Extremophiles (2004) 8:335–343
DOI 10.1007/s00792-004-0393-4

ORIGINAL PAPER

Tsuei-Yun Fang · Xing-Guang Hung · Tong-Yuan Shih
Wen-Chi Tseng

Characterization of the trehalosyl dextrin-forming enzyme from the thermophilic archaeon *Sulfolobus solfataricus* ATCC 35092

Received: 11 December 2003 / Accepted: 6 April 2004 / Published online: 19 May 2004

Abstract The trehalosyl dextrin-forming enzyme (TDFE) mainly catalyzes an intramolecular transglycosyl reaction to form trehalosyl dextrins from dextrins by converting the α-1,4-glucosidic linkage at the reducing end to an α-1,1-glucosidic linkage. In this study, the *treY* gene encoding TDFE was PCR cloned from the genomic DNA of *Sulfolobus solfataricus* ATCC 35092 to an expression vector with a T7 *lac* promoter and then expressed in *Escherichia coli*. The recombinant TDFE was purified sequentially by using heat treatment, ultrafiltration, and gel filtration. The obtained recombinant TDFE showed an apparent optimal pH of 5 and an optimal temperature of 75°C. The enzyme was stable in a pH range of 4.5–11, and the activity remained unchanged after a 2-h incubation at 80°C. The transglycosylation activity of TDFE was higher when using maltoheptaose as substrate than maltooligosaccharides with a low degree of polymerization (DP). However, the hydrolysis activity of TDFE became stronger when low DP maltooligosaccharides, such as maltotriose, were used as substrate. The ratios of hydrolysis activity to transglycosylation activity were in the range of 0.2–14% and increased when the DP of substrate decreased. The recombinant TDFE was found to exhibit different substrate specificity, such as its preferred substrates for the transglycosylation reaction and the ratio of hydrolysis to transglycosylation of the enzyme reacting with maltotriose, when compared with other natural or recombinant TDFEs from *Sulfolobus*.

Communicated by J. Wiegel

T.-Y. Fang (✉) · X.-G. Hung · T.-Y. Shih
Department of Food Science,
National Taiwan Ocean University,
2 Pei-Ning Rd., Keelung, 202, Taiwan
E-mail: tyfang@mail.ntou.edu.tw
Tel.: +886-2-24622192
Fax: +886-2-24622586

W.-C. Tseng
Department of Chemical Engineering,
National Taiwan University of Science and Technology,
Taipei, Taiwan

Keywords *Escherichia coli* · Starch · *Sulfolobus* · Trehalose · Trehalosyl dextrin-forming enzyme

Introduction

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a nonreducing sugar consisting of two glucose molecules linked by an α-1,1 linkage, and its sweetness is about half that of sucrose (Richards et al. 2002). Trehalose is widely distributed in nature, generally found in animals, plants, fungi, insects, and bacteria. The intracellular accumulation of trehalose in some microorganisms, insects, and desert plants can increase the resistances to various environmental stresses, such as salts, ethanol, freezing, heat, and desiccation (Page-Sharp et al. 1999; Potts 1994; Richards et al. 2002; Sharma 1997; Singer and Lindquist 1998). This sugar has many applications such as a sweetener component, preservative, or stabilizer for food, cosmetics, and medicines (Richards et al. 2002). Developing economical processes to increase the yield can lower the cost of trehalose production and accelerate the applications of trehalose.

Lama et al. (1990) first reported that the thermophilic archaeon *Sulfolobus solfataricus* MT4 could hydrolyze starch to produce trehalose. Afterwards, two thermostable enzymes, trehalosyl dextrin-forming enzyme (TDFE) and trehalose-forming enzyme (TFE) (also known as maltooligosyltrehalose synthase and maltooligosyltrehalose trehalohydrolase, respectively), were found to be involved in trehalose production from starch (Kato et al. 1996a, b; Nakada et al. 1996a, b). These two enzymes have been purified and characterized from several sources including *S. solfataricus* MT4, *S. solfataricus* KM1, *S. acidocaldarius* ATCC 33909, and *S. shibatae* DMS 5389 (de Pascale et al. 2001; Di Lernia et al. 1998; Gueguen et al. 2001; Kato 1999; Kato et al. 1996b; Nakada et al. 1996a). TDFE mainly catalyzes an intramolecular transglycosylation reaction to form trehalosyl dextrins from dextrins by converting the

α-1,4-glucosidic linkage at the reducing end to an α-1,1-glucosidic linkage. Moreover, TFE mainly cleaves the α-1,4-glucosidic linkage next to the α-1,1-glucosidic linkage of trehalosyl dextrins to produce trehalose and the dextrins with lower molecular weight. Both TDFE and TFE also catalyze a side reaction to hydrolyze dextrins to release glucose.

