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Role of the C-Terminal Domain of *Thermus thermophilus* Trehalose Synthase in the Thermophilicity, Thermostability, and Efficient Production of Trehalose

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Trehalose synthase (TS) from *Thermus thermophilus* (TtTS) is a thermostable enzyme that catalyzes the conversion of maltose into trehalose by intramolecular transglucosylation. It has a relatively higher thermophilicity and thermostability and a better conversion ratio for trehalose production than other known TSs from different sources at present. By amino acid sequences and the schematic motif alignment of trehalose synthase-related enzymes, it was found that TtTS (965 amino acid residues) contains a particular C-terminal fragment that is not found in most other TSs. To verify the function of this fragment, C-terminal deletion and enzyme fusion were respectively performed to explain the important role this fragment plays in the formation of trehalose. First, the C terminus (TtTSΔN, 415 amino acid residues) of TtTS is deleted to construct a TtTSΔC containing 550 amino acids. Furthermore, a novel cold-active TS was cloned and purified from *Deinococcus radiodurans* (DrTS, 552 amino acid residues) and then a fusion protein was created with TtTSΔN at the C terminus of DrTS (DrTS−TtTSΔN). It was found that the recombinant TtTS△C enzyme had a lower thermostability and a higher byproduct than TtTS in catalyzing the conversion of maltose into trehalose. On the other hand, the recombinant DrTS−TtTSΔN enzyme had a higher thermostability and a lower byproduct than DrTS in their reactions. The above-mentioned results allowed the inference that the C terminus of TtTS plays a key role in maintaining its thermostability and hence in modulating the side reaction to reduce glucose production at a high temperature. A new, simple, and fast method to improve thermophilicity by fusing this fragment with particular conformation to a thermolabile enzyme is offered.

KEYWORDS: Trehalose; *Thermus thermophilus*; *Deinococcus radiodurans*; C terminus; thermostability; trehalose synthase

INTRODUCTION

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is a nonreducing disaccharide that is widely found in various organisms (*1*−*8*). In the literature, trehalose itself can protect proteins against denaturation due to desiccation and freezing (*9*, *10*). In addition, its water-holding capability has been applied to the development of additives, stabilizers, and sweeteners, which are useful in the food, cosmetic, and pharmaceutical industries (*11*−*14*). Due to its desirable physical and chemical characteristics, investigations have been focused on searching for efficient synthetic processes and abundant raw sources for the production of trehalose (*15*, *16*). At present, many trehalose-synthesizing enzyme systems have been reported in microorganisms (*17*−*21*). It is mainly accepted that trehalose can be metabolized in several ways, which involve different enzyme systems including trehalose-6-phosphate synthetase and trehalose-6-phosphate phosphatase (*16*, *22*, *23*), trehalose synthase (*20*, *24*), maltose phosphorylase and trehalose phosphorylase (*7*), glycosyltransferase (*25*), and maltooligosyltrehalose trehalohydrolase and maltooligosyltrehalose synthase (*17*).

Trehalose synthase (TS; EC 5.4.99.16) uses a very simple disaccharide as its substrate and converts it into trehalose in the absence of a coenzyme. This enzymatic process has the advantages of simple reaction, high substrate specificity, high conversion yield, and low cost. Therefore, it has great potential

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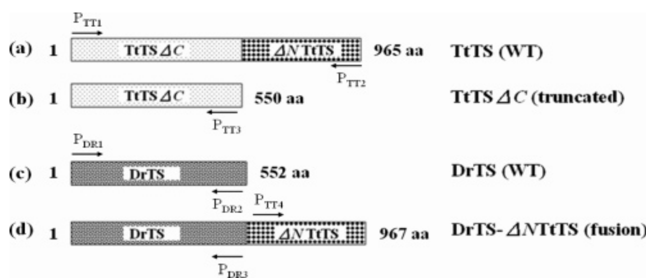

Figure 1. Expression cassettes of the enzymes TtTS, TtTSΔC, DrTS, and DrTS–TtTSΔN. The recombinant DNAs for TtTS (**a**), TtTSΔC (**b**), DrTS (**c**), and DrTS–TtTSΔN (**d**) were inserted into pET20b(+) and then were transformed into *E. coli* Tuner(DE3)pLysS to express recombinant enzymes as described under Materials and Methods.

in industrial application. However, starch bioprocessing usually involves two steps, namely, liquefaction and saccharification, which run at high temperatures. Thus far, the TS pathway has been identified in *Pimelobacter* sp. R48 (*20*), *Pseudomonas* sp. F1 (*21*), and a *Thermus* strain (*26*), and only TS from the *Thermus* strain was characterized as a thermostable enzyme. Three trehalose synthase genes from *Thermus thermophilus*, *Pimelobacter* sp. R48, and *Picrophilus torridus* have been cloned (*20*, *27*, *28*). Therefore, it is highly desirable to obtain thermostable TS from organisms or by engineering existing TSs to improve their performance.

In the present study, we have cloned and functionally expressed a novel cold-active TS from *Deinococcus radiodurans* (ATCC 13939) (DrTS) and a thermostable TS from *T. thermophilus* (ATCC 33923) (TtTS). We have elucidated the determinant that affects the thermostability of TtTS by protein engineering. The role of this determinant was further confirmed by fusing it with DrTS to improve the thermostability of DrTS.

MATERIALS AND METHODS

Materials. Deoxynucleotides and enzymes for DNA restriction and modification were purchased from Roche (Branchburg, NJ). All saccharides including maltose, trehalose, and starch were purchased from Sigma (St. Louis, MO). Protein assay reagents and dyes were from the Bio-Rad Laboratories (Richmond, CA). Columns for protein separation were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Other chemicals and reagents were of analytical grade.

