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Purification and Characterization of Extremely Thermostable β-Mannanase, β-Mannosidase, and α-Galactosidase from the Hyperthermophilic Eubacterium *Thermotoga neapolitana* 5068

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Thermostable and thermoactive β-mannanase (1,4-β-D-mannan mannanohydrolase [EC 3.2.1.78]), β-mannosidase (β-D-mannopyranoside hydrolase [EC 3.2.1.25]), and α-galactosidase (α-D-galactoside galactohydrolase [EC 3.2.1.22]) were purified to homogeneity from cell extracts and extracellular culture supernatants of the hyperthermophilic eubacterium *Thermotoga neapolitana* 5068 grown on guar gum-based media. The β-mannanase was an extracellular monomeric enzyme with a molecular mass of 65 kDa. The optimal temperature for activity was 90 to 92°C, with half-lives ($t_{1/2}$) of 34 h at 85°C, 13 h at 90°C, and 35 min at 100°C. The β-mannosidase and α-galactosidase were found primarily in cell extracts. The β-mannosidase was a homodimer consisting of approximately 100-kDa molecular mass subunits. The optimal temperature for activity was 87°C, with $t_{1/2}$ of 18 h at 85°C, 42 min at 90°C, and 2 min at 98°C. The α-galactosidase was a 61-kDa monomeric enzyme with a temperature optimum of 100 to 103°C and $t_{1/2}$ of 9 h at 85°C, 2 h at 90°C, and 3 min at 100°C. These enzymes represent the most thermostable and thermoactive versions of these types yet reported and probably act synergistically to hydrolyze extracellular galactomannans to monosaccharides by *T. neapolitana* for nutritional purposes. The significance of such substrates in geothermal environments remains to be seen.

Hyperthermophilic microorganisms (those capable of growth at temperatures above 90°C) are sources of extremely thermostable saccharolytic and hemicellulolytic enzymes which could either replace those currently used at less than optimal processing temperatures or be used in new biocatalytic applications (1). The most extensively examined sources of hyperthermophilic glycosyl hydrolases have been members of the eubacterial genus *Thermotoga* (6, 10, 21), which produce xylose isomerase (11, 46), β-galactosidase (15, 34), β-glucosidase (15, 41, 42), xylanase (12, 43, 47), amylase (24), cellobiohydrolase (41), and β-xylosidase (42). The cloning and expression of genes encoding several of these *Thermotoga* enzymes have been reported (12, 34, 46).

Given the natural abundance of hemicellulose (heteroglycans), it is not surprising that many microorganisms have enzyme systems for its hydrolysis (19). Moreover, given the variety and complexity of hemicellulases, several biocatalytic steps are typically required to hydrolyze specific polysaccharides completely into simpler sugars that can be readily used as carbon and/or energy sources by particular microorganisms. Hetero-1,4-β-D-mannans, one of the major constituents of hemicellulose, are hydrolyzed to mannose through endo-acting β-mannanases (1,4-β-D-mannan mannanohydrolase [EC 3.2.1.78]) (29), and exo-acting β-mannosidases (β-D-mannopyranoside hydrolase [EC 3.2.1.25]) (30, 31). Additional enzymes are required to remove side chain sugars that are attached at various points on mannans. For example, galactomannans have galactose residues bound to the mannan backbone, and their removal is effected through the action of α-galactosidases (α-D-galactoside galactohydrolase [EC 3.2.1.22]) (27). Figure 1 shows a galactomannan molecule, with the glycosidic bonds that are hydrolyzed by the three enzymes of interest in this study. β-Mannan-based natural polymers have wide-ranging industrial applications, such as those used in the processing of foods and the massive hydraulic fracturing of oil and gas wells. Enzymatic treatment of coffee beans (18) and the production of Konjac (36, 37) both require the hydrolysis of β-mannan-based oligosaccharides. Recent developments in the oil and gas industries have established a need for the in situ enzymatic hydrolysis of galactomannans used in well stimulation (32). In these applications, enzyme thermostability and thermoactivity are factors. In food processing, for reasons of asepsis and the need to process viscous materials, elevated temperatures provide obvious advantages. In oil and gas well stimulation, temperatures exceeding 100°C are characteristic of the deeper reaches of well bores (32). Thus, the availability of thermostable and thermoactive versions of enzymes involved in the hydrolysis of β-mannan-based natural polymers is necessary.

Here we report the purification and characterization of three hemicellulases from *Thermotoga neapolitana* 5068 which are involved in galactomannan degradation. The existence of these enzymes in this organism raises questions about the significance of this substrate in geothermal environments. In addition, the discovery of these thermostable activities expands the range of industrially important enzymes known to be produced by hyperthermophiles (1).

MATERIALS AND METHODS

Bacterial strain and culture conditions. *T. neapolitana* DSM 5068 (22) was obtained from Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany. Cells were cultured in an artificial seawater-based medium (11) supplemented with 0.1% yeast extract, 0.5% tryptone, 0.5% lactose, and 0.03% guar

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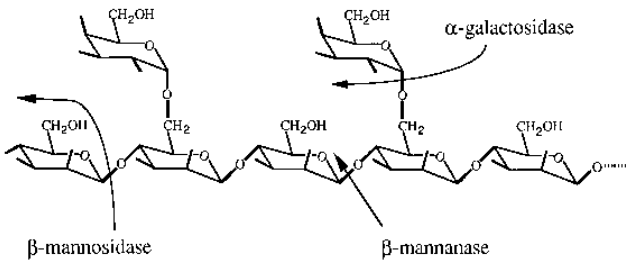

FIG. 1. Schematic galactomannan structure. The nonreducing end of guar gum is shown schematically. The polymannose chain (β-linkage) is substituted every 2 residues by a galactose molecule (α-linkage). The hydroxy groups are shown as thick bars in their correct equatorial or axial positions. The arrows represent the glycosidic links recognized by β-mannanase, β-mannosidase, and α-galactosidase. Based on references 28–30.

gum. The medium composition was (per liter) as follows: NaCl, 15.0 g; Na_2SO_4, 2.0 g; $MgCl_2 \cdot 6H_2O$, 2.0 g; $CaCl_2 \cdot 2H_2O$, 0.50 g; $NaHCO_3$, 0.25 g; K_2HPO_4, 0.10 g; KBr, 50 mg; H_3BO_3, 20 mg; KI, 20 mg; $Fe(NH_4)_2(SO_4)_2$, 15 mg; $Na_2WO_4 \cdot 2H_2O$, 3 mg; and $NiCl_2 \cdot 6H_2O$, 2 mg. Lactose, guar gum, K_2HPO_4, and $Fe(NH_4)_2(SO_4)_2$ were added after sterilization. Lactose, K_2HPO_4, and $Fe(NH_4)_2(SO_4)_2$ were filtered through a 0.2-μm-pore-size filter before being added to the mixture. Inocula were grown in closed bottles (125 ml) under anaerobic conditions. These cultures were prepared by heating the medium-containing bottles to 98°C for 30 min, sparging them with nitrogen, and adding $Na_2S \cdot 9H_2O$ (0.5 g/liter) from a 50-g/liter stock solution. Prior to anaerobic inoculation, the cultures were cooled to 80°C.