Producing trehalose from starch by the thermophilic enzymes has several advantages. The reaction can be carried out at high temperatures to accelerate the reaction rate, to decrease the degree of retrogradation of starch, and to decrease the risks of microbial contamination in the reaction mixtures (Kato 1999; Kobayashi et al. 1997; Leveque et al. 2000). On the other hand, using an inexpensive substrate, such as starch, could lower the production cost. A previous process combined these two thermophilic enzymes with a commercial debranching enzyme to produce trehalose from starch and obtained a yield of around 82% (Kato 1999; Kobayashi et al. 1997; Mukai et al. 1997). The process of producing trehalose from starch is similar to that of producing other sugars, such as glucose and maltose. The yields of glucose and maltose from starch were about 95 and 90%, respectively. Altering the substrate specificity of glucoamylase by mutagenesis was demonstrated to further enhance the glucose yield in saccharification up to 98% (Fang et al. 1998a, b; Ford 1999). Although the side hydrolysis reactions catalyzed by TDFE and TFE might be factors limiting the yield of trehalose to 82% (Kato 1999; Kobayashi et al. 1997), both TDFE and TFE with altered substrate specificities, such as the reduction in the side hydrolysis reactions, might be able to enhance the yield of trehalose production from starch similarly to the production of glucose (Kato 1999; Kobayashi et al. 1997). However, the substrate specificity of TDFE, especially regarding the hydrolysis reaction, has received little attention.

Recently, the complete genome of *S. solfataricus* ATCC 35092 has been sequenced (She et al. 2001), but the TDFE, encoded by the *treY* gene, from this strain has not been characterized yet. In this study, we PCR cloned the *treY* gene from the genomic DNA of *S. solfataricus* ATCC 35092 and expressed the cloned gene in *Escherichia coli*. In addition to characterizing the properties of this recombinant TDFE, we also investigated the substrate specificity of the enzyme on both the transglycosylation and hydrolysis reactions.

Materials and methods

Materials

The genomic DNA of *Sulfolobus solfataricus* ATCC 35092 was obtained from the American Type Culture Collection (Manassas, Va., USA). *Escherichia coli* BL21-CodonPlus (DE3)-RIL was obtained from Stratagene (La Jolla, Calif., USA). Plasmid pET-15b, and BENZONASE Nuclease were from Novagen (Madison, Wis., USA). Vent DNA polymerase and mung bean nuclease were purchased from New England BioLabs (Beverly, Mass., USA). T4 DNA ligase and restriction enzymes were supplied by Promega (Madison, Wis., USA). Maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, glucoamylase, 3,5-dinitro salicyclic acid (DNS), bovine serum albumin (BSA), and phenylmethylsulfonyl fluoride (PMSF) were from Sigma (St. Louis, Mo., USA). Ultrafree-15 and Microcon Centrifugal Filter U were obtained from Millipore (Bedford, Mass., USA). Sephacryl S-200 HR and protein low molecular weight standards were from Amersham Pharmacia Biotech (Piscataway, N.J., USA).

Amplification of the *treY* gene

The *treY* gene was amplified by the polymerase chain reaction (PCR). Two primers were designed on the basis of the *treY* sequence of *S. solfataricus* (GenBank accession number AE006815). In order to clone the *treY* gene into the pET-15b vector, the *Xho*I and *Bam*HI restriction sites were included in the forward and reverse primers, respectively. The primer sequences are as follows: 5XtreY (forward primer), 5′-CCC GGG TCG A**CT CGA G**AT GAT AAT AGG TAC GTA TAG GCT AC-3′; and 3BtreY (reverse primer), 5′-TTA GCA GCC **GGA TCC** CTA TAT AGG TAG TTC ACC CAC TTT T-3′ (the *Xho*I and *Bam*HI restriction sites indicated in boldface). The reaction was carried out in 100 μl reaction mixture containing the genomic DNA of *S. solfataricus* ATCC 35092, two primers, dNTP, Vent DNA polymerase, and Vent DNA polymerase buffer, and was performed by using a GeneAmp PCR system 2400 (PerkinElmer, Wellesley, Mass., USA) according to the following conditions in sequence: 95°C for 5 min, an amplification, and a final extension at 72°C for 10 min. The amplification profile was 1 min at 95°C, 1 min at 60°C, and 2 min at 72°C.

Construction of expression vector for TDFE

The 2.2-kbp PCR-amplified fragment was purified and then digested with *Xho*I and *Bam*HI. The digested fragment was inserted into the pET-15b vector, resulting in a recombinant vector designated as pET-15b-*treY*. The amplified *treY* gene was fused in frame with the His-tag coding sequence on pET-15b; therefore, the expressed recombinant TDFE possessed a His tag on its N-terminal region. The sequences of the entire *treY* gene and the fused His-tag coding region on pET-15b-*treY* were confirmed by DNA sequencing. To express a wild-type TDFE—which contains no extra His-tag coding sequence derived from the cloning steps—the pET-15b-*treY* was digested with *Nco*I and *Xho*I to remove the His-tag coding sequence, made blunt by mung bean nuclease, and ligated, resulting in a recombinant clone designated as pET-15b-ΔH-*treY*. DNA sequencing was also performed to ascertain the removal of the His-tag coding sequence.

