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Purification and Characterization of a Thermostable α-Galactosidase from *Thermoanaerobacterium polysaccharolyticum*

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Food ingredients containing α-1,6-galactoside bonds elicit gastrointestinal disturbances in monogastric animals, including humans. Pretreatment of such ingredients with α-galactosidase (EC 3.2.1.22) has the potential to alleviate this condition. For this purpose, a thermostable α-galactosidase from *Thermoanaerobacterium polysaccharolyticum* was purified by a combination of anion exchange and size exclusion chromatographies. The enzyme has a monomeric molecular weight of ∼80 kDa; however, it is active as a dimer. The optimum temperature for enzyme activity is 77.5 °C. Approximately 84 and 88% of enzyme activity remained after 36.5 h of incubation at 70 and 65 °C, respectively. Optimum activity was observed at pH 8.0, with a broad range of activity from pH 5.0 to 9.0. Different transition metals had weak to strong inhibitory effects on enzyme activity. The K_m and V_{max} of the enzyme are 0.29−0.345 mM and 200−232 μmol/min/mg of protein, respectively. Importantly, enzyme activity was only slightly inhibited by 75−100 mM galactose, an end product of hydrolysis. Enzyme activity was specific for the α-1,6-galactosyl bond, and activity was demonstrated on melibiose and soy molasses.

KEYWORDS: ***Thermoanaerobacterium polysaccharolyticum*; α-galactosidase; purification; thermostability**

INTRODUCTION

Intestinal bacteria that produce α-galactosidases induce gastrointestinal disturbances in humans and other monogastric animals that consume oligosaccharides containing α-1,6-galactoside bonds. To alleviate this condition, α-galactosidases (enzymes that hydrolyze α-1,6-saccharolytic linkages) have been exploited in processes aimed to degrade soy and leguminal oligosaccharides (*1*−*4*). Additionally, α-galactosidases have been described as a means of oligosaccharide synthesis by reversed hydrolysis or glycosyl transfer reactions (*5*). However, historically, α-galactosidases used in industrial applications lack thermostability, specificity, and activity at desired pH conditions or demonstrate end-product inhibition. Thus, each process would benefit from an enzyme or group of enzymes that is specific for that application.

Thermostable enzymes have many advantages for use in industrial processes. These include increased thermostability, chemical stability, and improved reactions rates at elevated temperatures (*6*−*8*). To this end, thermostable α-galactosidases were isolated and used to hydrolyze sugar beet oligosaccharides at moderately elevated temperatures (*9*−*12*). Thermostable α-galactosidases isolated from extreme thermophiles are also being investigated for use in the petroleum- and soy-processing industries (*13*−*15*). Interestingly, the α-galactosidase genes of the extreme thermophiles, *Thermotoga maritima* and *Thermotoga neapolitana*, were found to be flanked by other genes involved in galactoside breakdown and utilization (*16*, *17*).

The recently isolated thermophile, *Thermoanaerobacterium polysaccharolyticum* (*18*, *19*), was found to produce a wide variety of enzymes, including an α-galactosidase. We have isolated and characterized this enzyme to determine its potential in industrial applications, especially in the removal of α-1,6-galactoside linkages from soy products at elevated temperatures.

MATERIALS AND METHODS

Strain and Growth Conditions. The isolation and characterization of *T. polysaccharolyticum* have been described elsewhere (*19*). The organism was initially grown on melibiose on a small scale, and an aliquot of the cell extract was screened for α-galactosidase activity. For purification of the enzyme, the bacterium was grown at 68 °C in 20 L of an anaerobic medium (95% CO_2 and 5% H_2) containing 30 g of Trypticase peptone (Difco), 10 g of yeast extract (Difco), 1 g of cysteine hydrochloride per liter (Sigma), and melibiose at a final concentration of 0.5%.

Enzyme Purification. A late log phase (∼14−16 h) culture grown under constant sparging with CO_2 was harvested by centrifugation

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(10000*g*, 10 min., 4 °C). The cell pellet (15−20 g) was resuspended in 200 mL of lysis buffer [25 mM bis-Tris propane, pH 7.0, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 1.0 mM dithiothreitol (DTT)] and disrupted using a bead-beater (Biospec Products, Bartlesville, OK) with an equal volume of 0.1 mm glass beads. The cell debris and beads were pelleted by centrifugation (10000*g*, 10 min., 4 °C), and the cell extracts recovered in the supernatant were retained for purification of the α-galactosidase.

The steps below describe the purification process. Each column chromatography was performed using a Rainin HPLC system (Rainin Instruments Co., Woburn, MA), and all steps were performed at room temperature.