Biomass production. Biomass corresponding to 150 liters of culture was generated in the artificial seawater-based medium with 0.5% lactose and 0.03% guar gum as supplements. Cells were grown in a semi-batch fashion in an 8-liter fermentor (Bioengineering Lab Fermentor type 1 1523, Basel, Switzerland). Anaerobic conditions were established and maintained by a continuous flow of nitrogen at approximately 5 liters/min. Temperature and agitation were controlled at 85 ± 2°C and 150 rpm, respectively. Growth was monitored by cell density enumeration and determined by epifluorescence microscopy with acridine orange stain (20). Cells were harvested in the late exponential phase (1.5×10^8 to 2.0×10^8 cells/ml). The culture (150 liters) was chilled and concentrated to approximately 20 liters with a 0.45-μm-pore-size Pellicon cross-flow filter (Millipore Corp., Bedford, Mass.). The cells were then pelleted by centrifugation of the retentate at 10,000 × *g* for 30 min and were frozen at −20°C for later use. The spent medium (extracellular material, approximately 130 liters) was further concentrated to 1 liter with a 0.22-μm Pellicon cross-flow filter (Millipore Corp.).

Preparation of cell extract. Concentrated cell pellets of *T. neapolitana* 5068 were resuspended in 430 ml of 0.1 M sodium phosphate buffer (pH 7.4) and disrupted by one passage through a French pressure cell at 18,000 lb/in^2. NaN_3 (0.01%, wt/vol) was added at this stage to minimize contamination. Cell debris was removed by centrifugation at 10,000 × *g* for 30 min, and the soluble fraction was used as the crude enzyme preparation.

Enzyme assays. β-Mannosidase and α-galactosidase activities were determined by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-mannopyranoside or *p*-nitrophenyl-α-D-galactopyranoside, respectively (Sigma Chemical Co., St. Louis, Mo.). Spectrophotometric readings were taken with a Lambda 3 spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.) equipped with a thermostated and automated six-cell transport system. A liquid-circulating temperature bath (model 1130; VWR Scientific, Philadelphia, Pa.), containing a 1:1 mixture of ethylene glycol and water, was used to maintain the desired temperature in the cell holder, monitored by a thermocouple mounted in a cuvette that was placed in the cell transporter. The six-cell transporter was controlled and data were collected and analyzed by computerized spectroscopy software (Perkin-Elmer Corp.). Routine enzymatic assays for β-mannosidase or α-galactosidase were conducted as follows. For each assay, 1.1-ml aliquots of 10 mM substrate in 0.1 M sodium phosphate buffer (pH 7.4) were pipetted into capped quartz cuvettes (Uvonic Instruments, Inc., Plainview, N.Y.). The cuvettes were preincubated for at least 10 min to allow the substrate to reach the assay temperature. After the preincubation, 0.1 ml of sample was added to the cuvettes and mixed promptly. The release of the *p*-nitrophenol (PNP) was measured by monitoring the change in absorbance at 405 nm. A blank containing the same amount of sample in 0.1 M sodium phosphate buffer (pH 7.4) was used as a control. At temperatures below 100°C and pHs below 9.5, nonenzymatic release of PNP was found to be negligible. One unit of β-mannosidase or α-galactosidase activity was defined as the amount of enzyme releasing 1 μmol of PNP per min under the specified assay conditions.

β-Mannanase activity was determined by monitoring the hydrolysis of Azocarob galactomannan substrate (AZO-LBG); Megazyme, Sydney, Australia) and/or by monitoring the release of reducing sugar (26, 28). The AZO-LBG substrate is a derivative of locust bean gum (LBG) to which a dye, Remazol Brilliant Blue R, has been covalently bound to the galactomannan polymannose backbone at an average of 1 of 20 mannose units. Digestion of the substrate by mannanase breaks the mannose chain, releasing smaller, more soluble galactomannan pieces which, because of the chromogenic dye, can be monitored spectrophotometrically. The assay was performed as follows. A 0.25-ml portion of a 1% solution of AZO-LBG was added to a 1.5-ml microcentrifuge tube. The reaction was started by adding 0.25 ml of an enzyme sample to the tube placed in a bath heated to the desired assay temperature. After a 30-min incubation, the reaction was stopped by placing the tubes in ice-cold water. The nondegraded substrate was precipitated at −20°C for 20 min by adding 1 ml of 95% ethanol. After centrifugation at 10,000 × *g* for 10 min, the increase in absorbance of the supernatant was measured at 590 nm and compared with that of a blank run under the same conditions. One unit of activity for assay with this substrate was defined as the amount of enzyme that increased the absorbance by 1 unit in 1 min. This assay was used for screening column fractions during the purification of β-mannanase as well as for the characterization of pH and temperature optima of the enzyme. Conversion to reducing-sugar equivalents (micromoles of reducing-sugar equivalent released per minute per milligram of protein) was done by using a standard curve provided by the manufacturer (Megazyme).

β-Mannanase kinetics were estimated by monitoring the release of reducing sugar. Initially, LBG substrate (Megazyme) was prepared in 0.05 to 0.2% (wt/vol) solutions. A sample of β-mannanase (50 μl) was added to 0.5 ml of substrate solution preheated at the temperature tested. Degradation of the LBG was stopped by immersing the tube in ice-cold water. Two methods were used to measure the released reducing sugar. In one, the sample was treated with *p*-hydroxybenzoic acid hydrazide mixed with sodium citrate as described previously (26). The second method was an adaptation of the Somogyi-Nelson assay provided by the LBG manufacturer, Megazyme. In either case, standard curves for reducing equivalents were generated with mannose as the substrate. One unit of activity was defined as 1 μmol of reducing sugar released per minute per milligram of enzyme.

Protein determination was performed by two different methods. The method of Bradford (9), involving the standard assay kit from Bio-Rad (Richmond, Calif.) with bovine serum albumin as a standard, was used for assays on samples with a high protein concentration (cell extract). The bicinchoninic acid method (44), using the standard BCA kit (Pierce, Rockford, Ill.) with bovine serum albumin as the standard, was used for samples with a low protein concentration, as well as for samples containing detergent.

