

# Characterization of novel thermostable dextranase from *Thermotoga lettingae* TMO

Young-Min Kim · Doman Kim

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**Abstract** Biochemical properties of a putative thermostable dextranase gene from *Thermotoga lettingae* TMO were determined in a recombinant protein (TLDex) expressed in *Escherichia coli* and purified to sevenfold apparent homogeneity. The 64-kDa protein displayed maximum activity at pH4.3, and enzyme activity was stable from pH4.3–10. The optimal temperature was 55–60°C during 15 min incubation, and the half-life of the enzyme was 1.5 h at 65°C. The enzyme showed higher activity against  $\alpha$ -(1→6) glucan and released isomaltose and isomaltotriose as main products from dextran T2000. An unusual kinetic feature of TLDex was the negative cooperative behavior on the reaction of dextran T2000 cleavage. Enzyme activity was not significantly affected by the presence of metal ions, except for the strong inhibited by 1 mM Fe<sup>2+</sup> and Ag<sup>2+</sup>. TLDex may prove useful as an enzyme for high temperature sugar milling processes.

**Keywords** Thermostable dextranase · *Thermotoga lettingae* · Negative cooperative behavior · Thermophile · GH family 66

## Introduction

A number of glycosidase amino acid sequences have been analyzed. There are currently about 114 glycoside hydro-

lase (GH) families based on sequence similarities (Cantarel et al. 2009). GH family 66 includes endodextranase (EC 3.2.1.11) and cycloisomaltooligosaccharide glucanotransferase (CITase, EC 2.4.1.-). Dextranase hydrolyzes the predominant  $\alpha$ -(1→6) glucosidic linkages found in the glucose homopolymer dextran (Khalikova et al. 2005). Dextranases isolated from bacterial sources include eight extracellular dextranases from *Streptococcus* sp. (Lawman and Bleiweis 1991; Igarashi et al. 1995a, b, 2000, 2004), two *Bacillus circulans* T3040 CITases (Oguma et al. 1995; Yamamoto et al. 2006), and five *Paenibacillus* sp. dextranases (Finnegan et al. 2004a, b, 2005). Five dextranase-similar genes from *Thermoanaerobacter* and *Thermotoga* have also been updated in GH family 66 using full genome sequence analysis (Cantarel et al. 2009). Among them, *Tlet\_1229* from *Thermotoga lettingae* strain TMO (TMO, Balk et al. 2002), a thermophilic anaerobic gram-negative bacterium (GenBank accession number, CP000812), is similar to the gene encoding dextranase but has not been detected as the active enzyme. The present study is the first to clarify the biochemical properties of this protein.

An attractive industrial application of thermostable dextranase is sugar processing. *Penicillium* sp. and *Chaetomium gracile* dextranases that are active and stable above 55°C would improve processing of dextran-contaminated cane juice in the sugar industry (Khalikova et al. 2005) and *Chaetomium* or *Penicillium* dextranase is currently used in worldwide sugar mills (90%) to treat dextran. The remaining 10% of worldwide sugar mills, which are located in the USA, do not use the commercial dextranase (Lee et al. 2006) because US Food and Drug Administration requirements forbid the use of the crude enzymes from potential toxin producing microorganisms in food-related applications. Thus, there is still much effort for the development of alternate dextranase. GH family 66 enzymes are another source of the desired enzyme displaying thermal stability at

Y.-M. Kim  
School of Biological Sciences and Technology,  
Chonnam National University,  
Gwangju 500-757, Republic of Korea

D. Kim (✉)  
School of Biological Sciences and Technology & Research  
Institute for Catalysis, Chonnam National University,  
Gwangju 500-757, Republic of Korea  
e-mail: dmkim@jnu.ac.kr

70–80°C from thermophilic anaerobic bacteria (Wynter et al. 1995, 1997). However, the well-known dextranases from *Paenibacillus* or *Streptococcus* display an undesirably low optimal temperature of 35–50°C. Little attention has been given to use of these enzymes in industrial processes due to the difficulty in culturing the bacteria and in the preparation of large-scale quantities of enzyme (Khalikova et al. 2005). While the genome sequences of several thermophilic anaerobic bacteria have been deduced, there has been no description of the successful production and characterization of the thermophilic dextranases.