Step 1. Ammonium Sulfate Fractionation. The supernatant (cell extracts) was fractionated by addition of $(NH_4)_2SO_4$ in 5% increments at 4 °C. After each increment, the insoluble fraction was pelleted by centrifugation at 10000*g* for 20 min, resuspended in buffer A (25 mM bis-Tris propane, pH 7.0, containing 0.1 mM DTT), and stored at −80 °C until used.

Step 2. Octyl-Sepharose Chromatography. The $(NH_4)_2SO_4$ concentrations in the samples obtained in step 1 were adjusted to 1.0 M by further addition of buffer A. The active fractions were then pooled and applied to an octyl-Sepharose column (Pharmacia-LKB, Uppsala, Sweden) pre-equilibrated with buffer B [buffer A containing 1.0 M $(NH_4)_2SO_4$]. The proteins that bound to the matrix were eluted with a descending gradient of buffer B [1.0−0.0 M $(NH_4)_2SO_4$] at a flow rate of 1.0 mL/min, and 8 mL fractions were collected.

Step 3. Mono-Q Anion Exchange Chromatography. Active fractions from step 2 were pooled and dialyzed against buffer A, with four changes of 2 L each. The dialyzed fractions were concentrated using Centriprep concentrators with a 30 kDa molecular weight (MW) cutoff (Amicon). The samples were then applied to a Mono-Q (anion exchange) column (Pharmacia-LKB) pre-equilibrated with buffer A. The bound proteins were eluted with a linear gradient of NaCl (0−1.0 M) in buffer A at a flow rate of 0.5 mL/min, and 0.5 mL fractions were collected.

Step 4. Superose-6 Gel Filtration Chromatography. Active fractions from the anion exchange chromatography were pooled and concentrated as described in step 3. Next, the sample was applied to a Superose-6 gel filtration column pre-equilibrated with 25 mM bis-Tris propane, pH 7.0. The chromatography was developed with the same buffer at a flow rate of 0.3 mL/min, and 0.3 mL fractions were collected.

Step 5. Mono-Q Anion Exchange Chromatography. A second anion exchange chromatography step was introduced into the purification program to improve the purity of the final product. Active fractions from step 4 were pooled, dialyzed against buffer A, concentrated with Centriprep concentrators (30 kDa MW cutoff, Amicon), and applied to an anion exchange column (Mono-Q, Pharmacia-LKB) pre-equilibrated with buffer A. The proteins that bound to the matrix were eluted with a linear gradient (0−0.25 M NaCl) of buffer C (buffer A containing 1 M NaCl) at a flow rate of 0.5 mL/min, and 0.5 mL fractions were collected.

Enzyme Assays and Protein Determination. The α-galactosidase activity was measured by the amount of *p*-nitrophenol released from *p*-nitrophenyl α-D-galactopyranoside (pNP-α-Gal; Sigma Chemical Co., St. Louis, MO). The reaction mixture (1 mL) contained 10 μL of the fraction under investigation and 990 μL of 50 mM bis-Tris propane containing pNP-α-Gal at a concentration of 10 mM. The mixture was incubated at the desired temperature (65 °C for standard assay, pH 7.0) for 30 min, and the reaction was terminated by the addition of 2 mL of 0.1 M $NaHCO_3$. The color that developed as a result of the release of *p*-nitrophenol was measured at an absorbance of 405 nm. For buffer characterization assays, bis-Tris propane, HEPES, MES, or sodium phosphate buffer was used at a final concentration of 25, 50, or 100 mM. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute under assay conditions.

Protein Assays. Protein concentrations were estimated by the bicinchoninic acid (BCA) method (*20*).

Molecular Mass (M_r) Determination. The M_r of the purified enzyme was estimated by gel filtration with a Superose-6 size exclusion column (Pharmacia-LKB) as described above. The column was calibrated with high molecular weight markers prior to each protein fractionation (Pharmacia-LKB). In a separate experiment, Superdex-G75 (Pharmacia-LKB) was used to confirm the M_r of the enzyme. The column was equilibrated with 25 mM bis-Tris propane, pH 7.0, containing 0.15 M NaCl, the chromatography was developed with the same buffer at a flow rate of 0.3 mL/min, and 0.3 mL fractions were collected. Prior to running of the purified α-galactosidase, the column was calibrated with low MW markers (Pharmacia-LKB).

Electrophoresis. Enzyme purity was assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (*21*). Low-range molecular weight standards were used for gel calibration (Pharmacia-LKB), and proteins were visualized by either Coomassie Brilliant Blue or silver staining.