Purification protocol. β-Mannosidase and α-galactosidase were purified from cell extracts by fast protein liquid chromatography (Pharmacia, Uppsala, Sweden). β-Mannanase was purified from extracellular material. All procedures were performed at room temperature under aerobic conditions, and samples were performed at room temperature under aerobic conditions, and samples were stored at 4°C after processing. Prior to use, all buffers were filtered through a 0.22-μm-pore-size filter. All columns and column media used were obtained from Pharmacia unless otherwise stated.

(i) Purification of β-mannosidase. (a) Ion-exchange chromatography. Portions of the crude enzyme preparation (200 ml) were applied to a column (XK50/30; bed volume, 500 ml) packed with DEAE-Sepharose CL6B (Sigma) and preequilibrated with 0.1 M sodium phosphate (pH 7.4)–0.005% *N*-lauroylsarcosine (NLS) (buffer A). After application of the crude extract, the column was washed with the same buffer until protein could no longer be detected in the eluent. The column was then washed with a two-step linear NaCl gradient (0 to 0.2 M and 0.2 to 1.0 M) in the same buffer A. The fractions eluting at the 0.2 M NaCl plateau contained both β-mannosidase and α-galactosidase activities.

(b) Hydrophobic interaction chromatography (HIC). NaCl was added to the pooled fractions from the previous step to bring the sample to a final concentration of 4 M. This material was applied to a column (XK26/70; bed volume, 200 ml) packed with phenyl-650M HIC Sepharose (TosoHaas, Philadelphia, Pa.) and previously equilibrated with 4 M NaCl in buffer A in 100-ml aliquots. The column was washed with the same buffer until protein could no longer be detected in the eluent. Then, the column was eluted stepwise with a four-step decreasing NaCl gradient (4.0 to 3.1 M, 3.1 to 2.2 M, 2.2 to 1.6 M, and 1.6 to 1.0 M) to buffer A and separated by three plateaus at 3.1, 2.2, and 1.6 M NaCl, respectively, to optimize peak resolution.

(b) Sephacryl S-300 gel filtration chromatography. Pooled fractions from the previous step were concentrated approximately 10-fold with 10,000-molecular-weight-cutoff membranes on a 180-ml (Amicon, Beverly, Mass.) stirring cell. Fractions (3 ml) of the concentrated material were applied to a gel filtration column (XK16/100; bed volume, 180 ml; packed with Sephacryl S-300-HR) previously equilibrated with 0.2 M NaCl in buffer A. The column was washed with the same buffer until protein could no longer be detected in the eluent.

(c) Anion-exchange chromatography (Mono Q). Pooled fractions containing β-mannosidase activity were first dialyzed overnight against 0.1 M sodium phosphate buffer (pH 7.4) to eliminate NaCl and detergent. Fractions (3 ml) were applied to a 1-ml Mono Q column previously equilibrated with buffer A (without detergent). The column was washed with the same buffer until protein could no longer be detected in the eluent. The column was then eluted with a linear NaCl salt gradient (0 to 0.4 M) in the same buffer A. Fractions containing β-mannosidase activity were then separated and viewed by sodium dodecyl sulfate-poly-

acrylamide gel electrophoresis (SDS-PAGE). Fractions showing a single band on the gel were pooled and stored at 4°C.

(ii) Purification of α-galactosidase. (a) HIC. α-Galactosidase copurified with β-mannosidase in the first step of the β-mannosidase purification (ion-exchange chromatography). The two activities also copurified during HIC, whether phenyl-Sepharose or butyl-Sepharose was used. As such, DEAE and phenyl-Sepharose could be used to purify α-galactosidase as described for β-mannosidase. An alternative HIC step was used for purification of α-galactosidase. A column (XK26/70; bed volume, 200 ml) packed with butyl-Sepharose, previously equilibrated with 1 M $(NH_4)_2SO_4$ in sodium phosphate buffer 0.1 M (pH 7.4)–0.005% NLS, was used. Ammonium sulfate (1.0 M) was added to the pooled fractions containing α-galactosidase activity from the previous ion-exchange step; these were filtered through a 0.22-μm membrane to remove any precipitate. This material was applied to the preequilibrated column, which was washed with the same buffer until protein could no longer be detected in the eluent. The column was washed with a two-step gradient, decreasing the $(NH_4)_2SO_4$ concentration to 0.35 M first and holding at this point for 2 column volumes before resuming the gradient.

(b) Sephacryl S-300 gel filtration chromatography. The Sephacryl S-300 gel filtration chromatography step was run under the same conditions as for the equivalent step described above for β-mannosidase purification.

(c) Cation-exchange chromatography. A 1.0-ml ResourceS cation-exchange chromatographic bed was equilibrated with buffer containing 0.05 M potassium acetate and 0.005% NLS (pH 4.6). An aliquot of the concentrated sample was diluted threefold in the same buffer and applied to the column. The column was washed until no protein could be detected. α-Galactosidase was eluted in a two-step gradient with the same buffer containing 1.2 M potassium chloride (0 to 0.4 M and 0.4 to 0.6 M).

(d) Sephacryl S-200 gel filtration chromatography. The fractions containing α-galactosidase activity from the previous step were concentrated approximately fourfold with 10,000-molecular-weight-cutoff membranes on an Amicon 50-ml stirring cell. A Sephacryl S-200 column HiPrep 26/60, 320 ml, was equilibrated with buffer A containing 0.2 M NaCl; 6 ml of sample was applied. The column was washed until no protein was detected in the eluent.

(iii) Purification of β-mannanase. (a) HIC. Ammonium sulfate was added to the concentrated extracellular material of the *T. neapolitana* cell culture to a final concentration of 1.0 M. This material (150 ml) was applied to a column (XK26/70; bed volume, 200 ml) packed with butyl-Sepharose that was previously equilibrated with 1 M $(NH_4)_2SO_4$ in 0.1 M sodium phosphate buffer (pH 7.4)–0.005% NLS. The column was washed with the same buffer until protein could no longer be detected in the eluent. The column was washed with a two-step gradient, decreasing the $(NH_4)_2SO_4$ concentration to 0.35 M first and holding there for 2 column volumes before resuming the gradient.

(b) Anion-exchange chromatography (Mono Q). A sample (20 ml) of the pooled fractions containing β-mannanase activity from the previous step was loaded on a Mono Q column equilibrated in buffer A. The column was washed with the same buffer until protein could no longer be detected in the eluent. The column was then eluted with a three-step NaCl salt gradient (0 to 0.08 M, 0.08 to 0.20 M, and 0.20 to 1.0 M) in the same buffer A. Fractions containing β-mannanase activity were pooled and stored at 4°C.