In this study, we characterized the TLDex (a recombinant protein of Tlet-1229 in TMO) following over-expression in *Escherichia coli*. We report that TLDex is dextranase showing the negative cooperative behavior on dextran cleavage.

## Materials and methods

### Expression and purification of recombinant protein

*Tlet\_1229* was amplified by polymerase chain reaction using KOD DNA polymerase (Toyobo, Osaka, Japan), and the genomic DNA of TMO (BAA-301, American Type Culture Collection, Manassas, VA, USA) as a template, and the primer pair (forward primer, 5'-TTTCATATGAGAGCAATTTTCGACAAAGC-3' and reverse primer, 5'-GTGCTCGAGCTATTCCTCCTTTTATTTG-3') containing *NdeI* and *XhoI* sites (italicized), respectively. The amplified gene was digested with *NdeI* and *XhoI* and was inserted into the corresponding sites of pET-28a (+) (Novagen, Madison, WI, USA) containing the His-tag at the C-terminus. *E. coli* BL21(DE3)pTrx containing the constructed plasmid grown in lysogeny broth medium containing 100 µg/ml of kanamycin and 30 µg/ml of chloramphenicol at 37°C to an absorbance of 0.5 at 600 nm were then induced with 0.1 mM isopropyl β-D-thiogalactopyranoside for 16.5 h at 18°C. After collection by centrifugation at 7,000×g, the cells were suspended in 20 mM sodium phosphate buffer (pH7.2) containing 500 mM NaCl (buffer A). The suspended cells were sonicated and centrifuged at 15,000×g for 30 min. The supernatant was loaded on a Ni-Sepharose column equilibrated with buffer A. The column was washed with buffer A containing 40 mM imidazole, and the sample protein was eluted with 250 mM imidazole. The elution buffer was then changed to 20 mM sodium acetate (NaAc, pH4.5), and the protein was concentrated using an Amicon Ultra 10,000 molecular weight cut-off (Millipore, Billerica, MA, USA). Protein concentration was determined by Bradford's method (Bradford 1976) with bovine serum albumin (BSA) as standard. The molecular mass of the proteins was estimated by gel

filtration on Superose 6 HR 10/30 column (1×30 cm) using AKTA-fast protein liquid chromatography system with a Frac-950 fraction collector (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The eluting buffer used was 20 mM NaAc buffer (pH4.5) containing 50 mM NaCl, and the flow rate was 0.5 ml/min. The Superose column was calibrated using molecular size standards including thyroglobulin (660 kDa), alcohol dehydrogenase (150 kDa), BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa), and aprotinin (6.5 kDa).

### Sequence analysis

The protein homology searches were done against National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST; Altschul et al. 1997). Predictions of protein signal peptide were done using Signal P (<http://www.cbs.dtu.dk/services/SignalP/>; Bendtsen et al. 2004; Nielsen et al. 1997).

### Measurement of enzymatic activity

Dextranase activity was examined in 40 mM NaAc buffer (pH4.3) with 0.4% (w/v) dextran T2000 at 55°C. The amount of reducing sugars liberated was measured by the copper-bicinchoninate method (McFeeter 1980) using glucose as a standard. One unit (U) of activity was defined as the amount of enzyme that released 1 µmol of reducing power per minute under the assay conditions; glucose was used as the standard. For determination of the optimum pH, 18.4 µg/ml of TLDex was incubated at 55°C in 32 mM (pH 2–12; Britton and Robinson 1931) with 0.4% dextran T2000. For determination of the pH stability, 18.4 µg/ml of enzyme was kept at 4°C for 20 h in 40 mM Britton–Robinson buffer (pH2–12), and the residual dextranase activity was examined. For the determination of optimal temperature, 18.4 µg/ml of enzyme was incubated at 25–80°C for 15 min in 40 mM NaAc buffer (pH4.3) with 0.4% dextran T2000. For determination of the thermal stability, 18.4 µg/ml of enzyme was kept at 25–70°C for 4 h in 40 mM NaAc buffer (pH4.3), and the residual dextranase activity was examined at 55°C. For kinetic constants, the initial velocity (*v*) was measured using various concentrations (*s*) of dextran T2000 (0.75, 1, 1.5, 3, 6, 9, 10.5, 13.5, 15, 16.5, 18, 19.5, 21, and 24 µM) in 40 mM NaAc buffer (pH4.3) at 55°C.