N-Terminal Amino Acid Sequencing. The N-terminal amino acid sequence of the purified α-galactosidase was determined by the Genetic Engineering Core Facility at the University of Illinois at Urbana−Champaign.

Temperature and pH Optimum and Stability Assays. The optimum temperature for enzyme activity was determined by the standard assay described above with pNP-α-Gal as the substrate. Activity was monitored over 30 min at 5 min intervals. Temperature stability was determined by incubating enzyme samples in 25 mM bis-Tris propane buffer at each temperature (26, 37, 42, 55, 60, 65, 70, and 75 °C) for 2 and 4 h. Aliquots were taken from the mixture, and the residual activity was determined by the standard pNP-α-Gal assay. Half-life studies were performed by incubating samples in 25 mM bis-Tris propane, pH 7.0, for 36.5 h at the following temperatures: 65, 70, 72.5, 75, 77.5, and 80 °C. Aliquots were taken at various times and assayed for activity by the pNP-α-Gal assay at 55 °C. Arrenhius plots were performed to estimate inactivation energy of the enzyme. The pH optimum of enzyme activity was determined by the standard pNP-α-Gal assay in the following buffers: 25 mM MES, HEPES, or bis-Tris propane adjusted to a pH ranging from 4 to 10. The stability of the enzyme at different pH values was studied using 25 mM bis-Tris propane at pH values ranging from 4.0 to 10.0. The enzyme was preincubated in each of the buffers at 37 °C for 4 h. The remaining activity was then determined by the standard pNP-α-Gal assay.

Activator/Inhibitor Assays. The following compounds were tested in the standard pNP-α-Gal assay for activation or inhibition of α-galactosidase activity: $HgCl_2$, $(NH_4)_2SO_4$, ATP, AMP, EDTA, NAD, NADH, $CaCl_2$, $MgCl_2$, $MgSO_4$, $CdSO_4$, *p*-hydroxymercuric benzoate (pHMCB), KCl, sodium acetate, potassium acetate, *N*-ethylmaleimide, and iodoacetamide (0−10 mM); $CuCl_3$, $NiCl_2$, $MnCl_2$, $CoSO_4$, $FeSO_4$, and $FeCl_3$ (0−1 mM); galactose (0−100 mM); β-mercaptoethanol and dithiothreitol (0−200 mM); and NaCl and BSA (0−500 mM).

Enzyme Kinetics. K_m and V_{max} values were determined from Michaelis−Menten, Eadie−Hofstee, and Lineweaver−Burk plots. Subunit cooperativity and putative substrate-binding site number were determined by performing a Hill plot.

Substrate Specificity. Using the standard pNP-α-Gal assay conditions described above, the enzyme was tested for its ability to hydrolyze the following *p*-nitrophenyl analogue substrates: β-D-cellobioside, α-D-galactopyranoside, β-D-galactopyranoside, β-D-glucopyranoside, β-D-lactoside, β-D-maltoside, and β-D-xylopyranoside (Sigma Chemical Co.).

Natural α-Galactoside Hydrolysis. **Preparation of Soy Molasses and Melibiose.** Spray-dried soy molasses was obtained from Archer Daniels Midland (Decatur, IL). Melibiose was obtained from Sigma Chemical Co. Two percent soy molasses andor 3 mg/mL melibiose solutions (w/v) were prepared in 10 mM bis-Tris propane with a final pH of 8.0. These were used in the total carbohydrate and reducing sugar quantitation assays.

Total Carbohydrate Assays. Total carbohydrate was estimated using a phenol−sulfuric acid procedure (*22*). Three hundred microliters of soy molasses solution (2%) was added to 300 μL of 4% phenol. Concentrated H_2SO_4 (1.5 mL) was then added and mixed well by vortexing. The tubes were incubated at room temperature for 30 min, and absorbance was measured with a spectrophotometer at 485 nm. A glucose standard curve was plotted for each assay with the following concentrations: 0, 20, 40, 60, 80, and 100 μg/mL glucose.

Table 1. Summary of *T. polysaccharolyticum* α-Galactosidase Purification

purifn step	volume (mL)	protein (mg)	activity (units/mL)	total activity[a] (units)	specific activity (units/mg of protein)	purifn factor	recovery (%)
crude extract	180.0	2493.0	3.77	678.7	0.272	1.00	100.00
50–60% ammonium sulfate	22.0	432.3	18.1	397.6	0.922	3.39	58.6
octyl-Sepharose[b]	2.2	6.93	342.3	753.1	108.7	399.2	111.0
Mono-Q ion exchange	4.5	2.2	88.6	398.5	267.2	981.4	58.7
Superose-6	6.0	1.39	28.39	170.3	122.9	451.4	25.1
Mono-Q ion exchange	1.122	0.17	42.02	47.1	247.14	907.8	7.0

[a] One unit of enzyme activity is defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per milliliter per minute. [b] Step includes concentration and dialysis.