Gel electrophoresis. (i) SDS-PAGE. Cell extracts and purified proteins were boiled for 10 min with sample buffer (0.1 M Tris [pH 8], 1% SDS, 10 mM β-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, before being separated by SDS-PAGE, as described by Laemmli (23). Proteins were visualized by staining with Coomassie brilliant blue. The molecular mass was estimated with prestained markers (Bethesda Research Laboratories, Bethesda, Md.; Sigma). SDS-PAGE was also done with a Pharmacia LKB PhastSystem, using 8 to 25% gradient Phastgels and SDS buffer strips as specified by the manufacturer. Purified proteins were precipitated by addition of trichloroacetic acid to a final concentration of 10% [wt/vol]) and 30 min of incubation on ice. The precipitated protein was centrifuged, and excess trichloroacetic acid was removed by two acetone washes (0.5 ml). The dried, precipitated protein was resuspended in sample buffer (without glycerol) and separated on SDS-PAGE.

(ii) Native PAGE. Electrophoresis of native proteins was performed in the same SDS-PAGE gel system but in the absence of SDS and without sample pretreatment. Similarly, native Phastgels (Pharmacia) with native buffer strips were used on the PhastSystem, as specified by the manufacturer.

(iii) Activity gels. Taking advantage of the thermostability of the β-mannosidase and α-galactosidase, native gels were incubated at 80°C for 5 min in the presence of *p*-nitrophenyl-β-D-mannopyranoside or *p*-nitrophenyl-α-D-galactopyranoside in 0.1 M sodium phosphate buffer (pH 7.4). Incubation was performed under vacuum on a slab gel dryer (model SE1160; Hoefer Scientific Instruments, San Francisco, Calif.) preheated at 80°C. The gel and the substrate in solution were wrapped in a plastic film to prevent drying. Both activities were visualized by a yellow coloration corresponding to a protein band later visualized by Coomassie brilliant blue staining.

Determination of optimal pH. The pH optimum was evaluated by using the standard assay protocol for the particular enzyme, with substitution of the appropriate buffer. The following buffers (50 mM) were used: 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.5 to 6.7); 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 6.5 to 7.9); *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid) (EPPS) (pH 7.3 to 8.7); 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) (pH 8.6 to 10), and 3-(cyclohexylamino)propanesulfonic acid (CAPS) (pH 9.7 to 11.1). NaCl (50 mM) was also added to the buffer. The D-pyranoside substrates were prepared in the respective buffers. The AZO-LBG substrate was prepared as a 2% stock in water and diluted to 1% with the appropriate buffers (100 mM).

Determination of the isoelectric point. The isoelectric point was evaluated by isoelectrofocusing on a Phastgel IEF 3-9 (Pharmacia) against IEF markers (Sigma).

Determination of optimal temperature. The temperature optimum for each enzyme was evaluated by using the standard activity assay at different temperatures. The assay was performed at the optimal pH, determined previously. NaCl (50 mM) was also added to the buffer used for the assay. Before starting the assay, the β-mannosidase and α-galactosidase were preincubated at 70°C for 5 min to minimize temperature changes in the prewarmed cuvette with the substrate.

Thermostability measurements. The thermostability of the enzymes was measured at different temperatures. The enzymes were diluted in 50 mM MOPS buffer–50 mM NaCl (pH 7.5) and incubated in an oil bath. A sample of the enzyme was taken periodically and cooled on ice. Residual activity was then measured by the standard assay.

Kinetic parameters. The Michaelis-Menten kinetic parameters, V_{max} and K_m, were calculated for β-mannosidase and α-galactosidase. Activity was determined by the standard assays for each respective enzyme at their previously determined optimal pH. Lineweaver-Burk and Eadie-Hofstee plots were used to determine parameters, assuming that simple Michaelis-Menten kinetics were followed. The K_m and V_{max} values for β-mannanase were estimated by using either LBG or AZO-LBG substrate.

TABLE 1. Localization of galactomannan-degrading hemicellulases from *T. neapolitana* 5068

Hemicellulase	Extracellular activity (U/mg)[a]	Intracellular activity (U/mg)	Ratio of activities[b]
α-1,6-Galactosidase	0.27	0.43	0.63
β-1,4-Mannosidase	0.10	0.33	0.31
β-1,4-Mannanase	0.83	0.12	6.80

[a] U, micromoles of PNP or reducing-sugar equivalent released per minute.
[b] Extracellular activity/intracellular activity.

RESULTS

Localization of activities. β-Mannanase activity was found primarily in the culture media; the extracellular specific activity was about sevenfold greater than that found in the cell extracts (Table 1). The other two enzymes, α-galactosidase and β-mannosidase, were found mostly in cell extracts. The specific activity of α-galactosidase was approximately twice as high in the cell extract as in the culture, while the level of β-mannosidase activity was approximately three times as high in the extract as in the spent culture medium. No attempt was made to determine if any of these activities were membrane or toga associated; thus, this possibility cannot be discounted.

Enzymes purification and characterization. α-Galactosidase and β-mannosidase were purified from *T. neapolitana* 5068 cell extract, and the results are shown in Tables 2 and 3, respectively. The first purification step was common for β-mannosidase and α-galactosidase. The two activities also copurified in the subsequent hydrophobic chromatography step, whether phenyl-Sepharose or butyl-Sepharose was used. In either case, these two activities could be separated by gel permeation chromatography with Sephacryl S-300 as shown in Fig. 2. After the gel permeation step, β-mannosidase could be purified by an additional anion-exchange step. A decrease in specific activity after the anion-exchange step was observed, probably due to the removal of detergent from the enzyme preparation. In experiments where detergent was added prior to the anion-exchange step (Mono Q-buffer A), up to a twofold increase in specific activity was observed. However, since it was difficult to resolve this sample into homogeneous protein (data not shown), this approach was abandoned. *T. neapolitana* β-man-

TABLE 2. Purification of β-1,4-mannosidase from *T. neapolitana* 5068

Purification step	Amt of β-1,4-mannosidase		Cumulative purification (fold)	Cumulative yield (%)
	Total units	Units/mg[a]		
Cell extract	633	0.2	1	100
DEAE	593	0.9	4.1	93.7
Phenyl-Sepharose	703	1.9	9.2	111.1
Sephacryl S-300	463	47.9	228	73.1
Mono Q	224	34.0	162	35.4

[a] Units/mg refers to the number of micromoles of PNP released per minute per milligram of protein.

nosidase was purified 162-fold over the cell extract with a final yield of 35.4% (Table 2).