### Substrate specificity of TLDex

The rate of TLDex-mediated hydrolysis of dextran T10, T40, T70, T500, and T2000 from *Leuconostoc mesenteroides* B-512F (Sigma-Aldrich, St. Louis, MO, USA),

alternan [alternating  $\alpha$ -(1→6) and  $\alpha$ -(1→3) glucosidic polymer, kindly provided by Dr. GL Cote, US Department of Agriculture], pullulan (Sigma-Aldrich), or soluble starch was measured in a reaction mixture consisting of 26  $\mu$ g/ml enzyme, 0.4% (w/v) of each substrate, and 40 mM NaAc buffer (pH4.3) at 55°C for 16 h.

TLC analysis of TLDex final products from various glucans

The products of dextran hydrolysis were analyzed by thin layer chromatography (TLC) using a silica gel 60 plate (Merck, Darmstadt, Germany) developed in a solvent system consisting of nitromethan:1-propanol:water in a ratio of 4:10:3 (v/v/v) with glucose and isomaltooligosaccharides (isomaltose to isomaltoheptaose) as the standards. Carbohydrates were visualized on the TLC plate by dipping the plate into a solution containing 0.03 g *N*-(1-naphthyl) ethylenediamine and 5 ml of concentrated sulfuric acid in 95 ml methanol, followed by heating at 120°C (Bounias 1980).

## Results

### Sequence analysis

The similarity of the amino acid sequences of TLDex was investigated using the *blastp* program in NCBI BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The nine highest score proteins in GH family 66 enzymes are shown sequentially in Table 1. TLDex exhibited similarity to dextranase, especially a 29% identity with *Thermoanaerobacter pseudoethanolicus* ATCC33223. Amino acid sequence alignment analysis (Fig. 1) revealed that TLDex shared nine short sequence modules (SSM 1–9) that have

been previously proposed (Aoki and Sakano 1997). Like other dextranases, TLDex also lacked an insertion region of 130 amino acids, which corresponds to Ala416-Gly546 of CITase (Yamamoto et al. 2006). The amino acid sequence of TLDex contained two conserved acidic residues (Asp126 and Asp243), which correspond to Asp183 and Asp308 in *Bacillus* CITase (Yamamoto et al. 2006). From the prediction of protein localization site (PSORT; Nakai and Kanehisa 1991) and signal peptide (SignalP) (Bendtsen et al. 2004) servers, TLDex would be expected to be localized in the cytoplasm and lack a signal sequence.

