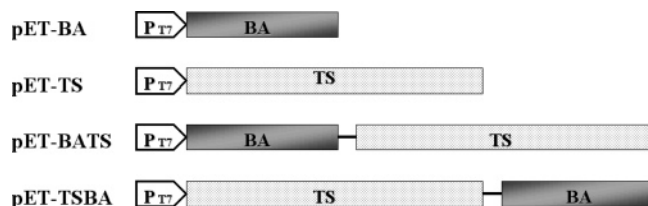
Figure 1. Expression cassettes of the enzymes BA, TS, BATS, and TSBA. The recombinant DNAs for the BA, TS, BATS, and TSBA were inserted into pET20b(+) and then transformed into Tuner(DE3)pLysS to express targets. The linker between BA and TS encodes sequence EFLGSRSAEL. The manners are described in the Materials and Methods.

molecular weight cutoff membrane (Amicon Ultra, Millipore) and then pooled as a purified enzyme preparation.

Protein Assay. The molecular masses of the purified recombinant proteins were determined in denaturing conditions by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed with a 12% separating gel. Proteins were visualized by staining with Coomassie Brilliant Blue R-250 according to standard procedures. Proteins were measured by the method of Bradford using a protein assay kit from Bio-Rad Lab with bovine serum albumin as the standard.

Enzyme Characterization. The activity of TS was assayed by measuring trehalose produced from maltose. A reaction mixture consisting of 50 mM sodium phosphate buffer (pH 6.0), 50 mM maltose, and the enzyme in a final volume of 100 μL was incubated at 60 °C for 2 h. The activity of BA was assayed by measuring maltose produced from soluble starch. A reaction mixture consisting of 50 mM sodium phosphate buffer (pH 6.0), 2% soluble starch, and the enzyme in a final volume of 100 μL was incubated at 60 °C for 2 h. The activities of fusion enzymes were assayed by measuring trehalose produced from soluble starch, in the same reaction buffer and condition as used for the BA assay. Then, this reaction mixture was heated at 100 °C for 10 min to stop the reaction. The trehalose or maltose produced was measured by a high-performance liquid chromatography (HPLC) system, as described below. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of trehalose per min.

Purified enzymes were used for kinetic study with different substrates at pH 6.0 and 60 °C. The enzyme activity was assayed with maltose or soluble starch as substrate, respectively, and determined by the production of trehalose as already mentioned. Then, the values of kinetic parameters and standard deviations were calculated from each triplicate sample with three independent experiments by nonlinear regression plots of the Michaelis−Menten equation.

Analysis of Carbohydrate. Quantitative analysis of sugar was carried out by a HPLC system equipped with refractive index detector (RID) and computerized processing unit (Chrom Manager multi-system, Version 5.2, Taiwan). The columns used include an APS2 analytical column (ThermoHypersil, 250 mm × 4.6 mm) and a guard column (ThermoHypersil, 10 mm × 4.6 mm). The solvent containing acetonitrile/water/formic acid (80:20:1) was used as a mobile phase with a flow rate of 1.0 mL/min.

RESULTS

Expression and Purification of the Bifunctional Fusion Enzymes BATS and TSBA. For the expression of the bifunctional fusion enzyme, genes encoding BA and TS were fused with a linker and expressed in *E. coli*. In the BATS fusion, the C terminus of BA was fused with the N terminus of TS in frame while the TSBA fusion enzyme was inverted, as shown in **Figure 1**. The linker was designed to expedite the recombinant DNA construction without deleting any amino acids of either protein moiety. This resulted in the insertion of 10 amino acids, EFLGSRSAEL, during construction.