Expression of TDFE by *E. coli*

The pET-15b-ΔH-*treY* vector was transformed into *E. coli* BL21-CodonPlus (DE3)-RIL to express TDFE. One single colony from a newly transformed culture plate was inoculated into 10 ml terrific broth medium supplemented with 100 μg/ml ampicillin plus 34 μg/ml chloramphenicol and grown at 37°C until the OD_{600} reached 0.6. Cells were collected by centrifugation and resuspended in 4 ml fresh medium. A volume of 3 ml resuspended culture was then added to 600 ml fresh medium containing 100 μg/ml ampicillin plus 34 μg/ml chloramphenicol and grown until the OD_{600} reached 0.6. The culture was then induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG). After a further culture at 20°C for 16 h, the cells were then harvested by centrifugation and stored at −70°C before further processing.

Preparation of cell-free extract

Frozen cells (10 g) were suspended in 100 ml lysis buffer containing 10 mM Tris-HCl (pH 8.0), 0.5 mg/ml lysozyme, 1 mM benzamidine, 0.05 mM PMSF, and 0.1% Triton X-100. This mixture was incubated at 25°C for 1 h, followed by the addition of 10 μl BENZONASE Nuclease (25 U/μl) and incubated for an additional 30 min. The cell-free extract was prepared by removing the insoluble fractions from the supernatant of the above mixture by centrifugation at 10,000 *g* for 30 min.

Purification of wild-type TDFE

The purification of TDFE was modified from the procedures described by de Pascale et al. (2001). Heat treatment was used to precipitate most of the undesired proteins by incubating the cell-free extract in an 80°C water bath for 6 h, followed by centrifugation to remove the heat-denatured proteins. The supernatant was concentrated and diafiltered with 10 mM Tris-HCl buffer plus 0.2 M NaCl at pH 8 in an Ultrafree-15 Centrifugal Filter Unit with Biomax-30 membrane (MWCO 30,000). After a second heat treatment was applied to the concentrated sample, the supernatant was loaded onto a Sephacryl S-200 HR column (1.6 × 60 cm) previously equilibrated with the same buffer. The eluted fractions containing enzyme activity were collected for characterization of TDFE.

Enzyme activity assay

The assay of TDFE transglycosylation activity was modified from the procedures described by Gueguen et al. (2001). The TDFE activity was assayed at 75°C for 10 min by using 1.4 mM of maltohexaose as substrate in 0.05 M citrate–phosphate buffer (pH 5). The amounts of residual reducing sugars—determined by the DNS method (Miller 1959) with maltohexaose as standards—were used to determine the activity of TDFE because TDFE converts maltooligosaccharides, the compounds with reducing power, to trehalosyl maltooligosaccharides, the compounds without reducing power. One unit of TDFE activity was defined as the amount of enzyme required to convert 1 μmol of maltohexaose to trehalosyl maltotetraose per minute.

HPLC analysis of the transglycosylation and hydrolysis products of TDFE

The transglycosylation and hydrolysis products of TDFE were measured by using a Hitachi HPLC L-7000 (Tokyo, Japan) equipped with a refractive index detector. The enzyme reactions were carried out at 75°C in 0.05 M citrate–phosphate buffer (pH 5) by using 14 mM maltohexaose as substrate. The reactions were stopped by incubating the mixture in a boiling-water bath for 10 min. For the detection of the transglycosylation product, glucoamylase was added to the cooled mixture at a final concentration of 5 U/ml, and the reaction was carried out at 40°C overnight. The reaction mixtures were then filtered through the Microcon Centrifugal Filter Unit with YM-3 membrane (MWCO 3000) to remove the enzyme. The filtrates were analyzed by a Vercopak Nucleosil 5 μm NH_2 column (4.6 × 250 mm), with a mobile phase consisting of 30% (v/v) H_2O and 70% (v/v) acetonitrile.

Protein concentration measurement

Protein concentration was determined by Bradford's method (Bradford 1976), with BSA as standards.

Hydrolysis reaction assay

Maltooligosaccharides of DP 3-7 (14 mM) were incubated with TDFE in 50 mM citrate–phosphate buffer at pH 5 and 75°C for 30 min. The reactions were stopped by adding 0.4 volume of 4 M Tris-HCl buffer at pH 8.0 and incubating the mixture in a boiling-water bath for 10 min. The released glucose was measured by the glucose oxidase method (Rabbo and Terkildsen 1960).

Results and discussion

Gene analysis and comparison of amino acid sequences of several TDFEs from the *Sulfolobus* genus

TDFE from *Sulfolobus solfataricus* ATCC 35092 possesses 732 amino acids and a calculated molecular weight of 86,609.4 Da, which was deduced from the nucleotide sequence of *treY* gene and analyzed by the Compute pI/Mw tool (http://tw.expasy.ch/tools/pi_tool.html; Wilkins et al. 1998), respectively. TDFE from

Table 1 The comparison of deduced amino acid sequences of trehalosyl dextrin-forming enzyme (TDFE) from *Sulfolobus solfataricus* ATCC 35092 with those of other TDFEs from *Sulfolobus* genus

Source	Accession number[a]	No. of residues	Identity[b] (%)
S. solfataricus ATCC 35092	P95869	732	100
S. solfataricus MT1	None[c]	732	99.7
S. solfataricus KM1	Q55262	728	95.7
S. shibatae B12	Q9UWN8	728	94.8
S. acidocaldarius ATCC 33909	Q53688	720	51.3