### Production and characterization of TLDex

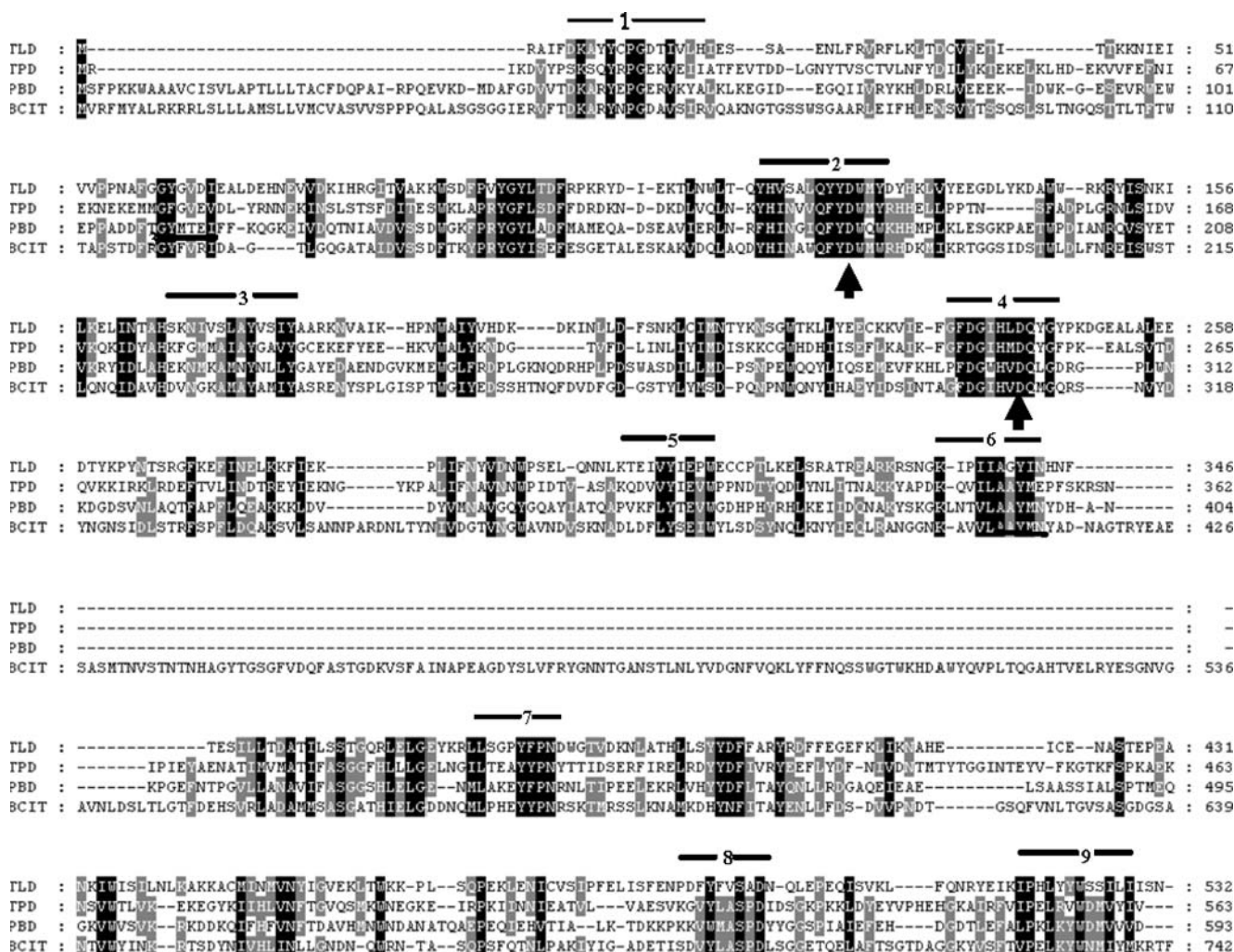
The recombinant TLDex produced in *E. coli* was isolated and purified to homogeneity in a one-step chromatographic procedure. From 1.2 l of culture broth, 2.6 mg of purified TLDex was obtained with a specific activity of 0.8 U/mg. The purified TLDex had a molecular mass of approximately 64 kDa as estimated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2). The native molecular mass was estimated to be approximately 70 kDa by gel filtration on Superose 6 column (data not shown). From these results, the enzyme was considered to be a monomer. The effects of pH and temperature on the purified TLDex were examined. The pH optimum for TLDex activity was 4.3, and the enzyme was stable over a broad pH range (4.3–10.0) after incubation at 4°C for 20 h (Fig. 3). The optimum temperature of TLDex was 55–60°C for 15 min incubation (Fig. 4a), and over 75% of the original activity was sustained at 60°C for 4 h (Fig. 4b). The half-life of TLDex was 1.5 h at 65°C with pH4.3 in the absence of added dextran. To examine the effect of metal ions on dextranase activity, the reaction mixture composed of 0.4% (w/v) dextran T2000, 40 mM NaAc buffer (pH4.3), 1 mM

**Table 1** Amino acid sequence comparison of TLDex to the GH family 66 proteins

Description	GenBank accession no.	Score	Identity (%)	Gap (%)
TLDex (536)	ABV33789			
<i>Thermoanaerobacter pseudoethanolicus</i> ATCC 33223 dextranase (566)	ABY93935	532	29 (158/533)	10 (58/533)
<i>Thermoanaerobacter</i> sp. X514 dextranase (566)	ABY92063	532	29 (158/533)	10 (58/533)
<i>Paenibacillus</i> sp. Dex50-2 dextranase (905)	AAQ91298	439	27 (162/588)	12 (75/588)
<i>Paenibacillus</i> sp. Dex40-8 dextranase (715)	AAQ91294	438	26 (157/591)	15 (89/591)
<i>Paenibacillus</i> sp. Dex70-1B dextranase (596)	AAQ91301	428	25 (146/570)	11 (67/570)
<i>Paenibacillus</i> sp. Dex70-34 dextranase (599)	AAQ91303	421	26 (148/566)	11 (63/566)
<i>Paenibacillus</i> sp. Dex50-2 dextranase (596)	AAQ91298	386	24 (143/573)	12 (69/573)
<i>Flavobacterium johnsoniae</i> UW101 dextranase (586)	ABQ07435	347	25 (143/566)	12 (69/566)
<i>Bacteroides thetaiotaomicron</i> VPI-5482 dextranase (592)	AAO78193	256	24 (123/501)	13 (69/501)