Recombinant enzymes produced in *E. coli* were purified to homogeneity as determined by SDS-PAGE analysis (**Figure 2**).
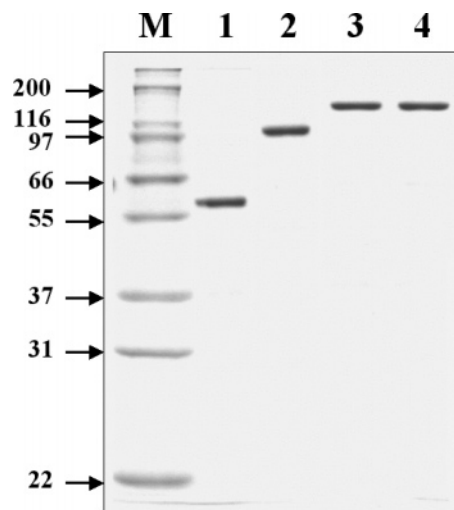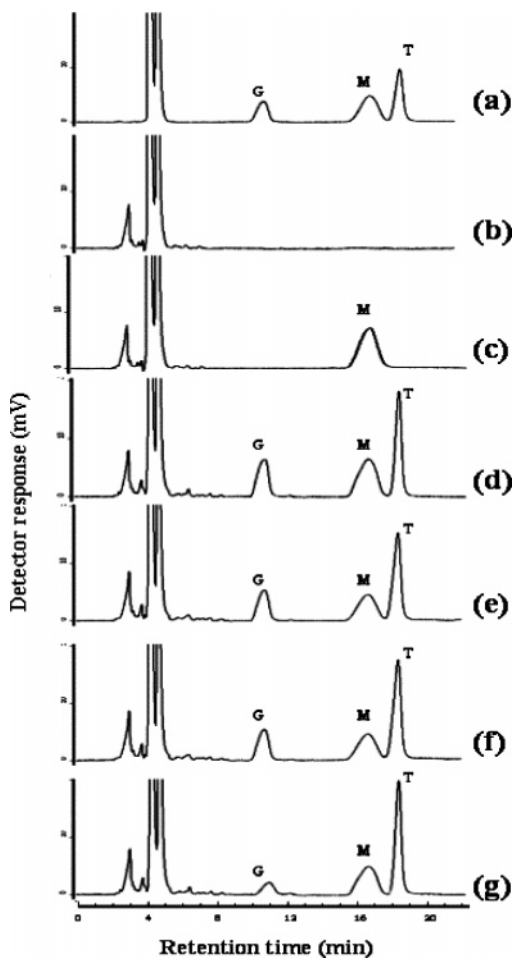

Figure 2. SDS-PAGE of the purified proteins BA, TS, BATS, and TSBA. The recombinant enzymes were purified by a succession of steps as described in the Materials and Methods. The purified proteins were analyzed by SDS-12% PAGE and stained with Coomassie brilliant blue. Lane M, protein size markers; lanes 1–4, 3 μg of purified BA, TS, BATS, and TSBA, respectively. The numbers on the left indicate the sizes of the markers (in kDa).

The estimated molecular masses of the recombinant BA, TS, BATS, and TSBA proteins were about 57, 106, 164, and 164 kDa, respectively. The sizes of the proteins observed on the gel were considered with those calculated from the deduced amino acid sequence (*40*, *41*).

Recombinant Enzyme-Catalyzed Trehalose Synthesis. Enzymatic activities of the purified recombinant BA, TS, BATS, and TSBA proteins were tested using maltose or starch as substrates. As shown in **Figure 3f,g**, both BATS and TSBA produced trehalose from starch. This demonstrated that BATS and TSBA were functional and able to catalyze two sequential reactions involving conversion from starch to maltose and then to trehalose. This result is consistent with the activity of the individual recombinant enzyme-catalyzed reactions. For example, the recombinant BA catalyzed the hydrolysis of starch to maltose, as shown in **Figure 3c**, and the recombinant TS catalyzed the synthesis of trehalose from maltose (**Figure 3d**). An equimolar mixture of these two enzymes produced trehalose from starch by catalyzing the sequential reactions (**Figure 3e**). The reactions catalyzed by the two enzymes are depicted in **Figure 4**. The rates of trehalose synthesis from starch by the BATS and TSBA fusion enzymes were at least 22 and 36% faster than that by an equimolar mixture of the individual enzymes (BA/TS), respectively (**Figure 5**).

Biochemical Properties of the Bifunctional Fusion Enzymes TSBA and BATS. Biochemical parameters of the recombinant TSBA and BATS were compared. The effect of temperature on the enzyme activity of trehalose formation was determined using starch as substrate at various temperatures ranging from 10 to 90 °C. As shown in **Figure 6**, the optimal temperature for all of the BATS, TSBA fusion enzyme, and the BA/TS mixture was 70 °C (**Figure 6a**). Their temperature dependence showed little difference even if the absolute activities of trehalose synthesis of BATS, TSBA, and BA/TS were different (data not shown). To determine differences in their stability against thermal denaturation, BATS, TSBA, and the equimolar mixture BA/TS were all incubated at various temperatures ranged from 10 to 90 °C for 30 min and cooled immediately (**Figure 6b**). At temperatures of 70 °C, both BATS