Bacterial Strains and Cultivation. *T. thermophilus* and *D. radiodurans* 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. *D. radiodurans* ATCC 13939 was grown in 86 Nutrient agar with 1% glucose (ATCC medium), pH 7.0, at 30 °C.

Construction of Recombinant Expression Plasmids. Genes encoding DrTS and TtTS were cloned from *D. radiodurans* and *T. thermophilus* genomic DNA, respectively. The ORF of *DrTS* (GenBank accession no. AE000513) was 1659 bp long and encoded 552 amino acids (*29*), whereas the ORF of *TtTS* (GenBank accession no. AY275558) was 2898 bp long and encoded 965 amino acid residues (*27*).

The TtTS gene was obtained from *T. thermophilus* genomic DNA by *Pfu* PCR with the primers P_{TT1} (5′-GGAATTCCAT**ATG**GAC-CCCCTCTGGTACAAGG-3′) and P_{TT2} (5′-GCCGACGTCGAC**CTA**G-GCTTTTCCGGCCTTGG-3′), which contain the translation initiation and termination codons (bold) of the *T. thermophilus* TS gene, respectively (**Figure 1a**). The *Nde*I and *Sal*I restriction enzyme sites (underlined) were introduced into P_{TT1} and P_{TT2}, respectively. The amplified DNA was ligated into *Nde*I- and *Sal*I-digested pET-20b(+) (Novagen, Madison, WI) to produce pET-TtTS.

To make an expression construct for the TtTSΔC truncated enzyme, the 415 amino acid C-terminal end of the *TtTS* gene was truncated. The TtTSΔC gene was amplified in the same manner with the primers P_{TT1} and P_{TT3} (5-GCCGACGTCGAC**CTA**GGAGGGGAGGTGGAGC-3′). The primer P_{TT3} additionally contained a translation termination codon (bold) (**Figure 1b**). The *Nde*I and *Sal*I restriction enzyme sites (underlined) were introduced into P_{TT1} and P_{TT3}, respectively. The amplified DNA was ligated into *Nde*I- and *Sal*I-digested pET-20b(+) to produce pET-TtTSΔC.

The DrTS gene was obtained from *D. radiodurans* genomic DNA by *Pfu* PCR with the primers P_{DR1} (5′-ATACAT**ATG**ACCCAGGCA-CACCCG-3′) and P_{DR2} (5′-CGGAATTC**TCA**ATTCAACCGCAG-3′), which contained the translation initiation and termination codons (bold) of the *D. radiodurans* TS gene, respectively (**Figure 1c**). The *Nde*I and *Eco*RI sites (underlined) were introduced into P_{DR1} and P_{DR2}, respectively. The amplified DNA was ligated into *Nde*I- and *Eco*RI-digested pET-20b(+) to produce pET-DrTS.

To make an expression construct for the DrTS−TtTSΔN fusion enzyme, the *DrTS* gene was fused with a TtTSΔN DNA fragment at its C-terminal end. The TtTSΔN DNA fragment encodes the 415 amino acid C-terminal end of the TtTS. The *DrTS* gene was amplified in the same manner with the primers P_{DR1} and P_{DR3} (5′-CGGAATTCAT-TCAACCGCAGCC-3′). The primer P_{DR3} had no termination codon of the *D. radiodurans* TS gene. The *Nde*I and *Eco*RI restriction enzyme sites (underlined) were introduced into P_{DR1} and P_{DR3}, respectively. The amplified DNA was ligated into *Nde*I- and *Eco*RI-digested pET-20b-(+) to produce pET-DrTSd. The TtTSΔN fragment was amplified with the primers P_{TT4} (5′-CCGGAATTCCCCGACTGGGCCGAGGAGCC-3′) and P_{TT2}, digested with *Eco*RI and *Sal*I and then ligated into *Eco*RI- and *Sal*I-digested pET-DrTSd to produce pET-DrTS−TtTSΔN plasmid (**Figure 1d**).

Expression of Recombinant Enzymes. The *Escherichia coli* strains Tuner(DE3)pLysS (Novagen) were used to express the recombinant enzymes. Tuner(DE3)pLysS, transformed with the expression cassette (pET-TtTS, -TtTSΔC, -DrTS, or -DrTS−TtTSΔN), was inoculated into 2 L of Luria−Bertani (LB) medium supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol in combination and then was grown at 37 °C. When the optical density of the culture media at 590 nm reached 0.8, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM to induce the protein expression. Incubation was continued for another 12 h at 20 °C before 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 the cells were disrupted with a French Pressure Cell Press (Thermo Spectronic) for three cycles. The soluble fractions of the lysated cells were collected using 30 min of centrifugation at 15000*g* at a temperature of 4 °C. The crude extract of the recombinant enzyme was collected for purification.

Purification of Recombinant Enzymes. The crude extracts of the recombinant enzymes were purified by using the following steps. To purify the recombinant DrTS, DrTS−TtTSΔN, TtTS, and TtTSΔC, solid $(NH_4)_2SO_4$ was added to the cell-free extract to achieve 70% saturation. The resulting precipitate was collected and dissolved in 50 mM sodium phosphate buffer (pH 7.0) and then was dialyzed against the same buffer. The dialyzed solution was loaded on a DEAE-Sepharose column (160 × 10 mm, Pharmacia Biotech) equilibrated with a 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 loaded on a butyl-Sepharose column (160 × 10 mm) equilibrated with a 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 1−0 M $(NH_4)_2SO_4$ in the same buffer. The active fractions were pooled and then dialyzed against a 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 × 10 mm) equilibrated with a 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 through ultrafiltration using a 30000 or 50000 molecular weight cutoff membrane (Amicon Ultra, Millipore, Bedford, MA), and then it was pooled as a purified enzyme preparation.