Protein homology searches were done against National Center for Biotechnology Information Basic Local Alignment Search Tool (Altschul et al. 1997). The values in parentheses indicate the number of amino acid residues. The percent identity and gap column values were defined as number of identified or gapped amino acid residues/number of aligned amino acid residues

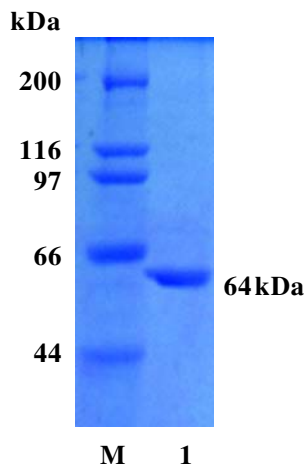




**Fig. 1** Multiple alignment of GH family 66 amino acid sequences. Multiple alignments were done by Clustal W. Numbers on the right indicate residue positions within each sequence. Amino acid identities (black) or similarities (gray) present in three or more of the sequences are indicated, respectively. Two catalytic amino acid residues of TLDex corresponding to CITase are indicated by arrows. Nine highly

conserved short sequence motifs (SSM 1–9) in these sequences are numbered (Aoki and Sakano 1997). TLD dextranase from TMO, TPD putative dextranase from *Thermoanaerobacter pseudoethanolicus* ATCC 33223, PBD dextranase from *Paenibacillus* sp. Dex70-1B (Finnegan et al. 2004b), BCIT CITase from *Bacillus circulans* T-3040 (Yamamoto et al. 2006)

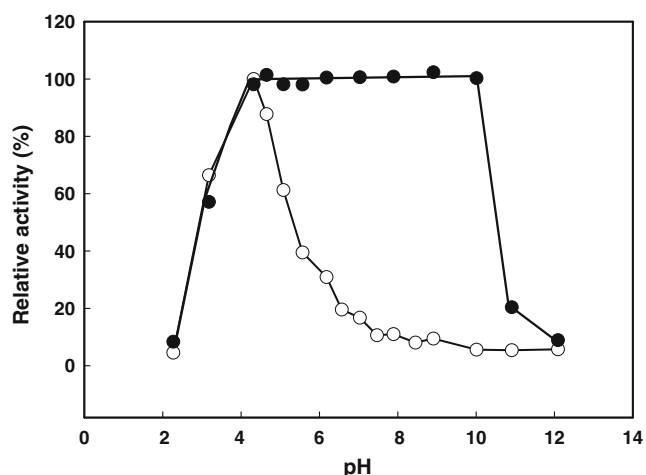
**Fig. 2** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified TLDex produced in *Escherichia coli*. SDS-PAGE of 5.2 μg of purified protein was performed using a 12% gel. Lane M contains size markers, and lane 1 contains purified TLDex



final concentration of metal ions, and 10.4 μg/ml of enzyme was incubated at 55°C for 30 min, and a reducing value was measured. Most of the tested metal ions such as Ca<sup>2+</sup>, Li<sup>2+</sup>, K<sup>+</sup>, and Na<sup>+</sup> (with chloride) did not show appreciable effects. Activity increased from 100% (without addition of metal ions) to 125% in the presence of Ni<sup>2+</sup> and Mg<sup>2+</sup>, while activity decreased from 100% to 10% in the presence of Ag<sup>2+</sup> and Fe<sup>2+</sup>.