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Figure 3. Analysis of the products of the enzymatic reaction by HPLC. The purified recombinant enzymes were incubated for 2 h at 60 °C in 50 μL of assay buffer containing 50 mM maltose or 2% soluble starch as substrates. (**a**) A 12.5 mM concentration of each standard glucose, maltose, and trehalose; (**b**) 2% of standard starch; (**c**) reaction product of BA with 2% starch; (**d**) reaction product of TS with 50 mM maltose; and (**e**–**g**) reaction products of BA/TS mixture, BATS, and TSBA with 2% starch, respectively.

and TSBA retained about 95% of their original activities, which were obtained after incubation for 2 h at 60 °C, but the residual activity of BA/TS was significantly reduced. The thermostabilities of the recombinant fusion proteins were better than the individual recombinant enzymes.

For the pH test, enzyme activities at various pH values were assayed. As shown in **Figure 7**, the optimal pH was 6.0 for the BATS, TSBA fusion enzymes, and the BA/TS mixture (**Figure 7a**). To examine pH stability, enzymes were incubated at various pH values, and the residual activities were measured at pH 7.0. All enzymes were stable from pH 5.0 to 7.0, as shown in **Figure 7b**. The effects of metal ions and chemical reagents on the activity are shown in **Table 1**. The enzyme activity of BATS or TSBA was inhibited by Cu^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, and Tris. It was not significantly inhibited by Ca^{2+}, Fe^{3+}, Mg^{2+}, DTT, or EDTA.

Kinetic Properties of the Bifunctional Fusion Enzymes BATS and TSBA. To depict the enzymatic properties of the bifunctional fusion enzymes BATS and TSBA, the kinetic parameters were determined as shown in **Table 2**. These values were obtained using the Michaelis−Menten equation with Hyper Software (Synergy Software). The kinetic parameters for the overall synthesis of trehalose from starch were determined.
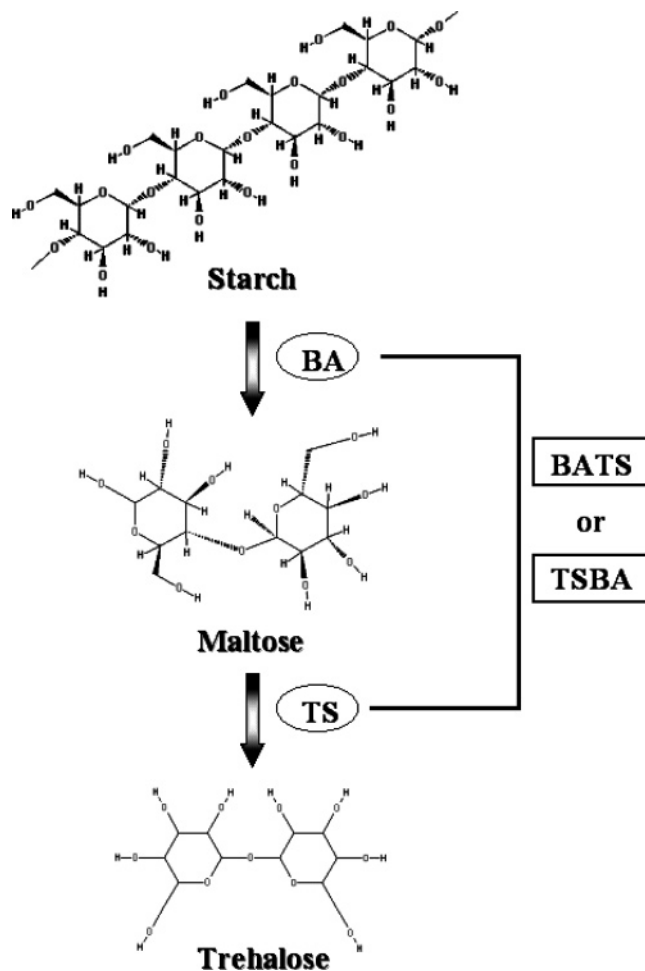