[a]The deduced amino acid sequences were obtained from TrEMBL protein sequence database
[b]The identity values were analyzed by SIM-Local similarity program (http://tw.expasy.org/tools/sim-prot.html; Wilkins 1998)
[c]The deduced amino acid sequence of TDFE from *S. solfataricus* MT1 was from de Pascale et al. (2001)

S. solfataricus ATCC 35092 shares more than 94% identity with other TDFEs from the *Sulfolobus* genus except *S. acidocaldarius* (Table 1). In addition, a 99.7% identity of amino sequences existed between TDFE from *S. solfataricus* ATCC 35092 and that from *S. solfataricus* MT4. Only residues 466D and 467V in TDFE from *S. solfataricus* ATCC 35092 were conservatively replaced by 466E and 467L in TDFE from *S. solfataricus* MT4, respectively, indicating that the properties of TDFEs from these two strains should be very similar.

TDFE from *Arthrobacter* sp. Q36 was previously classified in the α-amylase family based on the homology of its amino acid sequence and that of α-amylase (Maruta et al. 1996). TDFEs from *Sulfolobus* also contain the amino acid sequences corresponding to the four highly conserved regions in the α-amylase family enzymes (Fig. 1). With exception of four residues of TDFE from *S. acidocaldarius*, the amino acid sequences of the four highly conserved regions in these TDFEs are identical. Kubota et al. (2001) carried the site-directed mutagenesis of TDFE from *S. acidocaldarius* on residues Asp228, Glu255, and Asp443, which correspond to the catalytic residues of the α-amylase family enzymes, and all three individual mutations caused complete deactivation of TDFE. The results confirmed these three catalytic resides in TDFE and also supported the classifications of TDFE to the α-amylase family. According to a recently published three-dimensional structure of TDFE from *S. acidocaldarius*, residues Tyr50, Asp85, Arg226, Asp228, Glu255, His442, and Asp443 of TDFE from *S. acidocaldarius* are located around the catalytic cleft (Kobayashi et al. 2003). Except for Tyr50, six of these residues are also located at the four highly conserved regions (Fig. 1).

Fig. 1a, b Comparison of conserved amino acid sequences in the active sites of α-amylase family enzymes. **a** A generalized sequence of four conserved regions (MacGregor et al. 2001). *X* Hydrophobic residue, *B* hydrophilic residue, *O* Gly or Ala residue, *Z* residue important for specificity. **b** Trehalosyl dextrin-forming enzymes (TDFEs). The identical residues among these TDFEs are marked by *asterisks*. The three proposed catalytic residues, Asp, Glu, and Asp in regions 2, 3, and 4, respectively, are shown in *boldface*. Those residues existing in the active site of *Sulfolobus acidocaldarius* are *underlined*. The sources of the deduced amino acid sequences of TDFEs are identical to those shown in Table 1

	Region 1	Region 2	Region 3	Region 4
a. Generalized sequence	XDXXXNH	GXRXDXXZZ	XXXOEZZZ	XXBBHD
b. TDFE	*******	* *******	** *****	****
S. solfataricus ATCC 35092	84 QDIVPNH	237 GLRIDHIDG	265 IIYVEKIL	455 TLSTHD
S. solfataricus MT4	84 QDIVPNH	237 GLRIDHIDG	265 IIYVEKIL	455 TLSTHD
S. solfataricus KM1	84 QDIVPNH	237 GLRIDHIDG	265 IIYVEKIL	455 TLSTHD
S. shibatae B12	84 QDIVPNH	237 GLRIDHIDG	265 IIYVEKIL	455 TLSTHD
S. acidocaldarius ATCC 33909	84 QDIVPNH	224 GYRIDHIDG	251 IIIVEKIL	438 ATSTHD

Cloning, expression and purification of TDFE

Originally, we wanted to purify TDFE by metal-chelating chromatography; therefore, the amplified *treY* gene was fused in frame with the His-tag coding sequence on pET-15b. However, when we used the *Escherichia coli* cells carrying pET-15b-*treY* plasmid to express His-tagged TDFE under the control of a T7 *lac* promoter, we found that the enzyme was totally insoluble, and its transglycosylation activity could not be detected in the cell-free extract. We replaced the T7 *lac* promoter with the T5 and Tac promoters to express the His-tagged TDFE. However, still no transglycosylation activity could be detected in the cell-free extract. We tried to purify the His-tagged TDFE by metal-affinity chromatography under the denatured condition; however, the refolding of the enzyme was not successful. Therefore, the His-tag coding sequence on pET-15b-*treY* was removed, and the resulting pET-15b-Δ H-*treY* plasmid was transformed into *E. coli* cells to express wild-type TDFE under the control of a T7 *lac* promoter. The term wild-type TDFE used herein denotes the TDFE, which contains no extra His-tag coding sequence derived from the cloning steps and possesses the same coding sequence as that of the native TDFE produced