Reducing Sugar Assays. A 3,5-dinitrosalicylic acid (DNS) reducing sugar assay was used to estimate the release of reducing ends by α-galactosidase activity (*23*). The reaction mixture contained 100 μL of 2% soy molasses or melibiose solution (3 mg/mL), 80 μL (1 unit) of α-galactosidase enzyme solution, and 20 μL of 0.5 M bis-Tris propane, pH 8.0. The mixture (substrate) was preincubated at 70 °C before the enzyme was added, and incubation was continued for 1 h at the same temperature. Enzyme-containing controls were incubated at 4 °C. To terminate the reaction, 600 μL of DNS solution was added to the sample in each tube and incubated in boiling water for 10 min. The reaction mixture was cooled to room temperature, and 1.2 mL of double-distilled H_2O was added. After 20 min of incubation at room temperature, the absorbance at 550 nm was determined with a spectrophotometer, and for each assay a glucose standard curve was plotted and used to estimate the reducing sugars released.

RESULTS AND DISCUSSION

T. polysaccharolyticum cells, grown on melibiose, produced high levels of a thermostable α-galactosidase. The combination of ammonium sulfate precipitation and liquid chromatography based on hydrophobic interactions, anion exchange chromatography, gel filtration, and a second anion exchange chromatography resulted in a purification factor of 908 with 7.0% recovery of the α-galactosidase activity (**Table 1**). The addition of affinity chromatography as an early step may provide a better yield of purified enzyme.

The specific activity of the purified α-galactosidase is 247.1 units mg^{-1} of protein. This is about half of the value (515 units mg^{-1} of protein) determined for a thermostable recombinant α-galactosidase from *Bacillus stearothermophilus* (*24*).

Chromatograms and activity peaks of each fraction are shown in **Figure 1**. Preliminary gel electrophoresis (SDS-PAGE) demonstrated a predominant band of size ~80 kDa (**Figure 2**). However, the size of the protein was estimated to be 176 kDa with a Superose-6 size exclusion column, suggesting that the *T. polysaccharolyticum* α-galactosidase is a homodimer in solution. Contrary to our expectation, further analysis with a Superdex G-75 gel filtration column yielded a protein of size 88 kDa, suggesting the dissociation of the oligomeric form into monomers on this matrix. From the protein obtained from the Superose-6 gel filtration column, a homogeneous protein estimated to be 200 or 80 kDa when un-denatured or denatured (by boiling in SDS sample buffer), respectively, was visualized through non-denaturing polyacrylamide gel electrophoresis (**Figure 3** panel I, lanes 1 and 2). Activity gel analysis showed that whereas the undenatured product (~200 kDa) was active, the denatured protein (~80 kDa) lacked α-galactosidase activity (**Figure 3**, panel II, lanes 1 and 2, respectively). These results suggested that the *T. polysaccharolyticum* α-galactosidase was active in an oligomeric form, probably a dimer. Attempts to reconstitute the denatured 80 kDa protein from the Superdex G-75 chromatography were unsuccessful, and α-galactosidase activity was not detected from this sample, probably due to an irreversible denaturation. In agreement with the results from the Superose-6 gel filtration analysis, Hill plot data provided evidence of an enzyme with a positive cooperativity of $n = 1.4$ and a subunit number of 1.97 (~2), which suggests a complex possessing two binding sites.