**Substrate specificity of TLDex**

The substrate specificity of TLDex towards α-glucans was examined. Table 2 lists the relative hydrolytic activities on each of the substrates. TLDex exhibited the highest activity for dextran T70. TLDex preferred dextran more than



**Fig. 3** Optimum pH for activity and stability of TLDex. Activity profile for various pHs (*open circle*): 18.4  $\mu\text{g/ml}$  enzyme, 0.4% (*w/v*) dextran T2000 in Britton–Robinson buffer at 55°C for 20 min; pH stability profile (*closed circle*): 18.4  $\mu\text{g/ml}$  enzyme, 0.4% (*w/v*) dextran T2000 in Britton–Robinson buffer at 4°C for 20 h. The determination of reducing sugar liberated was performed using the copper-bicinchoninate procedure (McFeeter 1980) with glucose as standard

alternan and pullulan, and could not hydrolyze soluble starch. The enzyme also could not hydrolyze maltose, maltotriose, kojibiose, nigerose, sucrose, methyl  $\alpha$ -glucoside, trehalose, and panose (data not shown). From the hydrolysis products, the enzyme activity was very specific for  $\alpha$ -(1 $\rightarrow$ 6)-glucosidic glucan and produced isomaltose, isomaltotriose, and long isomaltooligosaccharides as final products for a 16-h reaction (Fig. 5). TLDex completely hydrolyzed the dextran T2000 during prolonged 4-day incubation. TLDex did not show any transglucosylation products with extremely high concentration (1%, *w/v*) of each isomaltooligosaccharide (isomaltotriose~isomaltoheptaose; substrates for TLDex) by TLC and high-performance liquid chromatography analysis (data not shown), suggesting that a main reaction on dextran T2000 is the hydrolysis. The  $v$  value for the cleavage of glucosidic linkage of dextran T2000 was the rate of hydrolysis (the liberation velocity of the reducing sugar). The maximum velocity for the cleavage of the dextran T2000 ( $V_{\text{max}}$ ) was estimated by extrapolating the convex curve of  $1/s$  versus  $1/v$  plots to the  $1/v$ -axis (Fig. 6a). The value of  $V_{\text{max}}$  was  $4.8 \mu\text{mol min}^{-1} \text{mg}^{-1}$ , and the  $K_m$  value ( $9.5 \mu\text{M}$ ) for the reaction was defined as the substrate concentration that resulted in half the  $V_{\text{max}}$ . To evaluate the degree of allosteric cooperativity (Wongchawalit et al. 2006), the Hill coefficient ( $n_H$ ) was calculated from the Hill plot (Fig. 6b) using the following equation:

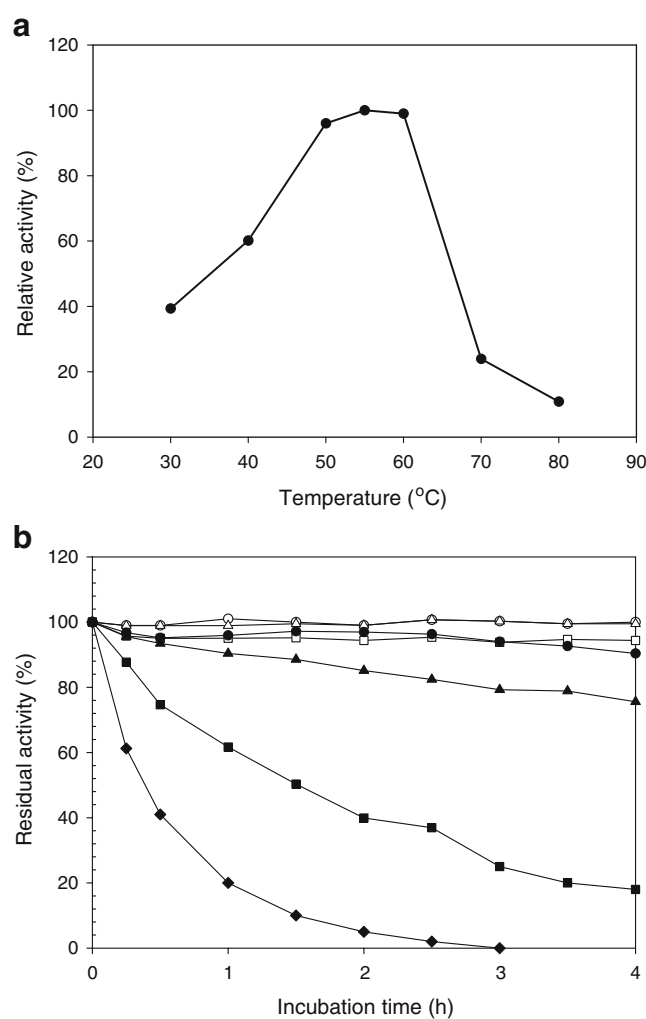
$$\log[v/(V_{\text{max}} - v)] = n_H \log s - \log K,$$

