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Comparison of a β-Glucosidase and a β-Mannosidase from the Hyperthermophilic Archaeon *Pyrococcus furiosus*

PURIFICATION, CHARACTERIZATION, GENE CLONING, AND SEQUENCE ANALYSIS*

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Two distinct exo-acting, β-specific glycosyl hydrolases were purified to homogeneity from crude cell extracts of the hyperthermophilic archaeon *Pyrococcus furiosus*: a β-glucosidase, corresponding to the one previously purified by Kengen *et al.* (Kengen, S. W. M., Luesink, E. J., Stams, A. J. M., and Zehnder, A. J. B. (1993) *Eur. J. Biochem.* 213, 305–312), and a β-mannosidase. The β-mannosidase and β-glucosidase genes were isolated from a genomic library by expression screening. The nucleotide sequences predicted polypeptides with 510 and 472 amino acids corresponding to calculated molecular masses of 59.0 and 54.6 kDa for the β-mannosidase and the β-glucosidase, respectively. The β-glucosidase gene was identical to that reported by Voorhorst *et al.* (Voorhorst, W. G. B., Eggen, R. I. L., Luesink, E. J., and deVos, W. M. (1995) *J. Bacteriol.* 177, 7105–7111; GenBank accession no. U37557). The deduced amino acid sequences showed homology both with each other (46.5% identical) and with several other glycosyl hydrolases, including the β-glycosidases from *Sulfolobus solfataricus*, *Thermotoga maritima*, and *Caldocellum saccharolyticum*. Based on these sequence similarities, the β-mannosidase and the β-glucosidase can both be classified as family 1 glycosyl hydrolases. In addition, the β-mannosidase and β-glucosidase from *P. furiosus* both contained the conserved active site residues found in all family 1 enzymes. The β-mannosidase showed optimal activity at pH 7.4 and 105 °C. Although the enzyme had a half-life of greater than 60 h at 90 °C, it is much less thermostable than the β-glucosidase, which had a reported half-life of 85 h at 100 °C. K_m and V_{max} values for the β-mannosidase were determined to be 0.79 mM and 31.1 μmol *para*-nitrophenol released/min/mg with *p*-nitrophenyl-β-D-mannopyranoside as substrate. The catalytic efficiency of the β-mannosidase was significantly lower than that reported for the *P. furiosus* β-glucosidase (5.3 *versus* 4, 500 s^{-1} mM^{-1} with *p*-nitrophenyl-β-D-glucopyranoside as substrate). The kinetic differences between the two enzymes suggest that, unlike the β-glucosidase, the primary role of the β-mannosidase may not be disaccharide hydrolysis. Other possible roles for this enzyme are discussed.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U60214.

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The hyperthermophilic archaeon *Pyrococcus furiosus* is an obligately anaerobic heterotroph, which grows optimally at 98–100 °C (1). It employs a fermentative type of metabolism (2), using polysaccharides, such as starch, glycogen, and pullulan (3), or disaccharides, such as maltose (3) and cellobiose (4), as carbon and energy sources. In order to utilize the different carbohydrates, *P. furiosus* synthesizes several intracellular and extracellular glycosyl hydrolases. Specifically, α-amylase (5), amylopullulanase (6), α-glucosidase (7), and β-glucosidase (4) activities have been purified and characterized. The α-amylase, amylopullulanase, and α-glucosidase are believed to work cooperatively to degrade α-linked polysaccharides, such as starch, glycogen, or pullulan (8). The endo-acting, α-specific amylase and amylopullulanase degrade α-linked polysaccharides to di- and trisaccharides (5, 6). α-Glucosidase presumably hydrolyzes these shorter oligosaccharides to glucose for use in a novel Embden-Meyerhof pathway (8, 9). Although *P. furiosus* cannot grow on cellulose or carboxymethylcellulose (4), it is not clear whether *P. furiosus* can utilize other β-linked complex carbohydrates as growth substrates. To date, no endo-acting, β-specific glycosyl hydrolases, such as cellulases, xylanases, or mannanases, have been identified in *P. furiosus*. However, when *P. furiosus* is grown on 5 mM cellobiose, a cell density of 7×10^8 cells/ml has been reported (4). Apparently, cellobiose is transported across the cell membrane and hydrolyzed to glucose by the intracellular β-glucosidase (9). Thus, the α- and β-glucosidases may play similar roles in the degradation of polysaccharides for the nutritional requirements of *P. furiosus*.