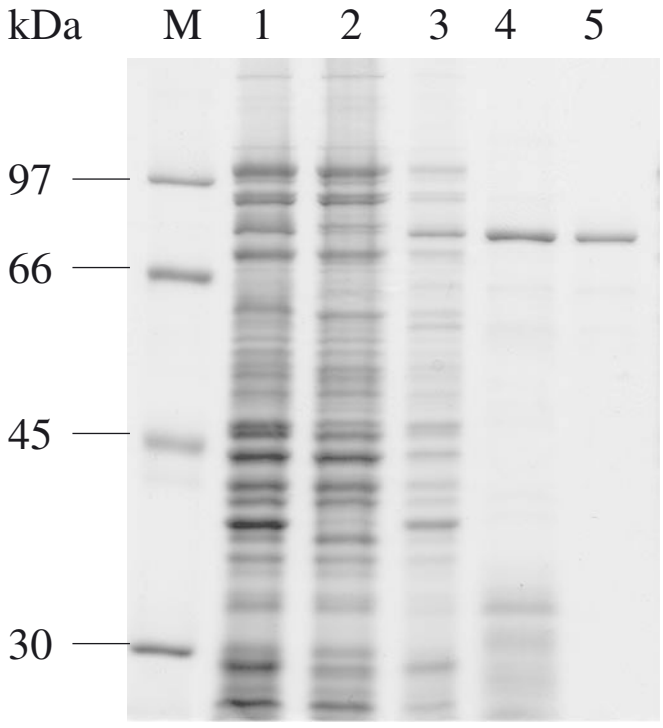

Fig. 2 SDS-PAGE analysis of expression and purification of TDFE expressed in *Escherichia coli* BL21-CodonPlus (DE3)-RIL carrying the pET-15b-ΔH-*treY* vector. *Lane M* Molecular weight standards, *lane 1* the total cellular proteins of the *E. coli* cells, *lane 2* crude cell-free extract, *lane 3* the insoluble proteins after cell lysis, *lane 4* partially purified fraction after the second heat treatment, *lane 5* the purified fraction after gel-filtration chromatography

from *S. solfataricus* ATCC 35092. The expression level of TDFE was higher in the absence of IPTG induction than in the presence of IPTG. The expressed TDFE, however, mostly remained insoluble, and only about 10% of the total expressed TDFE appeared in the cell-free extract (Fig. 2, lanes 1, 2 and 3).

The yield of active TDFE expressed in *E. coli* was 222 U/l and 8.9 U/g of wet cells, which was much higher than the previously reported yield of 0.1 U/g of wet cells for the natural TDFE from *S. solfataricus* ATCC 35092 (Kato et al. 1996b). TDFE was purified from the cell-free extract of *E. coli* by heat treatment, ultrafiltration, and gel filtration (Table 2); the purified TDFE showed a single band on SDS-PAGE, indicating that a high purity of TDFE was obtained (Fig. 2, lane 5). The molecular weight determined by SDS-PAGE was around 87 kDa, which is in a good agreement with the calculated molecular weight.

Effects of pH and temperature on the activity and stability of TDFE

The recombinant enzyme showed an optimal activity at pH 5 and remained stable in the pH range of 4.5–11 (Fig. 3a). The enzyme had an optimal activity at temperature of 75°C and remained stable at temperatures up to 80°C for 2 h (Fig. 3b). After a 2-h incubation at temperatures ranging from 45–80°C, the activity of the purified TDFE remained almost unchanged. The good thermostability of this enzyme indicated that the purified recombinant TDFE was well folded and should have the same structure as that produced directly from *S. solfataricus* ATCC 35092.

Effect of metal ions on the activity of TDFE

The addition of EDTA, Ca^{2+}, K^{+}, Mg^{2+}, or Na^{+} had no activating or inhibiting effect on TDFE activity, while the addition of Cu^{2+} and Zn^{2+} slightly inhibited the enzyme activity at a concentration of 4 mM (Fig. 4). Although the catalytic domain of the α-amylase family enzymes generally contained one conserved calcium ion to stabilize the interface of domain A and B, there is no metal ion bound to the domain interface in the three-dimensional structure of TDFE from *S. acidocaldarius* (Kobayashi et al. 2003). In addition, a conserved asparagine, Asn89, which corresponds to the calcium-binding residue of the α-amylase family enzymes, exists in the three-dimensional structure of TDFE from *S. acidocaldarius* (Kobayashi et al. 2003). The interface of domain A and B seems to be stabilized by the hydrogen bonds among OD1 of Asn89 (domain B), NH1 of Arg191 (domain B), and O of Gly232 (domain A) in the three-dimensional structure of TDFE from *S. acidocaldarius* (Kobayashi et al. 2003). These residues are all conserved in the amino acid sequences of TDFEs from the *Sulfolobus* genus. These results indicated that these metal ions were not required for TDFE activity.