Purification of α-galactosidase was ineffective when phenyl-Sepharose was used for the hydrophobic chromatography step. The enzyme copurified with another protein from which it could not be separated, even when preparative SDS-PAGE was tried (data not shown). However, butyl-Sepharose was effective for the HIC step in the α-galactosidase purification. After the gel permeation step, α-galactosidase required two additional steps for complete purification: cation exchange (ResourceS) and additional gel filtration (Sephacryl S-200). α-Galactosidase was purified 181-fold with a yield of 14% (Table 3).

β-Mannanase was purified directly from the cellular supernatant of *T. neapolitana*. An intracellular β-mannanase was detected but with significantly lower specific activity than the extracellular activity (data not shown). The low level of total protein in the extracellular material, compared with the cell extract, greatly simplified purification. Table 4 shows the results for the isolation of the extracellular β-mannanase, which was purified 4.8-fold in two steps with a 13.6% yield.

Molecular mass determination. The molecular mass of each protein was determined by SDS-PAGE and by gel filtration chromatography. β-Mannosidase separation on SDS-PAGE revealed it to consist of a single band at 200 to 220 kDa, when this enzyme was preincubated in the presence of 1% SDS or boiled for up to 1 min prior to electrophoresis. Boiling the enzyme sample for several minutes led to the breakdown of a single band into another single band, corresponding to a lower molecular mass, i.e., 95 to 100 kDa (Fig. 3). Activity gel staining showed that only the larger form was active, suggesting that the SDS treatment in the absence of heat was insufficient to dissociate the protein (data not shown). The molecular mass of β-mannosidase was also determined by Sephacryl S-300-HR gel filtration against molecular mass standards; here, the molecular mass was estimated to be approximately 200 kDa. No other fraction was found with β-mannosidase activity. These results indicate that β-mannosidase is a homodimer of approximately 200 kDa.

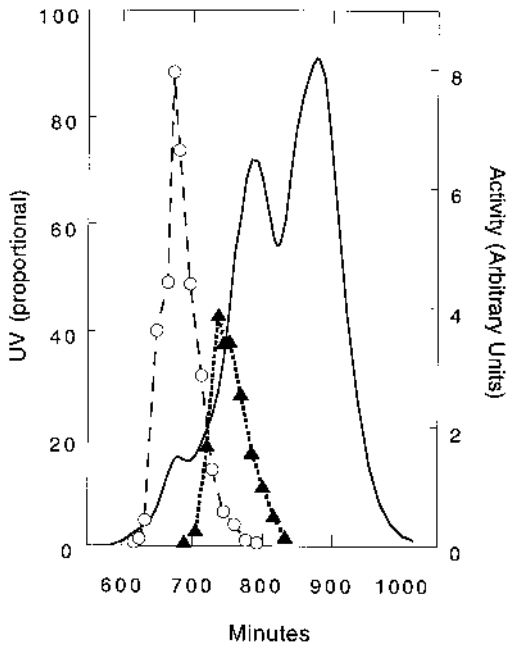

FIG. 2. Sephacryl S-300 elution profile. The solid line represents the protein profile detected at 280 nm. ▲ represents the elution profile of α-galactosidase activity, and ○ represents the elution profile of the β-mannosidase activity. All the numbers have been normalized to arbitrary units. The flow rate in this experiment was 0.2 ml/min.

The molecular mass of α-galactosidase was determined by both gel filtration chromatography and SDS-PAGE. Gel filtration on Sephacryl S-300 yielded a monomer of 66 kDa, which was in good agreement with the molecular mass determined by SDS-PAGE of the TCA-precipitated sample, which was found to be 61 kDa (Fig. 4).

SDS-PAGE of TCA-precipitated β-mannanase samples revealed a band corresponding to 65 kDa, as shown in Fig. 4. The result obtained from the Sephacryl S-200 analysis yielded a monomer of 55 kDa. The difference between these two estimates may be an artifact of the S-200 matrix, which has been reported by the manufacturer to retard the elution of very

TABLE 3. Purification of α-1,6-galactosidase from *T. neapolitana* 5068

Purification step	Amt of α-1,6-galactosidase		Cumulative purification (fold)	Cumulative yield (%)
	Total units	Units/mg[a]		
Cell extract	1,583	0.5	1	100
DEAE	1,635	2.0	4.2	103.3
Butyl-Sepharose	1,472	2.8	5.9	93.0
Concentration	1,277	2.8	5.9	80.7
Sephacryl S-300	1,114	3.5	7.4	70.4
ResourceS	381	28.3	60.3	24.1
Sephacryl S-200	228	85.0	180.8	14.4

[a] Units/mg refers to the number of micromoles of PNP released per minute per milligram of protein.

TABLE 4. Purification of β-1,4-mannanase from *T. neapolitana* 5068

Purification step	Amt of β-1,4-mannanase		Cumulative purification (fold)	Cumulative yield (%)
	Total units	Units/mg[a]		
Concentrated culture supernatant	38.5	0.8	1	100
Butyl-Sepharose	12.4	2.6	3.2	32.2
Mono Q	5.2	3.8	4.8	13.6

[a] Units/mg refers to the number of micromoles of PNP released per minute per milligram of protein.

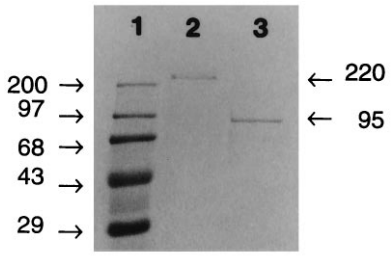

FIG. 3. SDS-PAGE of purified β-mannosidase from *T. neapolitana* 5068. Lanes: 1, molecular mass markers (from top to bottom in kilodaltons: 200, 97.4, 68, 43, 29, and 18.4); 2, purified β-mannosidase treated with SDS and β-mercaptoethanol; 3, purified β-mannosidase treated with SDS and β-mercaptoethanol and boiled for 5 min.

hydrophobic proteins, thus artificially lowering the estimated molecular mass. In support of the hydrophobicity of the β-mannanase, the enzyme was eluted from the butyl-Sepharose HIC matrix only after the $(NH_4)_2SO_4$ gradient was reduced to 0 M.

pH optima and isoelectric points. Figure 5 shows the pH optima for the three enzymes determined by activity assays at 85°C. All three enzymes show optimal activity near neutral pH (β-mannosidase at pH 7.7, α-galactosidase at pH 7.3, and β-mannanase at pH 6.9). Although some variation with temperature is expected for each of the buffers used, the pH range in which the optima were found corresponded to phosphate buffer, which showed little variation between room temperature and assay temperature (≤0.15 U). The pIs were 5.6 ± 0.1, 4.6 ± 0.1, and 5.1 ± 0.1 for β-mannosidase, α-galactosidase, and β-mannanase, respectively.

