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Biochemical Characterization of Thermophilic Dextranase from a Thermophilic Bacterium, *Thermoanaerobacter pseudethanolicus*

Park, Tae-Soon[1†], Hyung Jae Jeong[1†], Jin-A Ko[1], Young Bae Ryu[1], Su-Jin Park[1], Doman Kim[2], Young-Min Kim[1*], and Woo Song Lee[1*]

[1]*Infection Control Material Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Jeongeup 580-185, Korea*
[2]*School of Biological Sciences and Technology and the Research Institute for Catalysis, Chonnam National University, Gwang-ju 500-757, Korea*

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TPDex, a putative dextranase from *Thermoanaerobacter pseudethanolicus*, was purified as a single 70 kDa band of 7.37 U/mg. Its optimum pH was 5.2 and the enzyme was stable between pH 3.1 and 8.5 at 70°C. A half-life comparison showed that TPDex was stable for 7.4 h at 70°C, whereas *Chaetominum* dextranase (CEDex), currently used as a dextranase for sugar milling, was stable at 55°C. TPDex showed broad dextranase activity regardless of dextran types, including dextran T2000, 742CB dextran, and alternan. TPDex showed the highest thermostability among the characterized dextranases, and may be a suitable enzyme for use in sugar manufacture without decreased temperature.

Keywords: Thermostable dextranase, *Thermoanaereobacter*, sugar processing, thermostability

In the sugar cane industry, dextran, an expolysaccharide consisting mainly of α-1,6 glucosidic linkages of D-glucose, is a major by-product of sugar cane deterioration [9]. The dextran is produced by *Leuconostoc mesenteroides* and some *Lactobacillus* species during delays between harvesting and crushing of the cane. The presence of the highly viscous dextran in the cane juice entering the sugar factory has deleterious effects on raw sugar processing and quality [8]. This problem can be overcome through the addition of dextranase during processing. However, the enzymes from *Chaetomium* or *Penicillium* currently used in over 90% of sugar mills worldwide show optimal activity at 55°C, and the rapid inactivation of these enzymes at the optimal milling temperature of 70°C requires that the mill stream temperature be lowered before dextranase treatment. This cooling step leads to a new set of inefficiencies that could be eliminated by a thermoactive and thermostable dextranase.

Thermophilic anaerobic bacteria have the desired properties, because they thrive in the optimum temperature range of 70–80°C [13, 15]. However, thermostable enzyme preparation from several thermophilic anaerobic bacteria is very limited owing to its difficult culture system and low productivity. The genome sequences of bacteria such as *Thermoanaerobacter brockii* subsp. *finnii* Ako-1, *Thermoanaerobacter pseudethanolicus* ATCC 33223 39E, *Thermotoga lettingae* TMO [17], and *Thermoanaerobacter* sp. X513 or X514 have been deduced, but none of the above-mentioned active enzyme. Thermostable dextranase (TLDex) from *T. lettingae* TMO has an optimal temperature range of 55–60°C, but it is not sufficiently thermostable over the sugar cane processing temperature range (70°C) [10]. Furthermore, there were three types of *Thermoanaerobacter*-originated putative genes, expected to have similar structures, displaying 99% amino acid sequence identities. Therefore, we observed the non-characterized *Teth39_0264* gene from *T. pseudethanolicus* sp. ATCC 33223 39E.

In the present study, the biochemical properties of a thermostable dextranase (TPDex) from *T. pseudethanolicus* were determined and TPDex was compared with *Chaetomium* dextranase (CEDex), currently used in sugar manufacturing processes.

*Corresponding author
Y.M.K.
Phone: +82-63-570-5174; Fax: +82-63-570-5239;
E-mail: u9897854@kribb.re.kr
W.S.L.
Phone: +82-63-570-5005; Fax: +82-63-570-5239;
E-mail: wslee@kribb.re.kr

†Tae-Soon Park and Hyung Jae Jeong contributed equally to this work.