Table 2 Summary of purification procedure of TDFE expressed in *Escherichia coli* BL21-CodonPlus (DE3)-RIL

Purification step	Total protein (mg)	Total activity (U)	Activity recovery (%)	Specific activity (U/mg)	Purification fold
Crude extract	742	89	100	0.12	1
First heat treatment[a]	39	88	99	2.3	19
Ultrafiltration[b]	9.5	54	61	5.7	47
Second heat treatment[c]	8.2	54	61	6.6	55
Sephacryl S-200 gel filtration	1.2	23	26	19	160

[a]The crude extract was heated at 80°C for 6 h and then centrifuged to remove the precipitates
[b]The supernatant from the heat treatment was concentrated and diafiltered
[c]The concentrated, diafiltered sample was heated again at 80°C for 6 h and then centrifuged to remove the precipitates

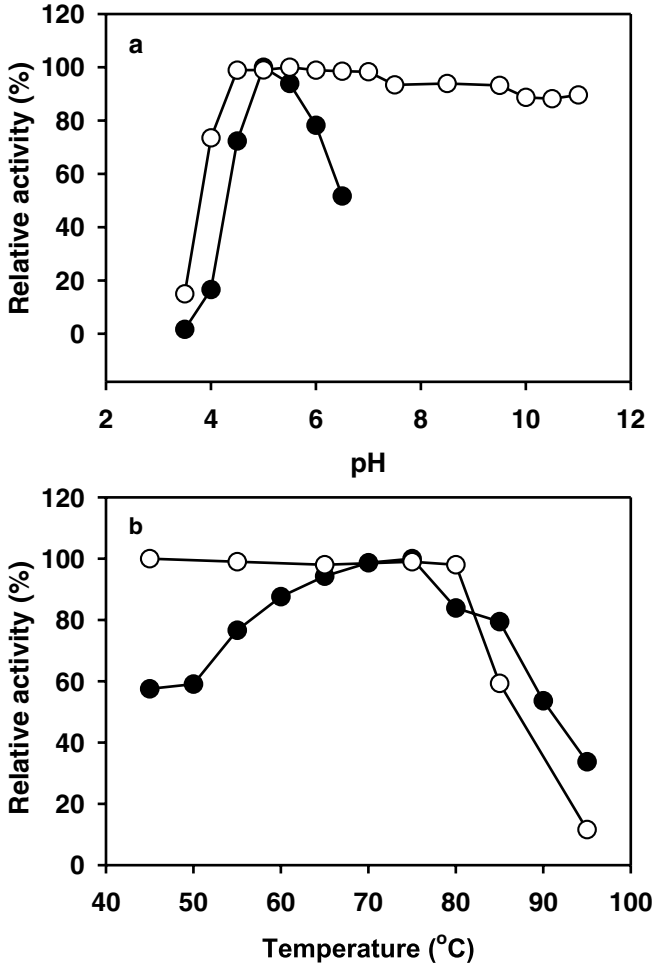

Fig. 3a, b Effects of pH and temperature on the activity and stability of TDFE. **a** Effects of pH. The following buffers with a concentration of 50 mM were used in different pH ranges: citrate–phosphate buffer (pH 3.5–7), Tris-HCl buffer (pH 7.5–8.5), and $NaHCO_3$–Na_2CO_3 buffer (pH 9.5–11). For determination of the optimal pH (*filled circle*), the enzyme (0.06 U/ml) was assayed at different pHs under standard conditions. For determination of pH stability (*open circles*), the enzyme (0.6 U/ml) was incubated at various pHs at 4°C for 24 h, and the remaining activities were assayed under standard conditions. **b** Effects of temperature. For determination of the optimal temperature (*filled circles*), the enzyme (0.06 U/ml) was assayed at different temperatures under standard conditions. For determination of thermostability (*open circles*), the enzyme (0.6 U/ml) was incubated at various temperatures at pH 5 for 2 h, and the remaining activities were assayed under standard conditions

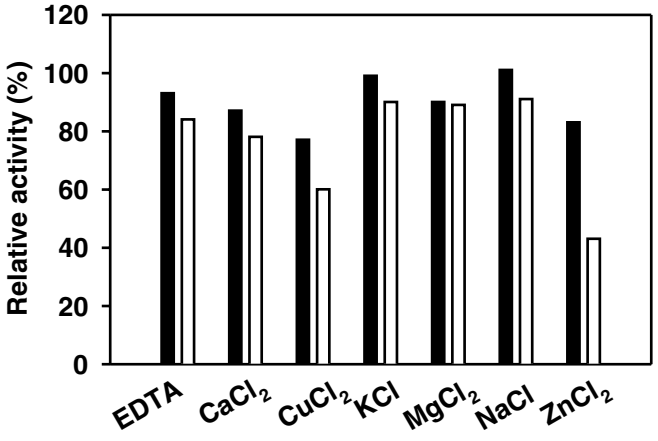

Fig. 4 Effect of metal ions on the activity of TDFE. The enzyme activity was assayed at 75°C, pH 5 in the presence of EDTA or different metal ions. Concentrations of EDTA and metal ions were 0.4 (*black bars*) and 4 mM (*white bars*), respectively. The relative activity was expressed as the percentage of activity in the absence of EDTA and metal ions