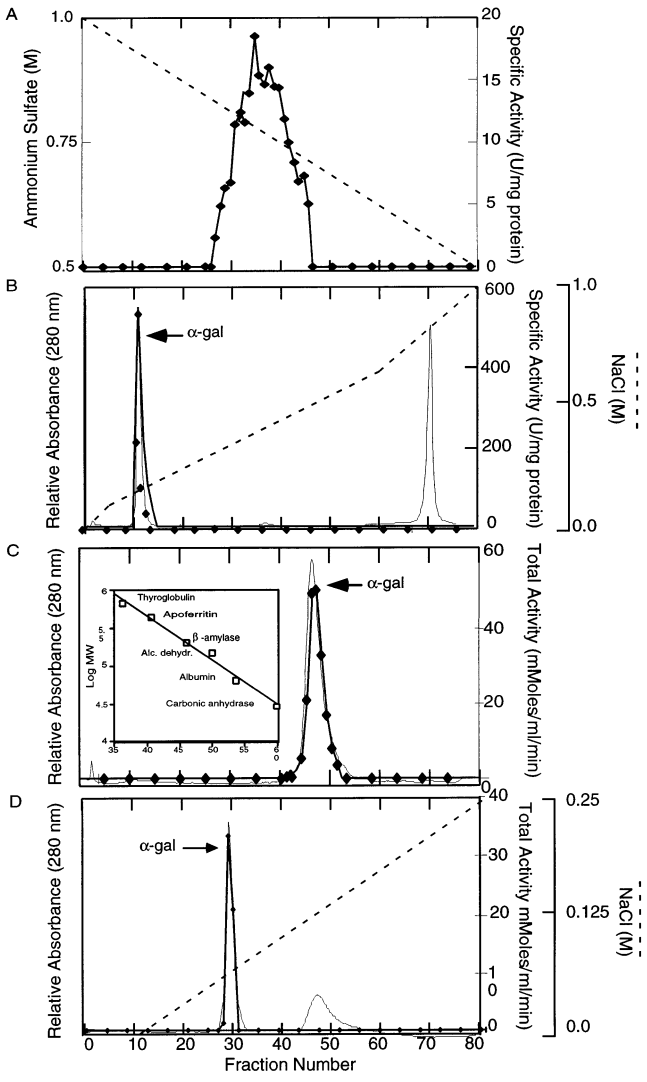

Figure 1. Chromatographic profiles of the α-galactosidase purification steps: octyl-Sepharose Cl-4B hydrophobic interaction (A), Mono-Q ion exchange using a wide NaCl gradient (B), Superose-6 gel filtration (C), and Mono-Q ion exchange using a narrow NaCl gradient (D). Relative absorbances (—), designated salt gradient (- - -), and activities (◆) are indicated.

As shown in **Table 2**, the N-terminal amino acid sequence of the *T. polysaccharolyticum* α-galactosidase was determined to be GIYYDSENRAFYLNA. Although this region does not

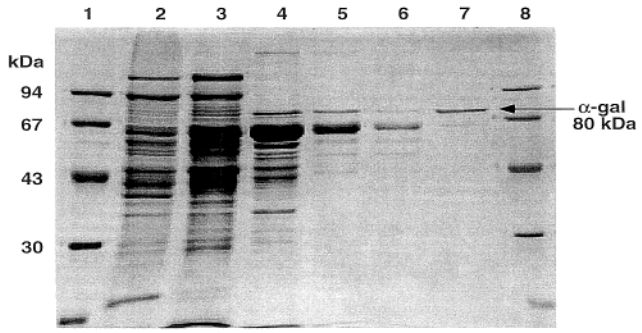

Figure 2. SDS-PAGE (10%) of *T. polysaccharolyticum* α-galactosidase purification steps: (lane 1) low MW marker (indicated); (lane 2) crude extract fraction (110.8 ng of protein); (lane 3) 50–60% ammonium sulfate fraction (137.5 ng of protein): (lane 4) octyl-Sepharose Cl-4B fraction (31.5 ng of protein); (lane 5) Mono-Q anion exchange fraction [broad gradient (6.1 ng of protein)]; (lane 6) Superose-6 gel filtration fraction (3.2 ng of protein); (lane 7) Mono-Q anion exchange fraction [shallow NaCl gradient (2.4 ng of protein)]; (lane 8) low MW marker. Protein gel was stained with Coomassie Brilliant Blue.