Materials and Methods

Gene Cloning and Production of Recombinant Enzyme

The gene encoding dextranase was amplified with the polymerase chain reaction using KOD DNA polymerase (Toyobo, Osaka, Japan), and the genomic DNA of *T. pseudethanolicus* sp. ATCC 33223 39E

(GenBank Accession No. ABY93933) as the template, and the primer pair (forward primer 5'-AAACCATGGGTAAAAAGATTA TTGCGTTAATAT-3' and reverse primer 5'-TTTCTCGAGTTTTTT AATAAATATCATGTCCCAG-3') containing the *Nco*I and *Xho*I restriction sites (underlined), respectively. The PCR product was subcloned into the pET23d (+) plasmid (Novagen, Madison, WI, USA) digested with the same restriction enzymes and then transformed into *E. coli* Rosetta (DE3). The recombinant *E. coli* cells for protein expression were cultivated with shaking at 180 rpm in a 3 L flask containing 1 L of Luria–Bertani (LB) medium at 37°C with 50 μg ampicillin/ml until the OD_{600} reached 0.5. Isopropyl β-D-thiogalactopyranoside was added to 0.2 mM to induce enzyme expression, and the culture was grown at 18°C for 18 h.

Enzyme Purification

Wet cells collected by centrifugation at 4,000 ×*g* for 5 min were suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl (buffer A). The suspended cells were sonicated and centrifuged at 10,000 ×*g* for 20 min, and the resulting supernatant was used as a crude enzyme solution. Purification of the TPDex was carried out with Ni-chelating Sepharose resin equilibrated with buffer A, using an AKTA-fast protein liquid chromatography system with a Frac-950 fraction collector (Amersham Pharmacia Biotech., Piscataway, NJ, USA). The column was washed with buffer A containing 20 mM imidazole, followed by elution with a linear gradient of 20–300 mM imidazole in buffer A. The fraction containing the purified TPDex was dialyzed against 20 mM sodium acetate buffer (NaAc, pH 5.2) and concentrated *via* ultrafiltration using an Amicon Ultra 10,000 Da molecular weight cut off (Millipore, Billerica, MA, USA). Approximately 14.4 mg of purified protein was obtained from 1 L of the culture medium. Protein concentration was determined by Bradford's method [1] with bovine serum albumin as standard, and enzyme purity was observed by SDS-PAGE. The resultant solution was used as the purified enzyme.

Measurement of Enzymatic Activity

Dextranase activity was examined in 30 mM NaAc buffer (pH 5.2) with 0.8% (w/v) dextran T2000 at 70°C. The amount of reducing sugars liberated was measured by the dintrosalicylic acid method [11] using glucose as a standard. One unit of activity was defined as the amount of enzyme that released 1 μmol of reducing power per minute under the assay conditions.

For determination of the optimum pH, 55 μg/ml of TPDex was incubated at 70°C in 32 mM Britton–Robinson (pH 2.0–12) [2] with 0.8% (w/v) dextran T2000. For determination of the pH stability, the enzyme was kept at 4°C for 20 h in 40 mM Britton–Robinson buffer (pH 2–12), and the residual enzyme activity was examined. For the determination of optimal temperature, the enzyme was incubated at 50–90°C for 10 min in 30 mM NaAc buffer (pH 5.2). For determination of the thermal stability, the enzyme was kept at 50–80°C for 4 h in 30 mM NaAc buffer (pH 5.2) and the residual enzyme activity was examined with 0.8% (w/v) dextran T2000 at 70°C.

Substrate Specificity of TPDex and Analysis of Final Products

The reaction velocity of TPDex-mediated hydrolysis of *L. mesenteroides* B-512F dextran T500 and T2000 (Sigma-Aldrich, St. Louis, MO, USA), *L. mesenteroides* 742CB dextran, *L. mesenteroides* 1299CB dextran, alternan (kindly provided by Dr. G. L. Cote, US Department of Agriculture), pullulan (Sigma-Aldrich), or soluble starch was measured in a reaction mixture consisting of 55 μg/ml enzyme, 0.8% (w/v) of each substrate, and 30 mM NaAc buffer (pH 5.2) at 70°C. For kinetic constants, the initial velocity (v) was measured using various concentrations (s) of *L. mesenteroides* B-512F dextran T500, dextran T2000, and *L. mesenteroides* 742CB dextran (1.125, 2.25, 4.5, 9, 13.5, 18, 22.5, 27, 31, 36, 40, 45 mg/ml) in 30 mM NaAc buffer (pH 5.2) at 70°C.