(a)

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TtTS       ..........MDPLWYKDAVIYQLHVRSFFDANNDGYGDFEGLRRKLPYLEELGVNTLWL   50
PsTS       MSIAESTVLGEEPEWFRTAVFYEVLVRSFRDPNAGGTGDFRGLAERLDYLQWLGVDCLWV   60
DrTS       .......MTQAHPEWYKSAVFYELSVRTFQDGNGDGKGDFPGLTSRLDYLKNLGVDCLWL   53
Consensus            p w   av y   vr f d n   g gdf gl    l yl   lgv   lw

TtTS       MPFFQSPLRDDGYDISDYYQILPVHGTLEDFKRFLDEAHGRGMKVIIELVLNHTSIDHPW  110
PsTS       PPFFSSPLRDGGYDVADYTGILPEIGTVEDFHAFLDGAHERGIRVIIDFVMNHTSDAHPW  120
DrTS       LPWFPSPLRDDGYDVADYRGIHPDLGTLDDFKVFLREAHARGLRVIGDLVTNHTSSDHPW  113
Consensus   p f splrd gyd  dy  i p  gt  df  fl  ah rg  vi   v nhts  hpw

TtTS       FQEARKPGSPMRDW.......YYWSDTPEKYKGVRVIFKDFETSNWTFDPVAKAYYWHRF  163
PsTS       FQASRSD...PDGPYGDF...YVWSDTDELYQDARVIFVDTEPSNWTWDQTRGQYYWHRF  174
DrTS       FQAARRGPTLPDGSPNEYHDYYVWSDEGKEYADTRIIFTDTEVSNWTLDEQAGKYYWHRF  173
Consensus  fq  r                yvwsd    y   r if d e snwt d      yywhrf

TtTS       YWHQPDLNWDNPEVEKAIHQVMFFWADLGVDGFRLDAIPYLYEREGTSCENLPETIEAVK  223
PsTS       FHHQPDLNFDNPKVQDAMLEAMAFWLDMGLDGFRLDAVPYLYERPGTNGENLPETHEMLK  234
DrTS       FASQPDLNYDNPKVVEELHGAARFWLDLGLDGFRVDAVPYLIEREGTSCENLPETHEILK  233
Consensus    qpdln dnp v            fw d g dgfr da pyl er gt  enlpet e  k

TtTS       RLRKALEERYGPGKILLAEANMWPEETLPYFGDGDGV.........HMAYNFPLMPRIFM  274
PsTS       RVRRFVDDNYPDRV.LLYEANQWPTDVVEYFGPEEREDGTVVGPESHMAFHFPVMPRIFM  293
DrTS       GFRAMVDREYPGRL.LLAEANQWPEEVVEYF.......GTEAEPEFHMCFNFPVMPRLYM  285
Consensus   r       y      ll ean wp     yf                hm   fp mpr  m

TtTS       ALRREERGPIETMLKETEGIPETAQWALFLRNHDELTLEKVTEEEREFMYEAYAPDPKFR  334
PsTS       AVRRESRFPISEIMEQTPAIPEGCQWGIFLRNHDELTLEMVTDEDRDYMWGEYAKDPRMK  353
DrTS       SLKREDTSSIREIMGRLPKIPSFGQWCTFLRNHDELTLEMVTDDERAFMYAAYAPDARMK  345
Consensus     re    i         ip   qw  flrnhdeltle vt   r  m   ya d

TtTS       INLGIRRRLMPLLGGDRRRYELLTALLLTLKGTPIVYYGDEIGMGDNPFLGDRNGVRTPM  394
PsTS       ANIGIRRRLAPLLDNDTNQIELFTALLLSLPGSPVLYYGDEIGMGDNIWLGDRDGVRTPM  413
DrTS       INVGIRRRLAPLLDNDRRRIELLNTVLLALPGSPILYYGDEIGMGDDLGLPDRNGVRTPM  405
Consensus   n girrrl pll  d    el    ll l g p  yygdeigmgd   l dr gvrtpm

TtTS       QWSQDRNAGFSRAPYHALFLPPVSEGPYSYHFVNVEAQRENPHSLLSFNRRFLALRNQHA  454
PsTS       QRTPDRNVGFSAATPGKLHLPTIQDPVYGYQSVNVEAQLENPSSLLHWTRRMIHIRRQRD  473
DrTS       QMNAGTSGGFSTAQPSDCFFPPIQDPVYGFGRVNVQSQLQDPSSLLKWTARQLELRRAHP  465
Consensus  q       gfs a       p     y    vnv  q   p sll    r     r

TtTS       KIFGRGSLTLLPVENRRVLAYLREHEGERVLVVA....NLSRYTQAFDLPLEAYQGLVPV  510
PsTS       A.FGLGTFEDLGGSNPAVLSYVRELPGDGGDDVILCVNNLSRFPQPVELDLRKYEGRVPV  532
DrTS       A.FAHGDLTFIETGNPAILAFTRQYDGETLLIVSNFAGNAQAGL....LDLAPFVGRAPV  520
Consensus    f  g          n   l   r   g     v     n          l l     g pv

TtTS       ELFSQQPFPPVEGRYR.LTLGPHGFALFALKPVEAVLHLPSPDWAEEPAPEEADLPRVHM  569
PsTS       ELIGGVPFPAVGELPYLLTLSGHGF.YWFRLTDPDTTGRPVL..................  573
DrTS       TLSGASPLPVVTGNGQYPVVMGKYDYYWLRLN............................  552
Consensus  l     p p v

TtTS       PGGPEVLLVDTLVHERGREELLNALAQTLKEKSWLALKPQKVALLDALRFQKDPPLYLTL  629
PsTS       ............................................................  573
DrTS       ............................................................  552
Consensus