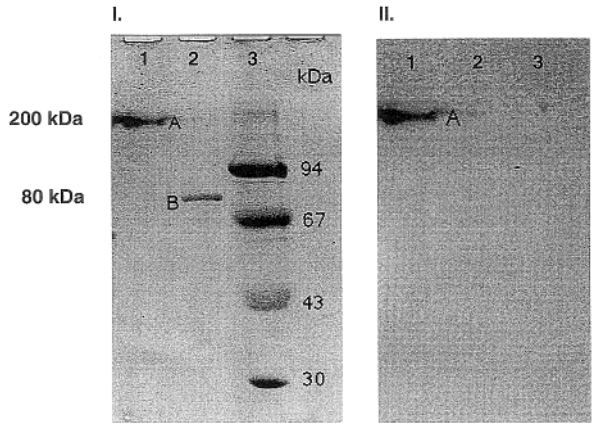

Figure 3. Zymogram-PAGE (10%) analysis of *T. polysaccharolyticum* α-galactosidase. Purified α-galactosidase was separated by electrophoresis: (lane 1) purified α-galactosidase (undenatured); (lane 2) purified α-galactosidase (denatured); (lane 3) low MW marker; (panel I) staining by Coomassie Brilliant Blue after X-α-gal staining; (panel II) staining of gel with X-α-gal. (A) indicates purified un-denatured α-galactosidase that migrates at ~200 kDa, appearing when stained with X-α-gal or Coomassie Brilliant Blue. (B) indicates purified heat-denatured α-galactosidase, which demonstrates no activity on X-α-gal in this form, appearing only when stained by Coomassie Brilliant Blue.

appear to be conserved among known α-galactosidases, the N-terminal amino acid sequence of *T. polysaccharolyticum* α-galactosidase shared some amino acid conservation (**Table 2**, underlined) with that of *Lactobacillus salivarius* (*3*), albeit at only a few positions.

A comparison of the properties of the *T. polysaccharolyticum* α-galactosidase with those of some hitherto reported α-galactosidases is presented in **Table 3**. The approximate molecular size (80 kDa) of the purified protein is consistent with that of family 36 glycosyl hydrolases such as the α-galactosidases of *Streptococcus mutans* and *Escherichia coli* Raf A (*25*, *26*). These α-galactosidases and others of monomeric MW of 80−85 kDa (*3*, *27*−*29*) represent group one of bacterial α-galactosidases (*30*). The members of group two have smaller molecular sizes, ranging from 53 to 65 kDa. Interestingly, most were isolated from extremely thermophilic bacteria (growth at temperature >80 °C), mainly of the genera *Thermus* and *Thermotoga* (*30*). To date, only the *T. polysaccharolyticum*, *T. maritima* GalA, and *E. coli* MelA α-galactosidases are reported to be active as dimers, whereas monomers, trimers, and tetramers have been reported for other organisms (**Table 3**). Heat treatment of the *T. polysaccharolyticum* α-galactosidase by boiling caused an irreversible loss of activity but no further dissociation into subunits of lower molecular weight.

Table 2. Comparison of *T. polysaccharolyticium* α-Galactosidase N-Terminal Sequence to α-Galactosidases of Mesophilic and Thermophilic Microorganisms

Organism	N-Terminal Sequence	Reference
T. polysaccharolyticum	GIYYDSENRAFYLNA	This paper
L. salivarius	VDLITVKENRVFHLHNEQL	3
T. ethanolicus	MGRDVLNFNVDWLYIPEDLN	40
T. neapolitana NS-E aglA	MEIFKRPFREGSFVLKEKDY	15
T. maritima	MEIFGKTFREGRFVLKEKNF	16
S. mutans	MGIBIKDNLFYIHDKDSSLI	26
E. coli melA	MMSAPKITFIGAGSTIFVKB	38
E. coli rafA	MISKYCRLSSPRSDLIIKTH	41
P. pentosaceus	MNGEYKTLANKSFESNVLFF	42
A. niger	MIQGLESIMNQGTKRILLAA	43

A 60 kDa protein was frequently found in the purified product (**Figure 2**, lane 7). A protein of a similar size was reported previously (*27*) during the purification of two α-galactosidases (80 and 85 kDa) from *Bacteroides ovatus*. This protein could be a contaminant or a truncated form of the α-galactosidase or a scaffolding protein that functions to stabilize the oligomeric form of the respective α-galactosidases. Further investigations into these hypotheses are required.

The K_m of the α-galactosidase is 0.29−0.35 mM, and the V_{max} is 200−232 μmol/min/mg of protein. These parameters are similar to those of *L. salivarus* α-galactosidase (K_m of 0.25 mM and V_{max} of 193 μmol/min/mg of protein). The enzyme is specific for hydrolysis of the α-1,6-galactosyl bond and showed little or no activity on *p*-nitrophenol-linked β-D-cellobioside, β-D-galactopyranoside, β-D-glucopyranoside, β-D-lactoside, β-D-maltoside, or β-D-xylopyranoside.

Of the buffers examined, MES buffer yielded the highest activity (**Figure 4**). However, bis-Tris propane was used during purification due to frequent precipitation problems associated with the MES buffer. The optimum pH of the α-galactosidase in 25 mM bis-Tris propane at 65 °C is 8.0. At least 50% of activity was retained between pH 5.0 and 9.0, which corresponded well to the pH limits for growth of *T. polysaccharolyticum* (*19*).