where  $V_{\text{max}}$  is the  $V_{\text{max}}$ , and  $K$  is the dissociation constant for a hypothetical binding of a certain number of

molecules of the ligand to the enzyme. The slope of the plots in coordinate logs versus  $\log [v/(V_{\text{max}} - v)]$  gave the Hill coefficient. The  $n_H$  values Hill coefficient was 0.436. From these findings, we could conclude that TLDex is a novel dextranase showing negative cooperative behavior.

## Discussion

A number of glycosidase amino acid sequences have been revealed by genome sequencing and classified into the GH family on the basis of their structural features (Cantarel et al. 2009). GH family 66 enzymes constitute CITases and



**Fig. 4** Optimum temperature (a) and thermal stability (b) of TLDex. Activity profile at various temperatures (*closed circle*): 18.4  $\mu\text{g/ml}$  enzyme in 40 mM NaAc buffer (pH4.3) for 20 min at various temperature; thermal stability: 18.4  $\mu\text{g/ml}$  enzyme in 40 mM NaAc buffer (pH4.3) at 25–70°C after 4 h incubation. After sampling from the reaction mixture at designated times, the remaining enzyme activity was assayed by a standard assay method. *Open circle*, 25°C; *open triangle*, 40°C; *open square*, 50°C; *closed circle*, 55°C; *closed triangle*, 60°C; *closed square*, 65°C; *diamond*, 70°C

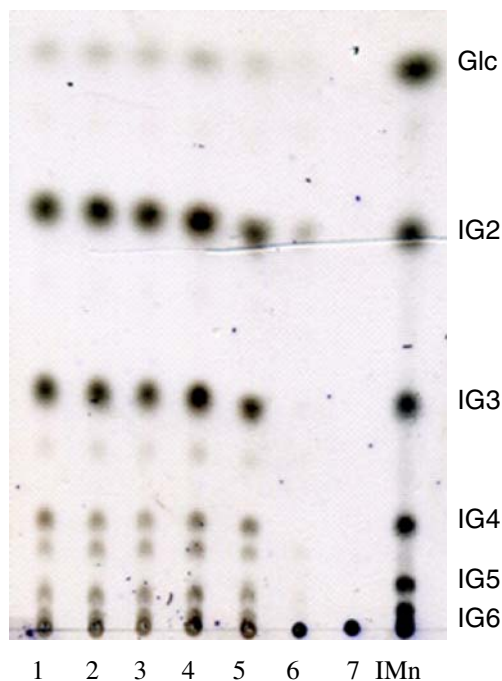
**Table 2** Substrate specificity of TLDex

Substrates	$\mu\text{mol (mg protein)}^{-1} \text{min}^{-1}$	Relative activity (%)
Dextran T10	0.19±0.02	73
Dextran T40	0.23	88
Dextran T70	0.26±0.02	100
Dextran T500	0.25±0.04	96
Dextran T2000	0.25±0.05	96
Alternan	0.04±0.01	15
Pullulan	0.38	4
Soluble starch	NA <sup>a</sup>	0

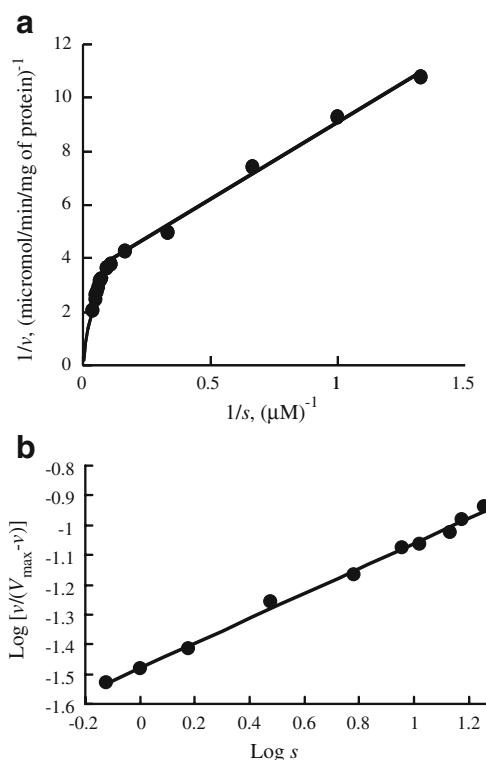
Determined in 40 mM NaAc buffer (pH4.3) at 55°C with 0.4% substrates. Assay was performed as described under “Materials and methods”. The protein amount is a relative value using bovine serum albumin. Relative activity was calculated based on 100% was obtained from dextran T70 hydrolysis