TPDex-mediated hydrolysis products were analyzed by thin-layer chromatography (TLC) using a silica gel 60 plate (Merck, Darmstadt, Germany) in a solvent system consisting of nitromethane, 1-propanol, and water in a ratio of 4:10:3 (v/v/v) with glucose and isomaltooligosaccharides standards, and the carbohydrates were visualized on the TLC plate described previously by Su and Robyt [12].

Results and Disccussion

Gene Cloning, Expression, and Purification of Dextranase (TPDex) from *T. pseudethanolicus*

A gene of 1,833 bp encoding a putative dextranase from *T. pseudethanolicus* sp. ATCC 33223 39E, with the same sequence as that reported in GenBank (Accession No. ABY93935), was expressed in *E. coli*. TPDex exhibited 99% and 30% identities with the amino acid sequences of three putative dextranases from *Thermoanaerobacter*-derived strains (X513, X514, and *brockii* subsp. *finnii* Ako-1) and TLDex from *T. lettingae* TMO, respectively. The enzyme was purified with a final purification yield of 82% and a specific activity of 7.37 U/mg. Moreover, 14.4 mg of active protein was obtained from 1 L of culture broth. The final purified enzyme showed a single band with a molecular mass of approx. 70 kDa in SDS-PAGE (Fig. 1),

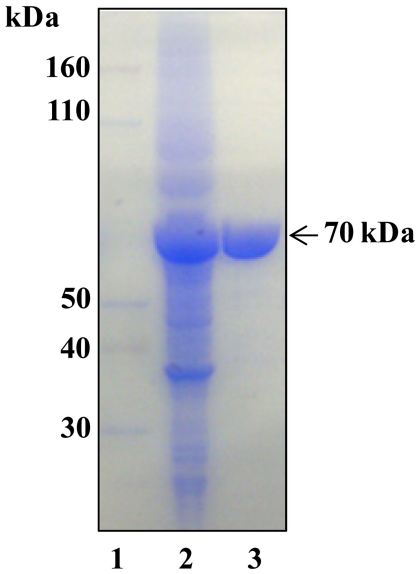

Fig. 1. SDS-PAGE analysis of purified TPDex from cell-free extract.
Lane 1, prestained marker proteins (160, 110, 50, 40, and 30 kDa); lane 2, crude extract; lane 3, purified enzyme.

consistent with the calculated value of 71,079 Da based on amino acid residues plus six histidine residues at the carboxyl terminus.

Effect of Temperature on the Activity and Stability

The effects of temperature on the purified TPDex were compared with CEDex, which is an enzyme currently used in the sugar milling industry. The optimum temperature of TPDex, observed at 76°C, was 21°C higher than that of CEDex at 55°C (Fig. 2A). When the half-lives of the two enzymes were compared, TPDex was stable for 7.4 h at 70°C, whereas CEDex was stable at 55°C. Furthermore, the half-lives of TPDex at 75°C and 80°C were approximately 109 min and 9 min, respectively (Fig. 2B). When TPDex was incubated in the presence of 1% (w/v) dextran T2000 at 80°C, it significantly changed half-lives from 9 min to 36 min. Similar with the 24% activity increase of thermophilic bacterium Rt364 dextranase [15], this increase may come from a strong folded structure between dextran and dextranase at the glucan binding site. On the other hand, CEDex did not show high recovery of original activity under the same conditions. These data suggest that TPDex is more effectively stable in high concentrations of deteriorated dextran during the sugar milling process. As shown in Table 1, the half-lives of thermostable dextranaser from the *Thermoanaerobacter* species [13, 14], *C. gracile* [5], and *T. lettingae* TMO [10] have been reported as 4 h at 55°C, 4 h at 75°C, and 90 min at 65°C, respectively. With the exception of Wynter's method [15] using blue dextran solid plate for thermostability with low accuracy, TPDex as described here exhibited the highest thermostability.