Figure 4. Overview of trehalose synthesis from starch by the fusion enzyme BATS or TSBA. BA catalyzes the hydrolysis of starch to maltose, which is further converted by TS to produce trehalose. The fusion protein, BATS or TSBA, consisting of two individual enzymes, catalyzes the sequential reactions.
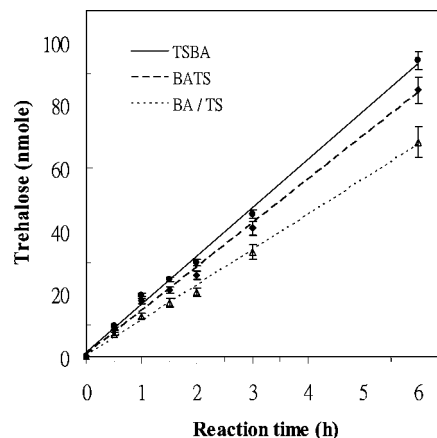
Figure 5. Time course for trehalose production from starch by the recombinant enzymes. Ten picomoles of TSBA (●), BATS (◆), or the BA/TS equimolar mixture (△) was incubated at 60 °C in a final 70 μL of assay buffer containing 2% soluble starch. Aliquots (10 μL) were withdrawn at the indicated times and quantified by HPLC.

These results were compared with those of a parallel assay with an equimolar mixture of BA/TS. The K_m values of BATS and TSBA for starch were 59 and 61%, respectively, lower than those of BA/TS. The k_{cat} value of TSBA for starch was 31% higher than that of BA/TS, but the k_{cat} value of BATS for starch

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(a)

(b)
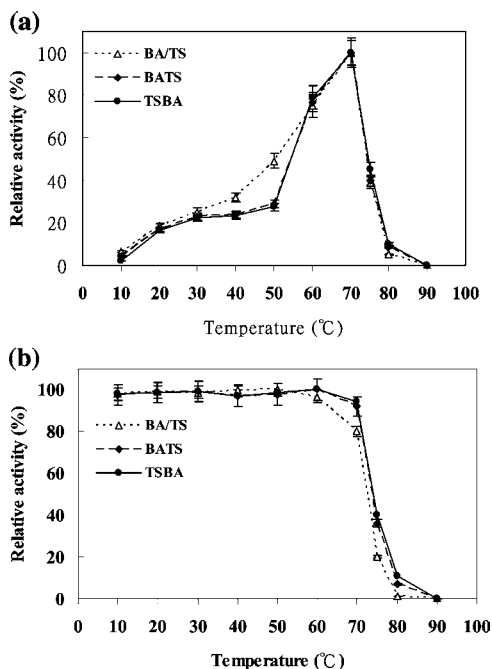
Figure 6. Effects of temperature on the activity and stability of TSBA, BATS, and the BA/TS equimolar mixture with starch as the substrate. (**a**) The enzyme activities at various temperatures (10–90 °C) were assayed. (**b**) To examine the thermal stability, the enzymes were incubated at various temperatures (10–90 °C) for 30 min and were immediately cooled. The residual activities were measured at 60 °C. TSBA, BATS, and the BA/TS equimolar mixture are symboled in ●, ◆, and △, respectively.

was similar to that of BA/TS. The catalytic efficiencies (k_{cat}/K_m) of the bifunctional fusion enzymes (BATS and TSBA) were 2.39 and 3.38 times greater than that of BA/TS, respectively.

The kinetic parameters for the TS activity were also measured at various concentrations of maltose. The catalytic efficiencies of TS and BATS were similar because they had the similar K_m and k_{cat} values. This suggested that the fusion pattern of BATS did not affect the kinetic properties of the TS activity. Interestingly, although the K_m value of TS was similar to that of TSBA, the k_{cat} value of TSBA was 31% higher than that of TS. The catalytic efficiency of TSBA for TS activity was 1.42 times greater than that of TS, implying that the fusion pattern of TSBA influenced the kinetic properties of the TS activity.

DISCUSSION

In the present work, we have successfully created a bifunctional fusion enzyme for efficient production of trehalose from starch at high temperature. First, we constructed the fusion enzymes, BATS and TSBA, by in frame fusion of structural genes for BA and TS (**Figure 1**) and overexpressed them in *E. coli*. Then, the capabilities of purified fusion proteins to produce trehalose at high temperatures were analyzed. Both fusion proteins for trehalose synthesis from starch were more efficient than an equimolar mixture of the two individual enzymes (**Figure 5**).