Temperature optima and thermostability. Figure 6 shows the temperature optima for the three enzymes. β-Mannosidase exhibited a temperature optimum at 87°C. Limitations in substrate stability precluded the measurement of activity at temperatures higher than 100°C. α-Galactosidase had a temperature optimum higher than that of β-mannosidase, at 100 to 105°C. β-Mannanase had a temperature optimum of 90 to 92°C as well as significant activity over a wide range of temperatures; e.g., it exhibited 30% of its maximum activity at 60°C. β-Mannosidase was extremely thermostable, with a half-life (based on residual activity) of approximately 18 h at 85°C, 42 min at 90°C, and 2 min at 98°C. α-Galactosidase was also extremely thermostable, with a half-life of 8.5 h at 85°C, 130 min at 90°C, and 3 min at 100°C. β-Mannanase was the most thermostable of the three, with a half-life of 34 h at 85°C, 13 h at 90°C, and 35 min at 100°C. For the calculated temperature at which each enzyme would have an activity half-life of 1 h and 24 h, see Table 6.

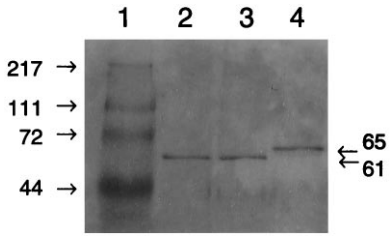

FIG. 4. SDS-PAGE of purified and TCA-precipitated α-galactosidase and β-mannanase from *T. neapolitana* 5068. Lanes: 1, molecular mass markers (from top to bottom in kilodaltons: 217, 111, and 71.5); 2 and 3, two different preparations of purified α-galactosidase; 4, purified β-mannanase.

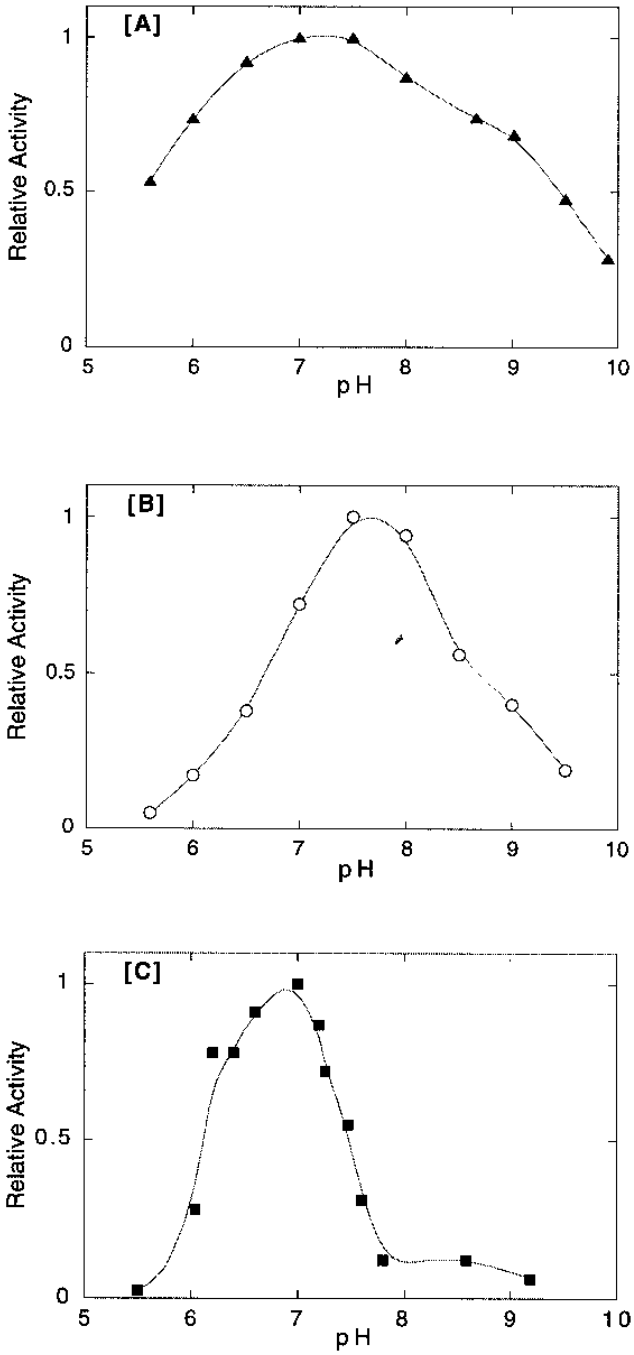

FIG. 5. Effect of pH on *T. neapolitana* 5068 β-mannosidase, α-galactosidase, and β-mannanase activity. The results are given as a normalized activity with respect to the pH optimum. [A] α-Galactosidase; [B] β-mannosidase; [C] β-mannanase.

Kinetic parameters. Assuming that Michaelis-Menten kinetics apply, parameters for *p*-nitrophenyl-β-D-mannopyranoside (β-mannosidase) and *p*-nitrophenyl-β-D-galactopyranoside (α-galactosidase) hydrolysis at 90°C are reported in Table 5. The values reported for k_{cat} was determined by assuming a molecular mass of 61 kDa for α-galactosidase with one active site and 200 kDa for β-mannosidase with one active site per monomer; these assumptions will need to be verified by more careful analysis. Kinetic parameters for the extracellular β-mannanase