Effect of pH on the Activity and Stability

TPDex had an optimum pH of 5.2 with a broad range of pH values (3.1–8.5), after incubation at 4°C for 20 h (Fig. 3). The optimum pH of TPDex was the same as that of the characterized thermostable dextranase (pH 5.5) [7], *P.* dextranase (pH 5.5) [3], but it was far different from that of *T. lettingae* TMO (pH 4.3) [10]. TPDex also exhibited a broad pH stability range compared with narrow *P.* dextranase (pH 6.5–8.0) [4] and *T. lettingae* TMO TLDex (pH 4.3–10) [10]. The clue of this broad pH ranged stability needs to be elucidated for the 3-dimensional structure of TPDex. Moreover, the broad pH stability of TPDex could be useful in sugar processing, which involves both acidic and alkaline conditions.

Effect of Metal Ions on the Activity

To examine the effect of metal ions on dextranase activity, the reaction mixture composed of 0.8% (w/v) dextran T2000, 30 mM NaAc buffer (pH 5.2), 1 mM final concentration of metal ions, and the enzyme was incubated at 70°C, and a reducing value was measured. Most of the tested metal ions such as Ca^{2+}, Li^{2+}, and Mg^{2+}, did not show appreciable

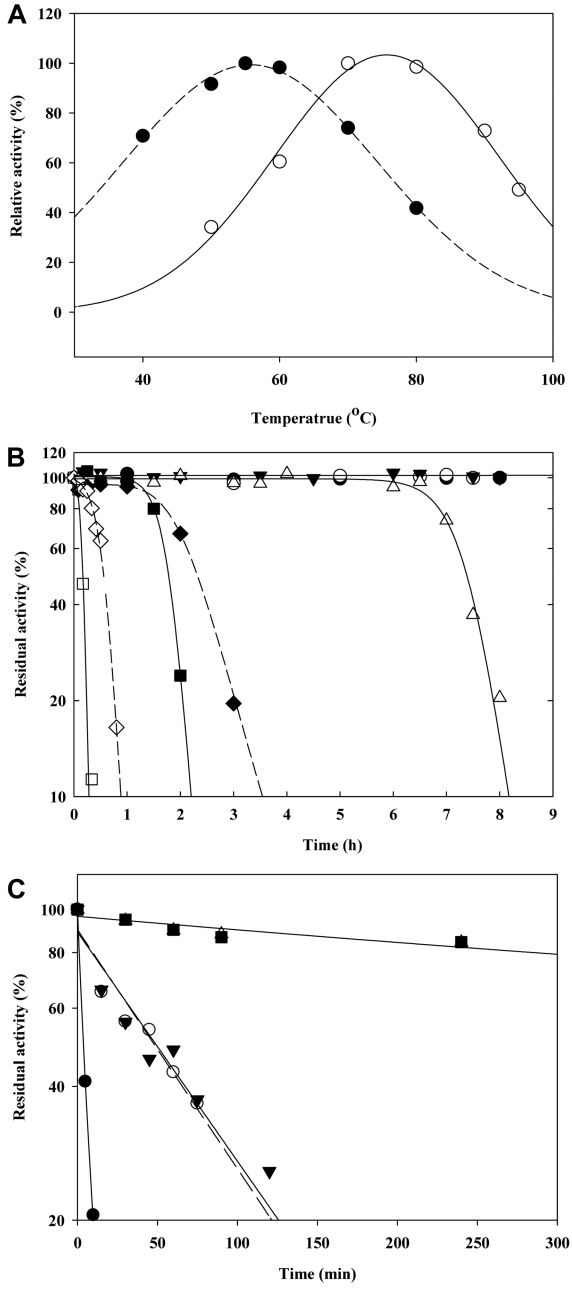

Fig. 2. Thermal studies on TPDex and CEDex.
(**A**) Effect of temperature on two thermostable dextranases at 30 mM NaAc (pH 6.0) or (pH 5.2). The CEDex reactions were performed with 0.8% (w/v) dextran T2000 for 10 min at 40 to 80°C (*filled circle*) and TPDex was observed with 0.8% (w/v) substrate for 10 min at 50 to 90°C (*open circle*). Thermal inactivation of TPDex(**B**) and CEDex (**C**). The TPDex was incubated at varying temperatures and time periods. Temperatures were 50 (*closed circle*), 60 (*open circle*), 65 (*filled upside down triangle*), 70 (*open triangle*), 75 (*closed square*), 75 with 1% (w/v) substrate (*closed diamond*), 80 (*open square*), and 80°C with 1% (w/v) substrate (*open diamond*). The CEDex was incubated at varying temperatures and time periods. Temperatures were 55 (*open triangle*), 55 with 1% substrate (*closed square*), 60 (*open circle*), 60 with 1% (w/v) substrate (*closed upside down triangle*), and 70°C (*closed circle*). A sample was withdrawn at each time interval and the residual activity was determined. After incubation, enzyme activity was measured with 0.8% (w/v) dextran T2000 at 70°C for TPDex, or 55°C for CEDex, for 10 min.

Table 1. Optimum pH and thermal stabilities of thermostable dextranases.