Kinetic parameters of the fusion enzymes BATS and TSBA were compared with those of a parallel assay with an equimolar mixture of BA/TS (**Table 2**). The catalytic efficiency, k_{cat}/K_m, of BATS and TSBA for both sequential reactions was 2.39 and 3.38 times the BA/TS mixture, respectively. The BATS catalytic efficiency was higher than that of BA/TS due to the reduced K_m value and an unchanged k_{cat} value. The improved catalytic activities of the BATS fusion enzymes might be due to a

(a)

(b)
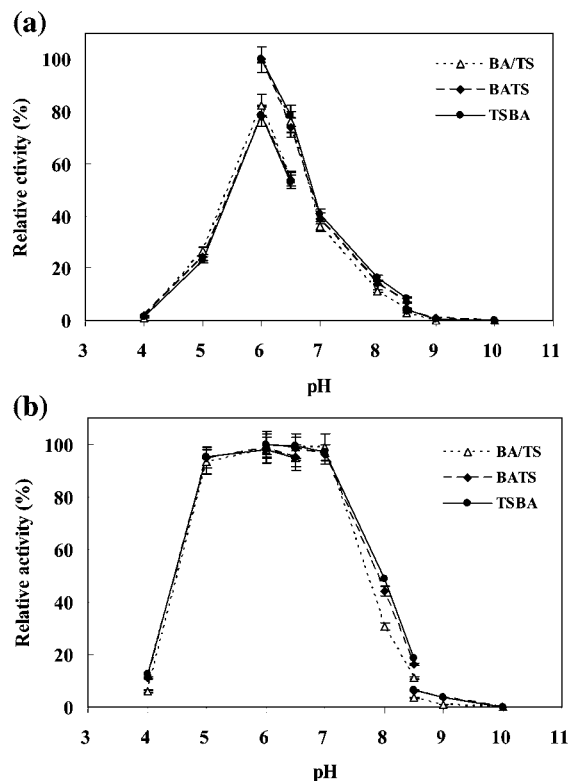
Figure 7. Effects of pH on the activity and stability of TSBA, BATS, and the BA/TS equimolar mixture with starch as substrate. Fifty millimolar concentrations of acetate buffer (pH 4.0–6.5), phosphate buffer (pH 6.0–8.5), and NaHCO$_3$–Na$_2$CO$_3$ buffer (pH 8.5–10.0) were used. (**a**) The enzyme activities at various pH values (pH 4.0–10.0) were assayed. (**b**) To examine pH stability, the enzyme was incubated at various pH values (pH 4.0–10.0) for 30 min at 60 °C. The residual activities were measured at pH 7.0. TSBA, BATS, and the BA/TS equimolar mixture are symboled in ●, ◆, and △, respectively.

Table 1. Effects of Metal Ions and Chemical Reagents on the Activity of the Fusion Enzymes BATS and TSBA with Starch as Substrate

| effector	concentration (mM)	relative activity (%)[a]	
		BATS	TSBA
none		100 ± 0.01	100 ± 0.01
AlCl$_3$	1.0	88 ± 0.08	90 ± 0.12
CaCl$_2$	1.0	96 ± 0.21	98 ± 0.18
CoCl$_2$	1.0	82 ± 0.01	82 ± 0.22
CuSO$_4$	1.0	16 ± 0.06	14 ± 0.02
FeCl$_3$	1.0	92 ± 0.03	96 ± 0.16
HgCl$_2$	1.0	22 ± 0.11	28 ± 0.09
MgCl$_2$	1.0	100 ± 0.38	102 ± 1.78
MnCl$_2$	1.0	86 ± 0.68	81 ± 0.68
NiCl$_2$	1.0	68 ± 0.12	70 ± 0.56
PbCl$_2$	1.0	54 ± 0.26	49 ± 0.02
ZnSO$_4$	1.0	41 ± 0.61	44 ± 0.14
DTT	1.0	100 ± 0.08	99 ± 0.28
EDTA	1.0	96 ± 0.18	98 ± 0.46
Tris	10.0	26 ± 0.09	28 ± 0.21

[a] The enzyme activity was measured in the presence of metal ions and chemical reagents under the standard assay conditions. The relative activity was expressed as a percentage of the enzyme activity in the absence of effectors. Data are the mean values and standard deviations of three independent experiments.

proximity effect. The proximity of enzymes allows the transfer of a reaction intermediate directly to the active site of the next enzyme in sequential reactions, and consequently, the overall reaction rate of the whole enzymatic process could be increased by preventing serious diffusion of intermediate (*37, 38*). It is