TtTS       LQLENHRTLQVFLPLLWSPQRREGPGLFARTHGQPGYFYELSLDPGFYRLLLARLKEGFE  689
PsTS       ............................................................  573
DrTS       ............................................................  552
Consensus

TtTS       GRSLRAYYRGRHPGPVPEAVDLLRPGLAAGEGVWVQLGLVQDGGLDRTERVLPRLDLPWV  749
PsTS       ............................................................  573
DrTS       ............................................................  552
Consensus

TtTS       LRPEGGLFWERGASRRVLALTGSLPPGRPQDLFAALEVRLLESLPRLRGHAPGTPGLLPG  809
PsTS       ............................................................  573
DrTS       ............................................................  552
Consensus

TtTS       ALHETEALVRLLGVRLALLHRALGEVEGVEGGHPLLGRGLGAFLELEGEVYLVALGAEKR  869
PsTS       ............................................................  573
DrTS       ............................................................  552
Consensus

TtTS       GAVEEDLARLAYDVERAVHLALEALEAELWAFAEEVADYLHAAFLQAYRSALPEEALEEA  929
PsTS       ............................................................  573
DrTS       ............................................................  552
Consensus

TtTS       GWTRHMAEVAAEHLHREERPARKRIHERWQAKAGKA                          965
PsTS       ....................................                          573
DrTS       ....................................                          552
Consensus
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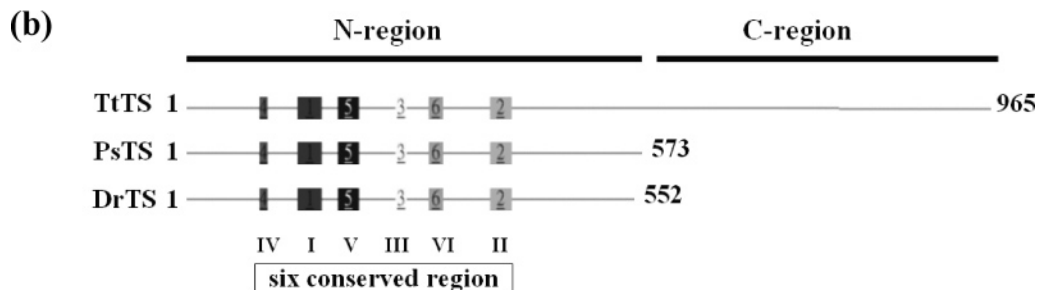

Figure 2. Sequence comparison of *D. radiodurans* trehalose synthase (DrTS) with other microorganisms' trehalose synthase. (**a**) Multiple alignment of trehalose synthases from *T. thermophilus* (TtTS), *Pimelobacter* sp. (PsTS), and DrTS. The alignment was performed with DNAMAN 4.1.1.1 software (Lynnon BioSoft). Residues that are identical are shaded in black boxes, whereas conserved residues are shaded in gray boxes. The dashed spaces represent gaps to maximize alignment. (**b**) Schematic alignment of the trehalose synthase-related enzymes. The alignment was performed with the program MEME (Multiple EM for Motif Elicitation) of Wisconsin Package (SeqWeb 2.01). There are six conserved motifs among these sequences.

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. The proteins were visualized by staining with Coomassie Brilliant Blue R-250 according to the standard procedures. Proteins were measured according to the method of Bradford using a protein assay kit from Bio-Rad Laboratories with bovine serum albumin as 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 6.5), 50 mM maltose, and the enzyme in a final volume of 100 μL was incubated at 65 °C (for TtTS), 40 °C (for TtTSΔC and DrTS−TtTSΔN), and 15 °C (for DrTS) for 2 h, respectively. Then this reaction mixture was heated at 100 °C for 10 min to stop the reaction. The trehalose produced was measured by the high-performance liquid chromatography (HPLC) system as described below. Then the values of the data and standard deviations were calculated from each of the triplicate samples with three independent experiments. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of trehalose per minute.

The effects of temperature on the enzyme activities of trehalose formation were determined at various temperatures ranging from 10 to 90 °C for TtTS and TtTSΔC and from 5 to 80 °C for DrTS and DrTS−TtTSΔN. To determine the stability against thermal denaturation, the enzymes were incubated at various temperatures for 30 min and then were cooled immediately to assay the residual activity. To examine the pH stability of the DrTS enzyme, the enzyme was incubated at various pH values, and the residual activity was measured at pH 7.0. The effects of temperature on glucose formation catalyzed by TSs were performed by incubating the enzyme reactions for 24 h at various temperatures ranging from 30 to 80 °C for TtTS and TtTSΔC and from 10 to 50 °C for DrTS and DrTS−TtTS△N in an assay buffer containing 50 mM maltose as substrate. The effects of metal ions and chemical reagents on the activity of the enzymes DrTS and TtTS were determined in an assay buffer containing 1 mM metal ions or 10 mM chemical reagents under the standard assay conditions.

Analysis of Carbohydrate. The quantitative analysis of sugar was carried out by an HPLC system equipped with refractive index detector (RID) and a computerized processing unit (Chrom Manager multisystem, version 5.2). The columns used included an APS2 analytical column (250 × 4.6 mm, ThermoHypersil, Bellefonte, PA) and a guard column (10 × 4.6 mm, ThermoHypersil). 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

Molecular Cloning of DrTS Gene. Bacterial genomic DNA containing a gene encoding a putative DrTS was cloned from *D. radiodurans*. The complete nucleotide sequence of the DrTS ORF was 1659 bp long and encoded 552 amino acid residues. The estimated molecular mass of the polypeptide was 60.7 kDa, and the predicted p*I* value was 4.94. The deduced amino acid sequence of DrTS showed 26 and 50% sequence identity to those of *T. thermophilus* and *Pimelobacter* sp., respectively (**Figure 2a**). Through bioinformatics analysis, it was noted that the deduced amino acid sequences encoded by the *DrTS* gene contained six motifs which were highly conserved with other trehalose synthases (**Figure 2b**).