Name of bacteria or enzyme	Optimum temperature (°C)	Thermostability half-life	Molecular mass (protein from 1 L culture)	References
T. thermosaccharolyticum	66–70	75°C (6 h)	200 kDa (ND)	[7]
Thermophilic bacterium, Rt364	80	75°C (12 h)	144 kDa as native form (1 mg)	[15]
T. sp. AB11Ad	70	75°C (6.5 h)	-	[14]
C. gracile	55	Stable at 55°C	77 kDa (7.5 mg)	[5]
Recombinant, *P.* Dex70-1B	60	57°C (70% for 9.5 h)	68.6 kDa (ND)	[3]
Recombinant, *T. lettingae* TMO	55–60	65°C (1.5 h)	64 kDa (2.6 mg)	[10]
Recombinant, *T. Pseudoethanolicus* sp.	76	70°C (7.4 h)	70 kDa (14.4 mg)	This study

ND, not described.

effects similar to those observed in TLDex from *T. lettingae* TMO [10]. Activity increased from 100% (without addition of metal ions) to 107% in the presence of Ni^{2+}, whereas the residual activity was 98%, 45%, and 8% in the presence of Ag^{2+}, Mn^{2+}, and Fe^{2+}, respectively. Compared with the cycloisomaltodextran glucanotransferase in the same glycoside hydrolase family 66 [16], TPDex did not require the Ca^{2+} ion. This difference of the two enzymes also solves the 3-dimensional structure of TPDex.

Substrate Specificity of TPDex

The substrate specificity of TPDex towards α-glucans was examined. Table 2 shows the specific activities and kinetic parameters of the enzyme for α-glucans. The enzyme exhibited broad substrate specificities for dextran T2000, dextran T500, 742CB dextran, and alternan. The relative activity of the dextranase was low for dextrans having a relatively high content of α-1.2 branched structures such as 1299CB dextran, and it could not hydrolyze α-1,4 glucosidic polymers, such as soluble starch and pullulan (Table 2). The values of k_{cat}/K_m for dextran T500 were 2.64-fold higher than those obtained with 742CB dextran, indicating that the enzyme preferred the less-branched dextran over the branched one. From the analysis of hydrolysis products, the enzyme activity was very specific for α-1,6 glucosidic glucan and produced isomaltose, isomaltotriose, and long isomaltooligosaccharides as the final products of a 7 h reaction (Fig. 4). TPDex completely hydrolyzed the dextran T2000 during a prolonged 5 day incubation. TPDex did not show any transglucosylation products, with an extremely high concentration (1%, w/v) of each isomaltooligosaccharide (isomaltotriose – isomaltoheptaose; substrates for TPDex) by TLC and HPLC (data not shown), suggesting that the hydrolysis is a main reaction on dextran T2000.