Table 2. Kinetic Parameters of Recombinant Enzymes TS, TSBA, BATS, and BA/TS with Different Substrates[a]

| | substrate | | | | | |
| | starch[b] | | | maltose | | |
enzyme	K_m (%)	k_{cat} (S^{-1})	k_{cat}/K_m ($\%^{-1} S^{-1}$)	K_m (mM)	k_{cat} (S^{-1})	k_{cat}/K_m ($mM^{-1} S^{-1} \times 10^{-3}$)
TS				37.63 ± 3.89	10.74 ± 1.02	285.40 ± 18.98
TSBA	3.66 ± 0.12	33.30 ± 2.66	9.10 ± 1.08	35.36 ± 2.96	14.40 ± 2.88	407.20 ± 38.12
BATS	3.89 ± 0.28	25.02 ± 1.26	6.44 ± 0.68	38.46 ± 0.86	11.07 ± 1.06	287.70 ± 16.89
BA/TS	9.49 ± 0.89	25.49 ± 2.04	2.69 ± 0.06			

[a] Data are the mean values and standard deviations of three independent experiments. [b] Parameters for overall sequential reaction from starch to trehalose.
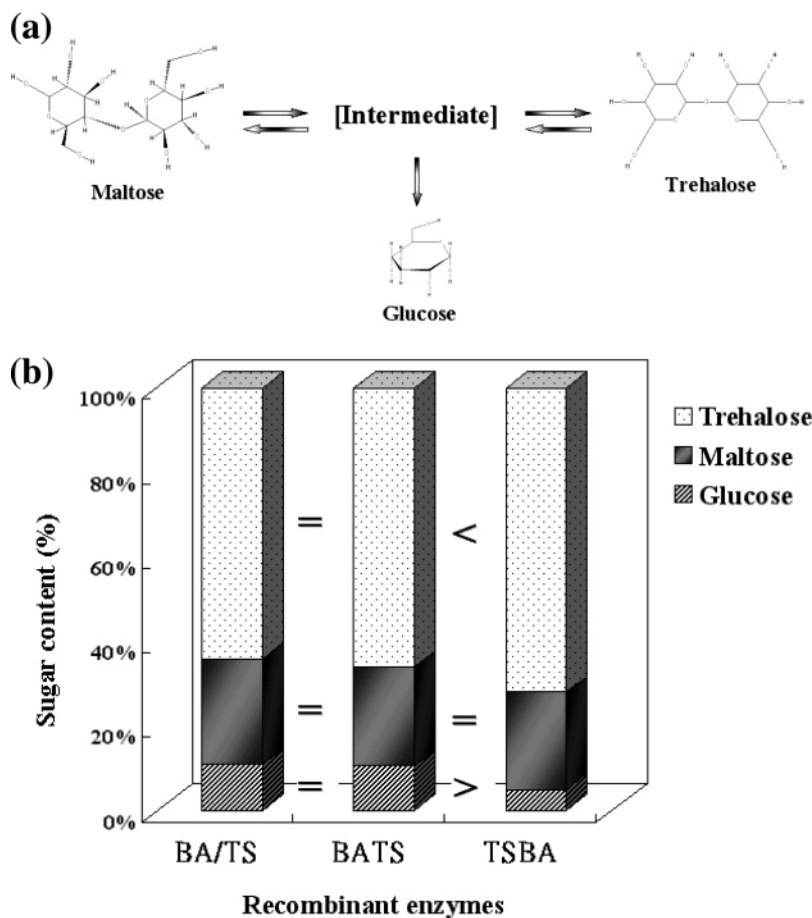
Figure 8. Overview of trehalose synthesis by TSBA, BATS, and the BA/TS equimolar mixture. (**a**) The formation of trehalose from maltose, which is related to intramolecular transglycosylation. TS converts maltose into trehalose in a reversible reaction, together with a small proportion of irreversibly formed glucose. (**b**) TSBA, BATS, and the BA/TS all had a weak hydrolytic activity, which increases at higher temperatures. In the trehalose synthesis reaction of TSBA, the glucose product was evidently reduced, and trehalose increased. These reactions were incubated for 24 h at 60 °C in assay buffer containing 2% soluble starch as substrate, and the reaction products were analyzed by HPLC.