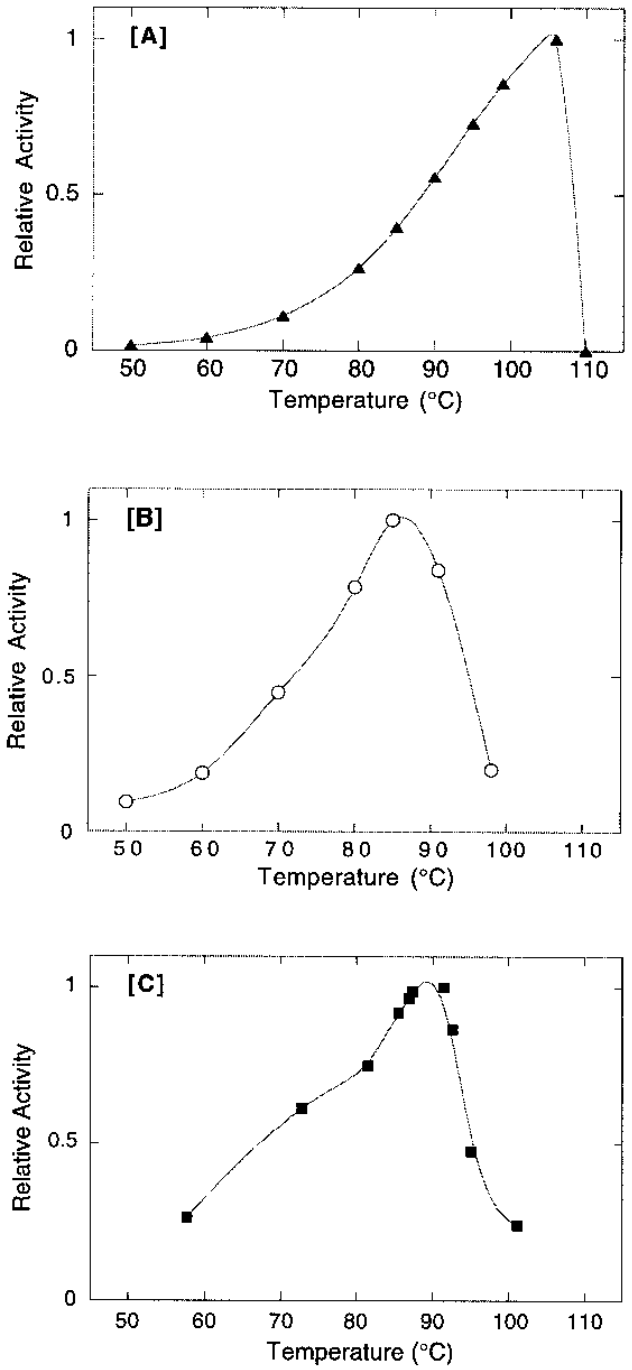

FIG. 6. Effect of temperature on *T. neapolitana* 5068 α-galactosidase, β-mannosidase, and β-mannanase. The results are given as a normalized activity relative to the temperature optimum of the particular enzyme. [A] α-Galactosidase; [B] β-mannosidase; [C] β-mannanase.

were also estimated, with LBG and AZO-LBG as substrates. A summary of the characteristics of the three enzymes is shown in Tables 5 and 6.

Substrate specificity of β-mannosidase. *T. neapolitana* 5068 β-mannosidase showed significant hydrolytic activity toward only one of the substrates examined: *p*-nitrophenyl-β-D-mannopyranoside. No activity was noted toward *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-maltoside, *p*-nitrophenyl-β-D-lactopyranoside, or *p*-nitrophenyl-β-D-mannobiose. Measurements were carried out by using the standard assay at 85°C and pH 7.5.

DISCUSSION

Described here is the purification and characterization of three extremely thermostable enzymes from *T. neapolitana* 5068: α-1,4-galactosidase (α-galactosidase), exo-1,4-β-D-mannanase (β-mannosidase), and endo-1,4-β-D-mannanase (β-mannanase). Presumably, these enzymes act in concert to provide simple saccharides to *T. neapolitana* growing on guar gum-based media. The cellular localization of these three activities suggests that the mannan backbone is cleaved by β-mannanase prior to the transport of smaller galactomannans to the site of the other two enzymes. It may be that one or more of these activities are associated with the cell membrane or with the characteristic sheath or "toga" common to members of the genus *Thermotoga* (21). Along these lines, Gherardini and Salyers (16) demonstrated that approximately 30% of the total cell-associated β-mannanase activity of *Bacteroides ovatus* partitioned with the cell membrane and that no extracellular activity was detectable for this microorganism.

The estimation of the molecular mass by SDS-PAGE and gel filtration chromatography yielded a value of approximately 200 kDa for the β-mannosidase. The molecular mass of the dimer is larger than those of other thermostable mannosidases previously reported. The β-mannosidases from an alkalophilic *Bacillus* sp. (94 kDa) (3), *Aspergillus awamori* (96 kDa) (35), and *Aspergillus niger* (130 kDa) (8) are all reported to be monomeric versions of the enzyme, although the *T. neapolitana* enzyme was found not to be active in a monomeric form.

The α-galactosidase from *T. neapolitana*, however, was active as a monomer of 61 kDa, a similar size and structure to those reported for several other thermostable, monomeric α-galactosidases: 45 kDa for *A. niger* (2, 5), 71 kDa for *Aspergillus ficuum* (49), 50 kDa for *Trichoderma reesei* (50), and 57 kDa for *Penicillium ochrochloron* (13). More complex structures for thermostable α-galactosidases have also been reported. *Bacillus stearothermophilus* has been found to produce an extracellular homotrimeric version of this enzyme with a monomeric subunit of 82 kDa (45). The α-galactosidase of *B. stearothermophilus* AT-7 is an intracellular, tetrameric isozyme of approximately 300 kDa (38). There was no evidence in this study, however, that *T. neapolitana* 5068 produces any additional intracellular or extracellular α-galactosidases or that the purified enzyme is active in any other form.

The monomeric, extracellular β-mannanase of *T. neapolitana* had a molecular mass of 65 kDa, as judged from SDS-PAGE. Comparison with other thermostable enzymes of this type shows that the *T. neapolitana* enzyme is slightly larger than the β-mannanase reported for *A. niger* (42 kDa) (14) but smaller than bacterial enzymes, such as those from *B. stearothermophilus* (a 162-kDa homodimer) (45) or the two β-mannanase forms from *Enterococcus casseliflavis* (monomers of 142 and 137 kDa) (36). The β-mannanase from *C. saccharolyticum* was found to be extracellular and to have a putative signal peptide involved in its secretion (7, 25). When this enzyme was cloned and expressed in *E. coli*, the recombinant protein was determined to be 34 kDa on SDS-PAGE, compared to 39 kDa deduced from the translated nucleotide sequence (25). The *T. neapolitana* enzyme appears to be extracellular, and presumably the secretion process also involves a signal peptide. Furthermore, the localization of secreted enzymes in *Thermotoga* species in relation to the toga has yet to be explored, and

TABLE 5. Kinetic parameters of the purified β-mannosidase, β-mannanase, and α-galactosidase from *T. neapolitana* 5068 at 90°C

Hemicellulase	V_{max} (U/mg)	K_m	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1} \cdot min^{-1}$)
β-Mannosidase[c]	36.9 ± 2.5[a]	3.1 ± 0.6 mM	4,070	1,454
α-Galactosidase[c]	150.0 ± 6[a]	0.50 ± 0.08 mM	9,150	18,300
β-Mannanase	3.8[b]	0.55 (LBG), 0.23 (AZO-LBG) mg/ml[d]	ND[e]	ND

[a] Micromoles of PNP per minute per milligram.
[b] Micromoles of reducing-sugar equivalent per minute per milligram.
[c] Kinetic parameters determined from Lineweaver-Burk plots.
[d] Substrate concentrations at which the rate was half maximum ($K_{0.5}$).
[e] ND, not done.

efforts that are underway to clone, sequence, and express the β-mannanase gene corresponding to this enzyme will address this issue. An intracellular β-mannanase activity identified in *T. neapolitana* cell extracts (data not shown) appears to have a different molecular mass than the extracellular enzyme, and a possible relationship between the two has not yet been established. Hemicellulases with multiple forms have been observed (4, 36). Winterhalter and Liebl (47) showed that *Thermotoga maritima* MSB8 produced two versions of a thermostable xylanase which were found to be independent moieties in that the smaller one (41 kDa) was not a proteolytic product of the larger enzyme (120 kDa). It remains to be seen how the two β-mannanases are related to each other.