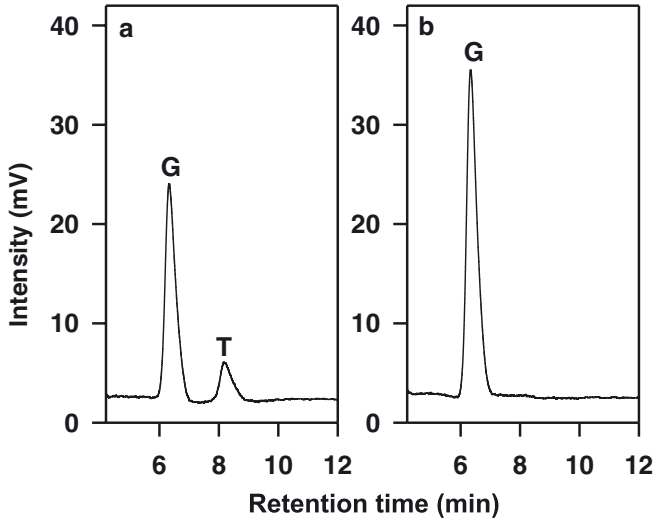

Fig. 5a, b HPLC analysis of transglycosylation products. **a** Contents of 14 mM maltohexaose after a reaction with TDFE (0.12 U/ml) at 75°C, pH 5 for 40 min, followed by a second reaction with glucoamylase at 40°C, pH 5 overnight. **b** Contents of 14 mM maltohexose after a reaction with glucoamylase at 40°C, pH 5 overnight. *G* and *T* represent glucose and trehalose, respectively

HPLC analysis of the transglycosylation and hydrolysis products

For the detection of the transglycosylation product, TDFE was incubated with 14 mM maltohexaose at 75°C for 40 min. After maltohexaose was converted to trehalosylmaltotetraose by TDFE, glucoamylase was added to digest the α-1,4 linkage in trehalosyl maltotetraose and in remaining maltohexaose. The digestion released trehalose and glucose as the final products (Fig. 5a). The detection of trehalose confirmed the existence of transglycosylation activity, which converts α-1,4 linkage to α-1,1 linkage, and the molar concentration of trehalose detected was equal to the molar concentration of trehalosyl maltotetraose produced by TDFE. Compared to the standard trehalose concentration, there was about 50% of maltohexaose that was converted to trehalosyl maltotetraose by TDFE at 75°C for 40 min (Fig. 5a). In the negative control containing only glucoamylase but no TDFE, glucose was detected as the only product (Fig. 5b).

For the detection of hydrolysis products, the released glucose was hardly detected when incubation time was

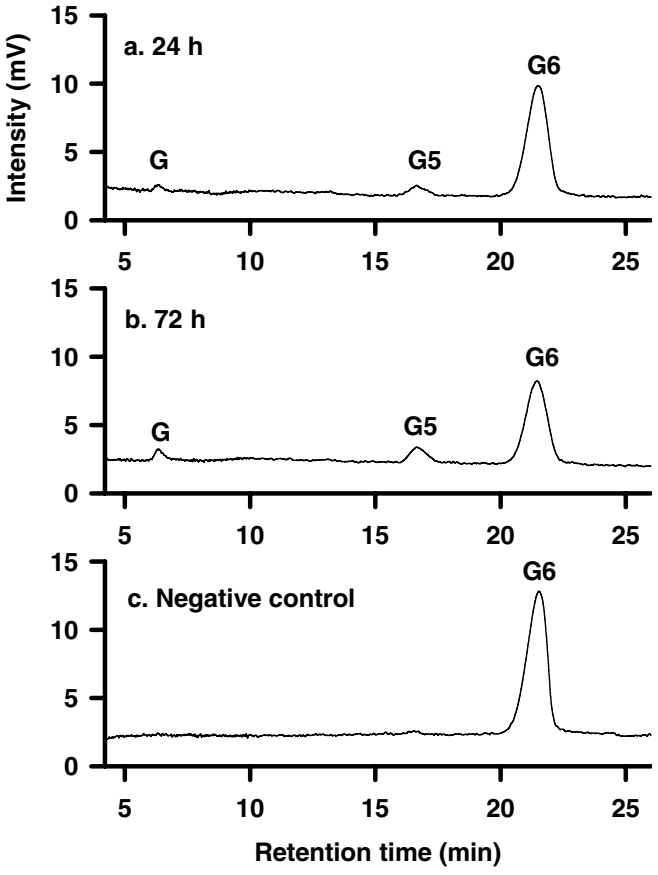

Fig. 6 HPLC analysis of hydrolysis products. A concentration of 14 mM maltohexaose reacted with TDFE (0.12 U/ml) at 75°C, pH 5 for the indicated time. The negative control containing only 14 mM maltohexaose but no enzyme was incubated at 75°C, pH 5 for 72 h. *G* Glucose, *G5* pentasaccharides including maltopentaose and trehalosyl maltotriose, *G6* hexasaccharides including maltohexaose and trehalosyl maltotetraose

less than 24 h. After the incubation time was prolonged to more than 24 h, the released glucose became detectable (Fig. 6a, b). The negative control containing no TDFE showed no changes in the substrate compositions during the prolonged incubation (Fig. 6c). Because only a small amount of glucose and pentasaccharides (about 2.5 and 13% of the total carbohydrates, respectively) can be detected after a 72-h incubation, the hydrolysis activity of the recombinant TDFE was relatively small in comparison to its transglycosylation activity when maltohexaose was used as substrate.