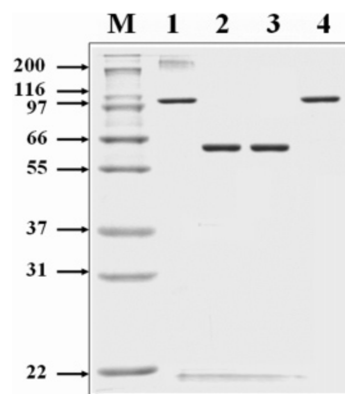

Figure 3. SDS-PAGE of the purified proteins TtTS, TtTSΔC, DrTS, and DrTS–TtTSΔN. The recombinant enzymes were purified by a succession of steps as described under Materials and Methods. The purified proteins were analyzed by SDS–10% PAGE and were stained with Coomassie Brilliant Blue. Lane M contains protein size markers. Lanes 1–4 represent 3 μg of purified TtTS, TtTSΔC, DrTS, and DrTS–TtTSΔN, respectively. The numbers on the left indicate the sizes of the markers (in kilodaltons).

Characterization of the Recombinant Trehalose Synthases. Recombinant enzymes produced in *E. coli* were purified to homogeneity as determined by SDS-PAGE analysis (**Figure 3**). The estimated molecular masses of the recombinant TtTS, TtTSΔC, DrTS, and DrTS−TtTSΔN proteins were about 106, 61, 61, and 106 kDa, respectively. The enzymatic activities of the purified recombinant TtTS, TtTSΔC, DrTS, and DrTS−TtTSΔN proteins were tested using maltose as substrates. As depicted in **Figure 4**, all TtTS, TtTSΔC, DrTS, and DrTS−TtTSΔN produced trehalose from maltose. This demonstrated that TtTS, TtTSΔC, DrTS, and DrTS−TtTSΔN are functional and able to catalyze the reaction involving conversion from maltose to trehalose and releasing a small amount of glucose. These results are consistent with the activity of other reported TS-catalyzed reactions (*20*, *24*).

The dependence of the DrTS activity on temperature ranging from 5 to 80 °C was assayed (**Figure 5a**). The optimal temperature for the DrTS enzyme was 15 °C. After incubation at the temperature of 60 °C for 2 h, the DrTS enzyme almost lost its activity. The biochemical characterization of the

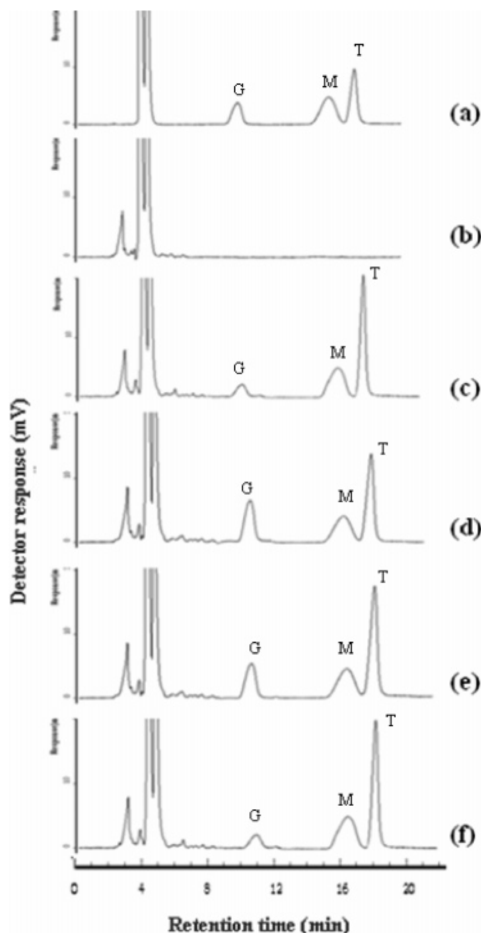

Figure 4. Analysis of the products of the TS-catalyzed reaction by HPLC. The purified recombinant enzymes were incubated for 2 h in 100 μL of assay buffer containing 50 mM maltose as substrate under the conditions described under Materials and Methods: (**a**) 12.5 mM of each standard glucose, maltose, and trehalose; (**b**) reaction buffer; (**c**, **d**, **e**, and **f**) reaction products of the TtTS, TtTSΔC, DrTS, and DrTS–TtTSΔN enzymes, respectively. G, glucose; M, maltose; T, trehalose.

recombinant DrTS expressed in *E. coli* showed that it was a highly cold-active enzyme, which can efficaciously convert maltose into trehalose at 5−20 °C. It displayed fine enzyme activity and a high yield of trehalose at low temperatures.

The pH dependency of the enzyme activity was measured. As shown in **Figure 5b**, the optimal pH was 6.5 for the DrTS enzyme. The enzyme was stable in a range of pH from 5.0 to 8.0. The effects of metal ions and chemical reagents on the activities of DrTS and TtTS are shown in **Table 1**. The activities of both enzymes were significantly inhibited by Cu^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+}. In particular, TtTS was highly inhibited by Tris, whereas DrTS was not sensitive to Tris.