The results in Table 5 give the kinetic parameters of the three enzymes at 90°C. The k_{cat}/K_m for the *T. neapolitana* α-galactosidase (18,300 $mM^{-1} \cdot min^{-1}$) is within the range estimated from kinetic data reported for other α-galactosidases (39,500 $mM^{-1} \cdot min^{-1}$ for the *A. nidulans* α-galactosidase [40] and 7,560 $mM^{-1} \cdot min^{-1}$ for the enzyme from *A. ficuum* [49]). Similarly, the k_{cat}/K_m for the *T. neapolitana* β-mannosidase (1,454 $mM^{-1} \cdot min^{-1}$) is comparable to those of versions of this enzyme isolated from *Aspergillus* species: the β-mannosidases from *Aspergillus niger* at 870 $mM^{-1} \cdot min^{-1}$ (8) and from *Aspergillus awamori* at 360 $mM^{-1} \cdot min^{-1}$ (35). In the case of the β-mannanase, kinetic comparisons on galactomannans are difficult since polymeric chains of mannose substituted with galactose, which are used as substrates, vary with the length and degree of substitution and are a function of the preparation method and the source of galactomannan. K_m values for different galactomannan substrates have been determined for the *C. saccharolyticum* β-mannanase and reported to be 0.127 mg/ml for LBG (7), which is close to the values (0.23 to 0.55 mg/ml) determined for the *T. neapolitana* enzyme at 90°C on two different LBG substrates (Table 5). The *C. saccharolyticum* β-mannanase, at 70°C, had a V_{max} of 63.2 U/mg on LBG but only 2.5 U/mg on guar gum, a result presumably related to the steric hindrance present in the more highly substituted mannan backbone associated with guar. The *T. neapolitana* enzyme had an approximate V_{max} of 3.8 U/mg on LBG at 90°C, which is similar to the value for the *C. saccharolyticum* β-mannanase on guar gum (7). Although this suggests a significantly lower rate of catalysis for the *T. neapolitana* β-mannanase, side-by-side comparisons of the two enzymes on substrates prepared under identical conditions need to be done to verify this difference.

The enzymes described here represent the most thermostable versions yet reported. The most thermostable β-mannosidases reported prior to this study were those from *A. awamori*, which had a maximum activity at 66°C with a half-life of less than 30 min at that temperature (35), and the version from *A. niger*, which had a half-life of 2 hours at 55°C (8). The *T. neapolitana* β-mannosidase described in this study, with a T_{opt} of 87°C and a half-life of 18 h at 85°C, is by far the most thermostable and thermoactive β-mannosidase to date. Two α-galactosidases with activity at high temperature have been reported. The enzyme from *Candida guilliermondii* is active at 75°C but has a half-life of only 20 min at that temperature (17). The most thermostable α-galactosidase previously reported is from *Bacillus stearothermophilus*, with a half-life of over 1 h at 68°C (45). Although the α-galactosidase from *T. neapolitana* is the least thermostable enzyme of the three enzymes described in this study ($t_{1/2}$, 8 h at 85°C), it is the most thermoactive of the three (T_{opt}, 100°C). While moderately thermostable versions of β-mannanase have been identified (4, 45), only the β-mannanase from *Caldocellum saccharolyticum*, with a T_{opt} of 80°C and a half-life of 48 min at 85°C, is comparable to the *T. neapolitana* enzyme (7). However, it is still far less stable than the *T. neapolitana* enzyme (T_{opt}, 92°C and a half-life of 34 h at 85°C).

The pH optima for the *T. neapolitana* enzymes described in this study are also noteworthy. The three enzymes have an optimum pH in the range 7.1 to 7.7, whereas the similar enzymatic activities reported for other organisms, except for those from *Bacillus* species (38, 45), are in the pH 3 to 6.5 range (4, 45). Moreover, the three *T. neapolitana* enzymes retain significant activity at pHs greater than 9 (Fig. 4).

In addition to the potential technological benefits derived from the use of these hyperthermophilic galactomannan-degrading enzymes for applications ranging from oil and gas recovery (32) to food processing and to pulp and paper processing (48), their production by *T. neapolitana* raises some

TABLE 6. Galactomannan-degrading hemicellulases from *T. neapolitana* 5068

Hemicellulase	Molecular mass (kDa) by:		pH optimum (50% activity range)	Isoelectric point	Temp optimum (50% activity range) (°C)	Temp (°C) where		Enzyme activity ($t_{1/2}$ at 85°C) (min)
	SDS-PAGE	Native mobility				$t_{1/2}$ = 1 h	$t_{1/2}$ = 24 h	
α-1,6-Galactosidase	61	66 (α)	7.3 (5.5–9.3)	4.6	100–105 (87–105)	91.5	82.5	530
β-1,4-Mannosidase	95	200 (α_2)	7.7 (6.8–8.8)	5.6	87 (72–96)	89.5	84	1,080
β-1,4-Mannanase	65	55 (α)	7.1 (6.1–7.5)	5.1	92 (67–95)	97.5	86	2,040

interesting questions concerning carbon and energy substrates in geothermal environments. Galactomannans are typically produced as storage polymers in plants (19, 33), although the existence of organisms such as *T. neapolitana* presumably predated the emergence of higher eukaryotes. These enzymes might reflect changes in the metabolism of this hyperthermophile as geothermal environments have changed and become more biodiverse. Rinker and Kelly (39) have observed that the hyperthermophilic archaeon *Thermococcus litoralis* (growth T_{opt}, 88°C) produces an exopolysaccharide consisting mainly of mannan when this organism is grown under certain conditions. The ability to use this hemicellulose, or others that might be produced by microorganisms in hyperthermophilic consortia, as a carbon and energy source would thus be advantageous. In any case, it remains to be seen how the genes encoding the *T. neapolitana* enzymes relate to other galactomannan-hydrolyzing enzymes and what physiological role this enzyme system plays in this hyperthermophile.

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