The enzyme has an optimum temperature of 77.5 °C and demonstrated maximal thermostability at 70 °C. The half-lives at 80, 77.5, 75, and 72.5 °C were 1.1, 2.7, 5.1, and 234 min, respectively. Approximately 84 and 88% of activity remained after incubation of the enzyme samples for 36.5 h at 70 and 65 °C, respectively. There was little or no loss of activity after 2 and 4 h of preincubation at ≤70 °C. Thus, the enzyme is stable at temperatures slightly above the temperature of optimum cell growth (65−68 °C) (*19*). An Arrenhius plot of activity levels after incubation at various temperatures gave an energy of enzyme deactivation of 11.9 kcal/mol. Enzymes isolated from thermophiles have been reported to have turnover rates similar to those found in their mesophilic counterparts at their optimum temperatures of activity (*8*, *31*). However, due to increased reaction rates at the higher temperatures, it may be advantageous to use thermostable enzymes.

The temperature stability (70 °C) of the *T. polysaccharolyticum* enzyme is slightly better than that of recombinant AgaN, an α-galactosidase of *B. stearothermophilus* strain NUB3621 (half-life at 70 °C of 19 h) (*24*). However, the optimum

Table 3. Comparison of α-Galactosidase Properties of Various Thermophilic and Mesophilic Bacteria

organism	subunits	subunit MW (kDa)	K_m (mM) mel	raf	PNPαG	pH optimum	temp optimum (°C)	ref
T. polysaccharolyticum	2	80	ND[a]	ND	0.29–0.35	7.5–8.0	77.5	this paper
T. maritima GalA	2	62	NR[b]	2.1	0.075	5.0–5.5	90–95	11
T. neapolitana 5068	1	61	NR	NR	NR	7.0–7.5	100–105	13, 14
T. neapolitana NS-E	1	61	NR	NR	NR	7.0	93	15
B. ovatus (α-gal I)	3	85	20.8	98.1	0.2	5.9–6.4	NR	27
B. ovatus (α-gal II)	3	80.5	2.3	5.9	0.4	6.3–6.5	NR	27
B. stearothermophilus	3	82	NR	NR	NR	7.0–7.5	60	28
B. stearothermophilus	4	80.3	12	16.4	0.38	6.0–7.5	75	24
L. salivarius	1	80	NR	NR	0.25	5.5–6.0	50	3
E. coli	NR	NR	2.33×10^{-3}	3.65×10^{-2}	1.07×10^{-4}	6.8	37	35
E. coli Raf A	4	81.2	NR	60	0.14	7.2	NR	25
E. coli MelA	2	50.6	10	NR	3	8.1	NR	36, 38, 39
S. mutans	4	82	9.1	197	4.4	6.5	NR	26

[a] ND, not determined. [b] NR, not reported.

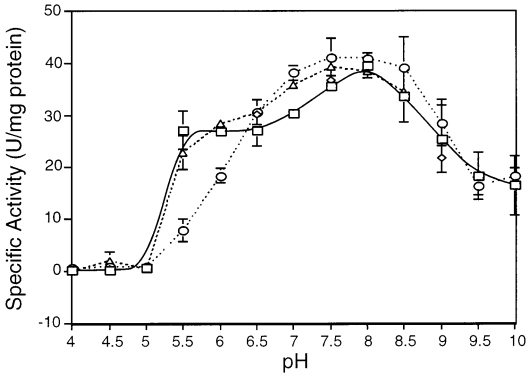

Figure 4. Effect of pH on *T. polysaccharolyticum* α-galactosidase activity. The squares represent bis-Tris propane buffer, the circles are for MES buffer, and the triangles are for citrate–phosphate buffer. The activity at each pH represents the mean ± SE of four measurements.

temperature of activity and stability is lower than that of α-galactosidases isolated from *Thermotoga* spp. (*13*−*15*). This may be an advantage depending on the temperature required for the processing of the α-galactoside, as the temperatures required for optimal activity of hyperthermophilic enzymes may not always be desirable for industrial applications.