Substrate specificity

A comparison of the substrate specificity among DP 3-7 maltooligosaccharides is shown in Table 3. For the transglycosylation activity, maltoheptaose was the most preferred substrate tested in this study, and the relative reactivity became higher when the DP of the substrate increased. However, the hydrolysis activity was highest when maltotriose was used as substrate and decreased as the DP of the substrate increased. The ratios of hydrolysis to transglycosylation were 14, 1.6, 0.8, 0.2, and 0.2% for DP 3-7 maltooligosaccharides, respectively. A previous report showed that the corresponding ratios were 80, 1.7, 1, 0.3, and 0.3%, respectively, for the same substrates that reacted with TDFE from *S. solfataricus* KM1 (Kato et al. 2000). These results suggested that the hydrolysis reaction towards the low DP substrate was one of the major reasons that caused the decreased yield of trehalose produced from starch. The relatively low ratio for maltotriose catalyzed by TDFE from *S. solfataricus* ATCC 35092 suggested that the use of this TDFE might be able to enhance the yield of trehalose production from starch. A further comparison of the enzymatic properties of TDFEs from the *Sulfolobus* genus is given in Table 4. In general, these TDFEs from different sources possess similar properties except for their preferred substrates for transglycosylation reaction and the ratio of hydrolysis to transglycosylation of the enzyme reacting with maltotriose.

Conclusion

The TDFE from *Sulfolobus solfataricus* ATCC 35092 was cloned and expressed in *Escherichia coli*. We characterized the enzymatic properties of this recombinant enzyme and compared these properties with other TDFEs from the *Sulfolobus* genus. Except for the substrate specificity, the recombinant TDFE that we cloned and expressed had very similar properties to other natural or recombinant TDFEs from the *Sulfolobus* genus. The differences in substrate specificity, especially the one in the ratio of hydrolysis to transglycosylation of the enzyme reacting with maltotriose, presumably affect the yield of trehalose production from starch. The results from this study also suggested that this recombinant TDFE could be used to produce trehalosyl dextrins at high temperature.

Table 3 Substrate specificity of TDFE from *S. solfataricus* ATCC 35092. [The substrate was incubated with the enzyme (0.1 U/ml) at 75°C, pH 5)]

Substrate (14 mM)	Trehalosyl dextrin formation rate (μM/min)	Glucose formation rate (μM/min)	Relative reactivity		Ratio of H/T[a] (%)
			Transglycosylation (%)	Hydrolysis (%)	
Maltotriose	64	8.8	10	1.4	14
Maltotetraose	230	3.7	38	0.6	1.6
Maltopentaose	470	3.8	77	0.6	0.8
Maltohexaose	550	0.9	90	0.2	0.2
Maltoheptaose	620	1.1	100	0.2	0.2

[a]Ratio of hydrolysis to transglycosylation

Table 4 Comparison of the enzymatic properties of TDFEs from *Sulfolobus* genus (strains *S. solfataricus* ATCC 35092, *S. solfataricus* MT4, *S. acidocaldarius* DSM639, *S. solfataricus* KM1, *S. acidocaldarius* ATCC 33909, and *S. shibatae* DSM 5389)

Properties	Recombinant			Natural		
	ATCC 35092[a]	MT4[b]	DSM639[c]	KM1[d]	ATCC 33909[e]	DSM 5389[f]
Optimum temperature (°C)	75	75	75	70–80	75	70
Optimum pH	5	5	5	5–6	5–5.5	4.5
Thermostability[g]	80°C, 2 h (96%) 85°C, 2 h (56%)	75°C, 2 h (~100%) 80°C, 2 h (~80%)	80°C, 72 h (60%) 90°C, 1 h (~70%)	85°C, 6 h (91%)	80°C, 1 h (~100%) 85°C, 1 h (~95%)	75°C, 2 h (~100%) 85°C, 2 h (~90%)
pH stability	4.5–11	3.5–11	NR[h]	4–10	4.5–9.5	4.5–9.5
Metal ion inhibition	Cu^{2+}, Zn^{2+}	NR	NR	Cu^{2+}	Cu^{2+}, Hg^{2+}	NR
Ca^{2+} activation	No	NR	NR	No	No	NR
Hydrolysis activity	Yes	NR	Yes	Yes	Yes	NR
Preferred substrate[i]	DP 7	NR	NR	DP 5	DP 6	NR
H/T ratio[j]	14%	NR	NR	80%	NR	NR

[a]This study
[b]de Pascale et al. (2001)
[c]Gueguen et al. (2001)
[d]Kato et al. (1996b, 2000)
[e]Nakada et al.(1996a)
[f]Di Lernia et al. (1998)
[g]The residual activities are shown in parentheses
[h]Not recorded in the published literature
[i]The preferred substrate among DP 3-7 maltooligosaccharides on transglycosylation reaction
[j]The ratio of hydrolysis to transglycosylation of the enzyme reacted on maltotriose

Acknowledgements This work was supported by grant NSC 91-2313-B-019-039 from the National Science Council at Taiwan.

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