Effects of the C-Terminal Domain of TtTS (TtTSΔN) on Thermophilicity and Thermostability. The effects of temperature on the enzyme activities of trehalose formation were assayed. As shown in panels **a** and **b** of **Figure 6**, the optimal temperatures for TtTS, TtTSDC, DrTS, and DrTS−TtTSDN were 65, 40, 15, and 40 °C, respectively. For the stability against thermal denaturation, after incubation at 80 °C, TtTS retained about 88% of the activity at 65 °C. However, the residual activity of TtTSDC at the same temperature (80 °C) was significantly reduced, as shown in **Figure 6c**. Obviously, the C-terminal truncation not only lowered the optimal temperature but also significantly decreased the tolerance of TtTS to thermal denaturation. At the temperature of 60 °C, DrTS−TtTSDN retained about 83% of the activity at 20 °C, whereas the residual activity of DrTS at the same temperature was nearly nonexistent as shown in **Figure 6d**. Therefore, the TtTSΔN fusion increased both the optimal temperature and the thermostability of the DrTS enzyme. These results indicated that the C-terminal domain (TtTSΔN) of TtTS might play an important role in the thermostability of TtTS and DrTS−TtTSΔN.

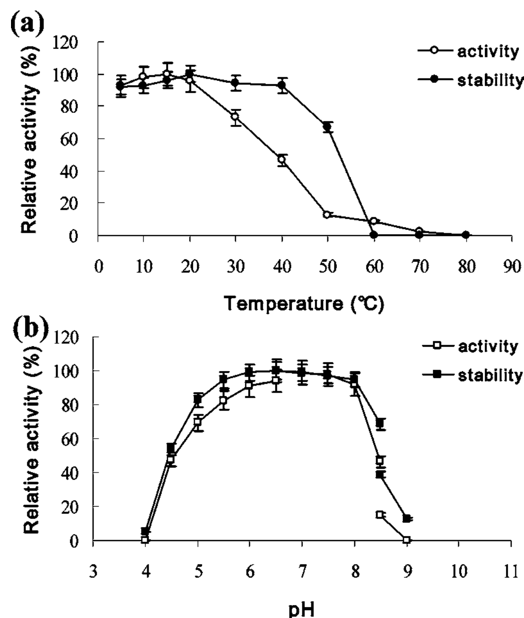

Figure 5. Effects of temperature and pH on the activity and stability of DrTS with maltose as substrate. (**a**) Enzyme activities at various temperatures (5–80 °C) were assayed. To examine the thermal stability, the enzymes were incubated at various temperatures (5–80 °C) for 30 min and then were immediately cooled. The residual activities were measured at 15 °C: (○) activity; (●) stability. (**b**) The 50 mM acetate buffer (pH 4.0–6.5), phosphate buffer (pH 6.0–8.5), and $NaHCO_3$–Na_2CO_3 buffer (pH 8.5–9.0) were used to assay the enzyme activities at various pH values (pH 4.0–9.0). To examine pH stability, the enzymes were incubated at various pH values (pH 4.0–9.0) for 30 min at 15 °C. The residual activities were measured at pH 7.0: (□) activity; (■) stability.

Table 1. Effects of Metal Ions and Chemical Reagents on the Activity of the Enzymes DrTS and TtTS with Maltose as Substrate

effector	concentration (mM)	relative activity[a] (%) DrTS	TtTS
none		100 ± 0.01	100 ± 0.01
$AlCl_3$	1.0	88 ± 0.28	91 ± 0.16
$CaCl_2$	1.0	96 ± 0.62	97 ± 0.19
$CoCl_2$	1.0	87 ± 0.11	85 ± 0.52
$CuSO_4$	1.0	16 ± 0.46	18 ± 0.32
$FeCl_3$	1.0	91 ± 0.15	97 ± 0.81
$HgCl_2$	1.0	26 ± 0.19	32 ± 0.16
$MgCl_2$	1.0	100 ± 0.71	98 ± 0.38
$MnCl_2$	1.0	88 ± 0.28	82 ± 0.67
$NiCl_2$	1.0	65 ± 0.17	72 ± 0.58
$PbCl_2$	1.0	56 ± 0.37	53 ± 0.12
$ZnSO_4$	1.0	43 ± 0.56	48 ± 0.54
DTT	1.0	100 ± 0.18	100 ± 0.22
EDTA	1.0	98 ± 0.27	98 ± 0.96
Tris	10.0	91 ± 0.19	26 ± 0.41

[a] 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. The data represent the mean values and standard deviations of three independent experiments.