A number of compounds were screened to determine their effect on the activity of the enzyme. Only AMP and NAD^+ at 10 mM concentration slightly activated enzyme activity (>10%). DTT and β-mercaptoethanol had no effect on dialyzed samples. Interestingly, galactose at concentrations of 75 and 100 mM had only slight inhibitory effects (up to 16% reduction in activity) on activity. This lack of galactose inhibition at moderate concentrations is likely to be an advantage for industrial applications of the *T. polysaccharolyticum* α-galactosidase. Most of the compounds tested were found to be inhibitory to enzyme activity (**Table 4**). Transition metals, which are likely to oxidize sulfhydryl groups, particularly have inhibitory effects on α-galactosidase activity. In the present experiment, pHMCB, a sulfhydryl group affector, totally inhibited enzyme activity, providing evidence of the presence of a sensitive sulfhydryl group(s) in this enzyme. Mercuric chloride is also known to be a strong thiol-specific inhibitor (*32*). As shown in **Table 4**, $HgCl_2$ at 1.0 mM had a strong inhibitory effect on activity and likewise $CdSO_4$. These results were similar to those reported for other α-galactosidases hypothesized as possessing sensitive sulfhydryl groups (*3*, *26*, *33*, *34*).

The total carbohydrate assay gave a total carbohydrate content of the 2% soy molasses as 5922 μg/mL. It has been reported

Table 4. Compounds Inhibiting Strain KM-THCJ α-Galactosidase Activity

compound	% inhibition 0.25 mM	1.0 mM	10.0 mM
	Weak Inhibitors		
$CuCl_2$	4.8 ± 1.9	30.8 ± 3.1	
$FeCl_3$	21.5 ± 1.5	46.2 ± 1.5	
$FeSO_4$	9.2 ± 1.2	50.7 ± 1.9	
$MnCl_2$	1.0 ± 0.8	18.4 ± 5.1	
$NiCl_2$	9.7 ± 0.5	21.7 ± 0.4	
$CaCl_2$		3.1 ± 0.5	19.9 ± 1.5
$MgCl_2$		1.7 ± 1.2	16.4 ± 1.4
N-ethylmaleimide		7.2 ± 1.8	12.9 ± 1.8
	Strong Inhibitors		
$CdSO_4$	99.9 ± 15.4	99.9 ± 28.0	
$CoSO_4$	8.3 ± 3.17	84.4 ± 1.6	
pHMCB	97.8 ± 15.5	98.0 ± 18.2	
$HgCl_2$		88.2 ± 16.9	84.3 ± 14.4
$ZnCl_2$		99.3 ± 13.7	99.4 ± 12.6

that α-1,6-containing oligosaccharides constitute slightly >50% of the total sugar content of soy molasses (*3*). Thus, the estimated total α-1,6-oligosaccharide content is ~3000 μg/mL. Incubation of the *T. polysaccharolyticum* α-galactosidase with soy molasses released 17 μg/mL of reducing sugar/h, equaling 0.6% of the total α-1,6-oligosaccharide content. The same amount of enzyme released 112.5 μg/mL of reducing sugar/h from melibiose (3.75% of total α-1,6 content). Only 1 unit (~5 ng of enzyme) of enzyme was used in these assays, yielding 0.6 and 3.8% hydrolyses of α-1,6-oligosaccharide in soy molasses and melibiose, respectively, in 1 h at 70 °C.

The temperature of incubation was chosen because it represents the temperature at which the enzyme exhibits long-term stability while maintaining an acceptable level of activity. At this temperature, the *T. polysaccharolyticum* α-galactosidase will exhibit 25% less activity than that observed at its optimum temperature (77.5 °C). The *B. stearothermophilus* α-galactosidase was tested using milligram quantities of enzyme. Eighty-seven percent of raffinose used as substrate (2.4% raffinose solution) was hydrolyzed after 6.6 h of incubation with *B. stearothermophilus* α-galactosidase (*9*). The *T. polysaccharolyticum* α-galactosidase was tested at much lower (nanogram) concentrations. If these values are scaled up for application optimization, the *T. polysaccharolyticum* α-galactosidase would likely demonstrate a better substrate hydrolyzing effectiveness and be comparable to or more favorable than that of the *B. stearothermophilus* enzyme.

Observed also in this study was the 7-fold increase in α-galactoside hydrolysis of the melibiose versus the soy molasses. The soy molasses may contain a number of the compounds shown to be inhibitory to the α-galactosidase. The large-scale isolation of *T. polysaccharolyticum* α-galactosidase and the optimization of its application to soy molasses processing should be a future research focus.

On the basis of the results obtained from the isolation and characterization of this enzyme, it would appear that the enzyme has numerous potential applications. For commercial application, where cost of production is an issue, the purification could be ended at the first Mono-Q step and still have a fairly purified and active enzyme. The neutral to alkaline activity and thermostability of *T. polysaccharolyticum* α-galactosidase are well suited for applications in the sucrose industry (*9*). Furthermore, the thermostability, lack of significant inhibition by galactose, and soy molasses hydrolysis warrant further studies on this enzyme for application to soy α-galactoside processing.

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