<sup>a</sup>No hydrolysate was detected by assay method

endodextranases. The present putative dextranase coding gene (*Tlet\_1229*) from TMO has been recently updated (GenBank accession number, CP000812) and remains unidentified functionally. TLDex is the first recombinant thermostable dextranase belonging to GH family 66 groups obtained from thermophilic and anaerobic bacteria. Our



**Fig. 5** Thin layer chromatography (TLC) analysis of TLDex hydrolysis product. TLDex (10.4  $\mu\text{g/ml}$ ) was incubated with 0.4% (*w/v*) substrates [lane 1, dextran T10; lane 2, dextran T40; lane 3, dextran T70; lane 4, dextran T500; lane 5, dextran T2000; lane 6, alternan; lane 7, pullulan; lane M, isomaltooligosaccharide standards (DP2–DP7) in 40 mM NaAc buffer (pH4.3) at 55°C for 16 h]. The reaction products were analyzed by TLC using nitromethane/1-propanol/ $\text{H}_2\text{O}$  in a volume ration of 4/10/3



**Fig. 6** Lineweaver–Burk plot (a) and Hill plot (b) for TLDex reaction on dextran T2000. TLDex (18.4  $\mu\text{g/ml}$ ) was incubated with 0.5–30  $\mu\text{M}$  of dextran T2000 in 40 mM NaAc buffer (pH4.3) at 55°C for 20 min. The initial velocity (*v*) was expressed as micromole ( $\mu\text{mol}$ ) reducing power liberated from substrate per minute per milligram of protein. The protein amount is a relative value using bovine serum albumin

observations indicate that TLDex resembles endodextranase on the basis of substrate specificity and hydrolysis products. However, TLDex exhibits unusual catalytic kinetic phenomena from *Paenibacillus* or *Streptococcus* dextranases. The unique feature of TLDex was presently described by the Hill equation. The Hill factor calculated for TLDex was lower than 1, indicating a negative cooperative behavior in the TLDex reaction (Wongchawalit et al. 2006). Similar to a previously reported thermostable dextranase (Arnold et al. 1998), TLDex also appears to be a monomeric enzyme, but one that is capable of allosteric behavior. To our knowledge, this is the first dextranase known to display negative cooperative behavior. Study of the cooperative behavior of TLDex monomeric enzyme is in progress through analysis of the structural changes induced by substrate molecules.

TLDex displayed an optimal activity at 55°C and retained more than 50% maximal activity after incubation at 65°C for 1.5 h incubation. This thermostability is reminiscent or even marginally greater than that of *Paenibacillus* Dex 70-1B or Dex 70–34 enzyme (70% of initial activity remaining after incubation at 57°C for 9.5 h) (Finnegan et al. 2004b). Interestingly, the pH optimum of TLDex, 4.3, is the lowest



pH of the characterized thermostable dextranase (pH5.5; Hoster et al. 2001), *Paenibacillus* dextranase (pH5.5; Finnegan et al. 2004b), and *Streptococcus rattus* dextranase (pH5.0; Igarashi et al. 2004) in the GH family 66 enzymes. TLDex exhibited a broad pH stability range (pH4.3–10.0) compared with *Paenibacillus* dextranase (pH6.0–8.0; Finnegan et al. 2005), especially on the alkaline side. The broad pH stability of TLDex could be useful in sugar processing, which involves both acidic and alkaline conditions. The successful production and biochemical observation of TLDex highlights the potential value of four other candidates from thermophilic anaerobic bacteria in GH family 66. Furthermore, the directed evolution studies aimed at increasing the thermostability (Hild et al. 2007) and broad pH-activity range of TLDex are currently in progress, with the goal of maximizing the utility of the enzyme in sugar processing.

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