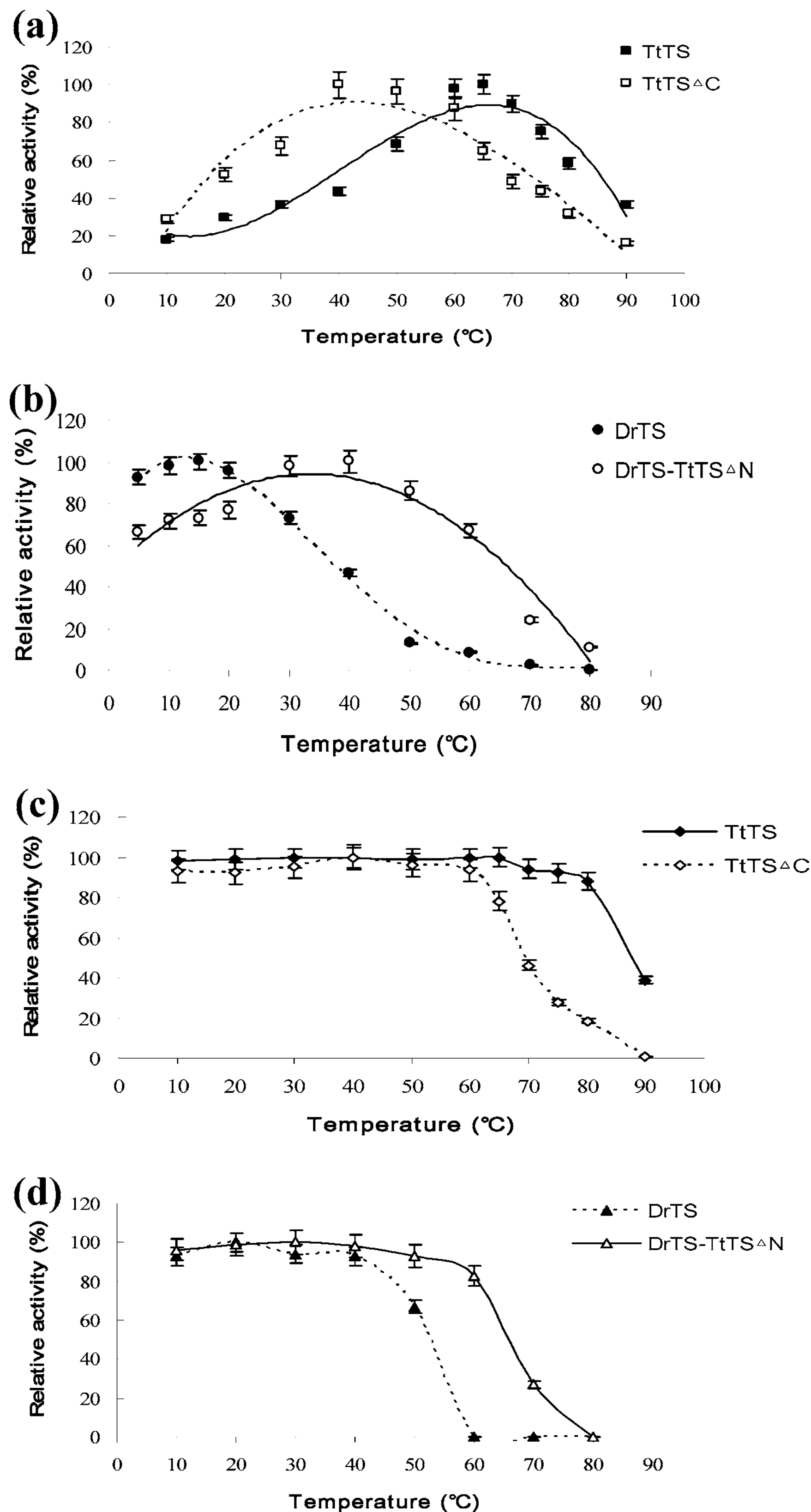

Figure 6. Effects of temperature on the activities and stabilities of TtTS, TtTSΔC, DrTS, and DrTS–TtTSΔN with maltose as substrate: (**a**) enzyme activities of TtTS (■) and TtTSΔC (□) at various temperatures (10–90 °C) were assayed; (**b**) enzyme activities of DrTS (●) and DrTS–TtTSΔN (○) at various temperatures (5–80 °C) were assayed; (**c** and **d**) to examine thermal stability, enzymes were incubated at various temperatures (10–90 °C) for 30 min and were immediately cooled. The residual activities of TtTS (◆) and TtTSΔC (◇) were measured at 65 and 40 °C, respectively. The residual activities of DrTS (▲) and DrTS–TtTSΔN (△) were measured at 15 and 40 °C, respectively.

Effects of the C-terminal domain of TtTS (TtTSΔN) on the Glucose Formation of TS-Catalyzed Reaction. The conversion of maltose into trehalose by TS is related to intramolecular transglucosylation, and it is a reversible reaction. However, TS also catalyzes a small proportion of irreversible glucose formation (*30*). TtTS, TtTSΔC, DrTS, and DrTS−

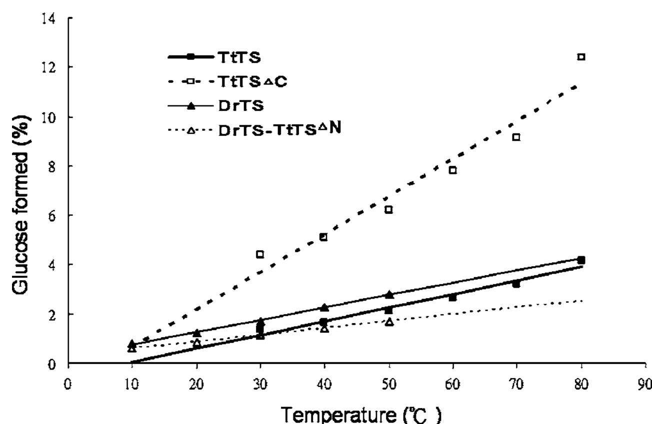

Figure 7. Effects of temperature on the glucose formation catalyzed by trehalose synthase. TtTS, TtTSΔC, DrTS, and DrTS–TtTSΔN catalyzed hydrolytic reactions for 24 h at various temperatures in an assay buffer containing 50 mM maltose as substrate, and the glucose product was analyzed by HPLC.

Table 2. Comparison of the Molecular and Enzymatic Properties of Trehalose Synthases from Different Sources

property	trehalose synthase		
	TtTS	PsTS[a]	DrTS
molecular mass (kDa)	106	62	61
optimum temperature[b] (°C)	65	20	15
optimum pH[c]	6.5	7.5	6.5
thermal stability[b]	up to 80 °C (88 ± 2.6%)[d]	up to 30 °C	up to 40 °C (92 ± 4.7%)[d]
pH stability[c]	pH 6.0–9.0	pH 6.0–9.0	pH 5.5–8.0
yield of trehalose from maltose (%)	80 ± 5.2[e]		92 ± 3.1[e]
to contain I–VI conserved regions	yes	yes	yes

[a] The data on PsTS properties were obtained from ref *20.* [b] The enzyme activity was measured at pH 7.0 for 2 h. [c] The activities of TtTS and DrTS were measured for 2 h at 65 and 15 °C, respectively. [d] The percentages indicated the residual activities of TtTS and DrTS at 80 and 40 °C relative to those measured at 65 and 15 °C, respectively. [e] The percentages indicated the yield of trehalose from maltose by TtTS and DrTS and were obtained at 30 and 5 °C, respectively, and at pH 7.0 for a 48 h reaction.