interesting to note that the catalytic efficiency of TSBA with starch as substrate was 1.41 times that of BATS due to the increased k_{cat} value. The catalytic efficiency of TSBA with maltose as substrate for TS activity was also 1.43 times greater than that of TS due to the increased k_{cat} value (**Table 2**). Therefore, in addition to the proximity effect, the higher catalytic efficiency of TSBA over BATS might be due to the conformation change of TS in the TSBA construct, resulting in further improvement of k_{cat} and catalytic efficiency. Further analyses showed that the increase of TSBA catalytic efficiency over BATS is due to the reduction of glucose formation (**Figure 8b**). In another aspect, the yield of trehalose from maltose by TS decreased with increasing reaction temperature. The reason appears to be that TS has a weak maltose hydrolytic activity (**Figure 8a**) (*41*), which increases at higher temperatures (*44*). The thermostable TS from *T. thermophilus* catalyzes two-step

reactions that cleave the α-(1-4)-linkage in maltose and form the (1-1)-linkage in the nonreducing disaccharide trehalose. It also readily converts maltose into trehalose in a reversible reaction, together with a small proportion of irreversibly formed glucose. In this study, glucose of the product was evidently reduced, and trehalose increased in the trehalose synthesis reaction of TSBA, in which the C terminus of TS was fused with the BA in frame, as shown in **Figure 8b**. We suggest that the TSBA configuration, which might have a more rigid structure that was formed by BA fusing the C terminus of TS, might decrease the minor glucose formation side reaction and enhance efficiency of the major trehalose formation reaction.

Thermal stability experiments suggested no great differences in the structures of each enzyme moiety in the fusion enzyme and the individual enzymes even though BATS and TSBA were slightly more stable than the BA/TS mixture. The molecular

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Table 3. Comparison of the Molecular and Enzymatic Properties of Recombinant Enzymes BA/TS, BATS, and TSBA

property	BA/TS	BATS	TSBA
molecular mass (kDa)	57/106	164	164
optimum temperature (°C)[a]	70	70	70
optimum pH (pH)[b]	6.0	6.0	6.0
thermal stability[a]	up to 70 °C	up to 70 °C	up to 70 °C
	(80 ± 1.7%)[c]	(92 ± 4.1%)[c]	(95 ± 2.2%)[c]
pH stability [b]	pH 5.0–7.0	pH 5.0–7.0	pH 5.0–7.0
sugar composition of product (%)[d]	64:25:11	66:23.5:10.5	72:23:5

[a] The enzyme activity was measured at pH 7.0 for 2 h. [b] The enzyme activity was measured at 60 °C for 2 h. [c] The percentages indicated the relative residual activities at 70 °C to that measured at 60 °C. [d] The data indicated the ratio of trehalose, maltose, and glucose products and were obtained at 60 °C and pH 6.0 for 24 h of reaction.

and enzymatic properties of the recombinant enzymes BA/TS, BATS, and TSBA were quite similar, as shown in **Table 3**. These results indicated that fusion of the two enzymes did not cause significant structural perturbation or changes in physico-chemical properties. We therefore inferred that two thermally stable proteins joined by a suitable linker might form a harmonious structure that provides more steady conformation in reacting with substrates at high temperatures.

In conclusion, construction of a recombinant bifunctional fusion enzyme catalyzing sequential reactions can increase the efficiency of the enzymes and simplify the expression and protein purification in one process, as demonstrated with BATS and TSBA in this study. It also provides a convenient and efficient way to produce trehalose for industrial applications. The fusion BA and TS proteins significantly improves trehalose productivity, increasing the reaction rate, as a result of one single polypeptide chain, which has bifunctionality. Additionally, this thermostable and thermophilic fusion enzyme has highly at-tractive advantages when operating at high temperatures, thus solving some severe problems, such as microbial contamination and lower medium viscosity in the carbohydrate industry. In combination with our previous invention, which produces high-maltose syrup and a high-protein product simultaneously from low-price raw materials such as rice (*45, 46*), this fusion enzyme can be further used to efficiently produce high-value trehalose and nutritious high-protein food from staples such as rice at a low cost. Furthermore, the bifunctional genes can be transformed directly into rice or other plants. In our previously reported transgenic plant harboring a bifunctional thermostable amylop-ullulanase gene (*47, 48*), the seeds are anticipated to produce trehalose and protein-enriched flour for industrial uses and human consumption. These works are currently underway.

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