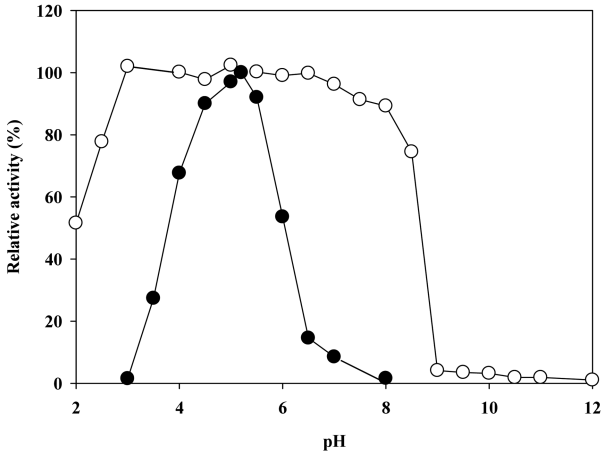

Fig. 3. pH inactivation of TPDex.
For determination of the optimum pH (*closed circle*), TPDex was incubated at 70°C in 32 mM Britton–Robinson buffer (pH 2-12) [2] with 0.8% (w/v) dextran T2000. For determination of the pH stability (*open circle*), the enzyme was kept at 4°C for 20 h in 40 mM Britton–Robinson buffer (pH 2–12), and the residual enzyme activity was examined.

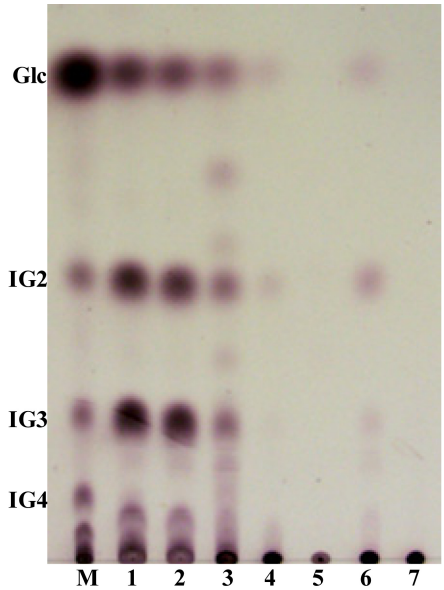

Fig. 4. Thin-layer chromatography (TLC) analysis of TPDex hydrolysis product.
TPDex (165 μg/ml) was incubated with 0.8% (w/v) substrates [lane M, isomaltooligosaccharides standards (isomaltose–isomaltoheptaose); lane 1, dextran T2000; lane 2, dextran T500; lane 3, 742CB dextran; lane 4, 1299CB dextran; lane 5, soluble starch; lane 6, alternan; lane 7, pullulan; in 30 mM NaAc buffer (pH 5.2) at 70°C for 7 h]. The reaction products were analyzed by TLC using nitromethane/1-propanol/H_2O in a volume ratio of 4:10:3.

Table 2. Specific activity and kinetic parameters of TPDex.

Substrate	Linkage composition				Specific activity (U/mg)	K_m (mg/ml)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} ml mg^{-1})
	α-1,6	α-1,3	α-1,2	α-1,4				
Dextran T2000[a]	95%	5%			7.37 ± 0.08	6.39 ± 0.02	612 ± 3	95.8 ± 2
Dextran T500[a]	95%	5%			6.17 ± 0.07	2.45 ± 0.05	487 ± 4	198 ± 3
742CB dextran[a]	67%	33%			2.68 ± 0.04	2.94 ± 0.05	221 ± 2	75 ± 0.9
Alternan	50%	50%			0.52 ± 0.01			
1299CB dextran[a]	65%		35%		0.01			
Pulluan	25%			75%	ND			
Soluble starch	5%			95%	ND			

[a]Originated from *L. mesenteroides* strains. ND, not detected.

In conclusion, the non-characterized TPDex from *T. pseudethanolicus* was overexpressed in the *E. coli* system and its biochemical properties were characterized. TPDex showed broad dextranase activity regardless of dextran types (dextran T2000, 742CB dextran, and alternan) and it showed dramatically high stability, sustaining a 7 h half-life at the industrially required temperature (70°C) and pH (5.0) of cane juice in the sugar factory. These broad substrate specificities and high thermostability suggest that this enzyme may be useful in the industrial sugar factory. Furthermore, directed evolution studies aimed at increasing the thermostability [6] of TPDex are currently in progress with the goal of maximizing the utility of the enzyme in sugar processing.

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