TtTSΔN all had a weak hydrolytic activity, which increased with elevated temperature (**Figure 7**). There was a significant relationship between the glucose formed and the elevated temperature. The slope of each line indicated the hydrolytic activity of TS enzymes. The slope of TtTS was similar to that of DrTS. Interestingly, the slope of TtTSΔC was higher than that of TtTS, and the slope of DrTS−TtTSΔN was smaller than that of DrTS. These results suggested that the truncation and fusion patterns of the wild-type TSs do affect the hydrolytic activity properties and implied that the TtTSΔN fragment plays a key role in reducing the hydrolytic activity.

DISCUSSION

The thermostable TtTS particularly has an extra C-terminal domain that is missing in many other TSs including DrTS. In the present work, the experimental data verified that this C terminus could improve thermostability, thermophilicity, and trehalose yield by reducing the uneconomical side reactant (glucose) in the whole reaction.

A comparison of the properties of TSs from different sources is shown in **Table 2**. As a matter of fact, TtTS had a sufficiently greater molecular mass due to the existence of an extra C-terminal region and showed higher optimal temperature and thermostability. To understand the role of the C-terminal domain of TtTS, a deletion form of wild-type TtTS in the C-terminal region (TtTSΔC) and a fusion form of wild-type DrTS (DrTS−TtTSΔN) were generated. The C-terminal deletion spanning residues 551−965 of TtTS, not comprising the six conserved regions (**Figure 2b**) and the TS active site, apparently did not affect the structural integrity and enzyme activity of TtTS (**Figure 4d**). However, this deletion significantly reduced the optimal temperature and thermostability of TtTS (**Figure 6a**,**c**). Therefore, the existence of the C-terminal domain in TtTS could stabilize the enzyme. This stabilization effect was further proven by fusing it at the C-terminal end of the cold-active DrTS. Notably, the thermophilicity and thermostability of the DrTS−TtTSΔN fusion were significantly improved compared with those of DrTS (**Figure 6b**,**d**).

On the other hand, all TSs had a weak hydrolytic activity to produce a byproduct of glucose, which decreased the trehalose yield when the reaction temperature increased. The existence of the C-terminal domain of TtTS caused the recombinant TtTS and DrTS−TtTSΔN enzymes to have a lower byproduct than TtTSΔC and DrTS (**Figure 7**). We suggest that the C-terminal domain of TtTS would change the overall enzyme conformation and provide a more rigid structure when reacting with substrates at high temperatures. This effect decreased the side reaction of glucose formation and enhanced the efficiency of the major trehalose formation.

Table 3. Melting Temperatures (T_m) of Different Recombinant Protein Sequences As Predicted by the T_m Predictor Using Statistical Methods (http://tm.life.nthu.edu.tw/)

enzyme	T_m index (TI)	T_m prediction[a]
TtTS	1.173289	>65 °C
TtTSΔC	0.905936	55–65 °C
TtTSΔN	1.467069	>65 °C
DrTS	0.172792	55–65 °C
DrTS–TtTSΔN	0.754230	55–65 °C

[a] T_m index >1 means that the predicted T_m is >65 °C; T_m index = 0–1 means that the predicted T_m is 55–65 °C; T_m index <0 means that the predicted T_m is <55 °C.

There are many possible mechanisms of protein thermostabilization including amino acid composition, contribution of all types of forces (e.g., disulfide bridges, hydrophobic interactions, aromatic interactions, hydrogen bonds, and ion pairs), prolines, decreasing entropy of unfolding, intersubunit interactions, and oligomerization, and so on (*31*). Through bioinformatics analysis, the melting temperatures (T_m) of TtTS, TtTSΔC, TtTSΔN, DrTS, and DrTS−TtTSΔN were predicted by the T_m Predictor (http://tm.life.nthu.edu.tw/) (*32*). Usually, thermostable proteins have a higher predicted T_m index (TI) than their mesothermophilic counterparts. As shown in **Table 3**, the predicted TI value of TtTS (1.173) was higher than that of DrTS (0.173) as expected. TtTS also showed a higher TI than the C-terminal truncated TtTSΔC (0.906), in which a C-terminal domain (TtTSΔN) with a high TI value (1.467) was deleted. Interestingly, the C-terminal fused DrTS (DrTS−TtTSΔN) had a higher TI value (0.754) than the wild-type DrTS. These predictions perfectly matched the experimental data and indicated that the C-terminal domain of TtTS might stabilize TtTS as well as DrTS.

At present, thermophilic enzymes available for industrial application are very few. The ever-growing number of enzymes characterized from thermophilic organisms and the recent advent of powerful protein engineering tools suggest that the applica-

tions of thermophilic and hyperthermophilic enzymes will be more realistic. Enzyme thermostability can be improved by protein engineering such as site-directed mutagenesis (*33*−*36*), computational methods in the design of stabilizing strategies (*37*−*40*), and directed evolution (*41*−*43*). In this study, we adopted the thermostable determinant of TtTS to create a recombinant chimeric DrTS (DrTS−TtTSΔN) that showed improved enzyme properties including increased thermostability and low side reaction. This method also provided another new, simple, convenient, and fast way to improve the thermostability and thermophilicity of an enzyme. Furthermore, in combination with our previous invention, which simultaneously produces high-maltose syrup and a high-protein product from low-priced raw materials containing starch and protein such as rice (*44*, *45*), engineered thermostable TS can be further used to efficiently produce high-value trehalose and nutritious high-protein food from high-maltose syrup at a low cost.

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