In addition to the physiological role of these glycosyl hydrolases within *P. furiosus*, it is also interesting to examine their relationship to similar enzymes from the other domains of life. This can be done on the basis of substrate specificity. However, many glycosyl hydrolases have a broad range of specificities (10). Henrissat (10) proposed an alternate and complementary classification scheme for glycosyl hydrolases based on amino acid sequence similarities. For example, glycosyl hydrolase family 1 is composed of exo-acting, β-specific enzymes with similar amino acid sequences. Based on substrate specificity, enzymes in this family have been characterized as β-glucosidases (EC 3.2.1.21), β-galactosidases (EC 3.2.1.23), phospho-β-gluco/galactosidases (EC 3.2.1.86/85), lactase-phlorizin hydrolases (EC 3.2.1.108/62), and thioglucosidases (EC 3.2.3.1). Family 1 glycosyl hydrolases provide a favorable framework for comparative studies of mesophilic and thermophilic enzymes for a number of reasons. First, the enzymes in this family function over a wide range of temperatures from mesophilic (11–22, 24–26) to moderately thermophilic (27–31) to hyperthermophilic (4, 32, 33). Second, enzymes in this family have been isolated from all three domains (bacteria, eucarya, and archaea), allowing the analysis of possible evolutionary relationships. Finally, crystal structures have been determined for

some family 1 enzymes (34, 35), facilitating structural comparisons among these enzymes.

For this report, crude cell extracts of *P. furiosus* were examined for the presence of exo-acting glycosyl hydrolases. In addition to the α-glucosidase (7) and β-glucosidase (4) reported previously, a β-mannosidase activity was isolated and characterized in relation to the other glycosyl hydrolases of *P. furiosus*. In order to investigate the molecular basis for substrate specificity differences between the β-mannosidase and the β-glucosidase, the genes for both of these enzymes were isolated from a genomic library by expression screening. A search for homology between the deduced amino acid sequences of the β-mannosidase and the β-glucosidase and other glycosyl hydrolases was completed. Based on the relative catalytic efficiencies of the two enzymes, it is likely that they play different physiological roles in *P. furiosus*. Several possible functions for the β-mannosidase are discussed.

MATERIALS AND METHODS

Purification of β-Mannosidase from P. furiosus

P. furiosus (DSM 3638) was grown on maltose-based medium in a 600-liter batch fermentor, and cell-free extract was prepared as described previously (36, 37). All purification steps were carried out at room temperature using an FPLC system (Pharmacia Biotech Inc.). The purification protocol for β-mannosidase was as follows.

DEAE-Sepharose Fast Flow Chromatography—The cell-free extract was applied directly to a column (10 × 20 cm) of DEAE-Sepharose (Pharmacia). After washing the column with 7 liters of buffer (50 mM Tris/HCl, pH 8, containing 2 mM sodium dithionite, 10% glycerol (v/v)), the adsorbed proteins were eluted with a linear gradient from 0 mM to 115 mM NaCl (90 ml) and 115 mM to 376 mM NaCl (5000 ml) at 12 ml/min. β-Mannosidase activity eluted between 264 mM and 288 mM NaCl. These fractions were pooled and concentrated using an Amicon Ultrafiltration Cell 202 with a YM10 membrane (Amicon, Beverly, MA) and a pressure of 55 p.s.i.g.

Phenyl-Sepharose 650M Chromatography—The concentrated fractions from the previous column were equilibrated to 50 mM sodium phosphate buffer, pH 7.0, containing 243 g/liter ammonium sulfate (buffer A). About 10% of the equilibrate pool (268 ml) was applied to a column (5 cm × 50 cm) of phenyl-Sepharose 650 M (Toso Haas, Montgomeryville, PA) previously equilibrated with buffer A. The column was washed with 600 ml 100% buffer A followed by 1000 ml of 25% buffer A. The remaining adsorbed proteins were eluted with a 2000-ml linear gradient from 25% to 0% buffer A. β-Mannosidase activity eluted at 0% buffer A. Fractions containing β-mannosidase activity were combined, concentrated as described above, and equilibrated to 25 mM potassium phosphate buffer, pH 7.0.

Hydroxyapatite Chromatography—The concentrated pool from the previous column was applied at 10 ml/min to a column (5 cm × 30 cm) of hydroxyapatite (Calbiochem, Sunnyvale, CA) previously equilibrated with 25 mM potassium phosphate buffer, pH 7.0. After the column was washed with 900 ml of 25 mM potassium phosphate, adsorbed proteins were eluted with a 600-ml linear gradient from 25 mM to 100 mM, a 400-ml linear gradient from 100 mM to 250 mM, and a 300-ml linear gradient from 250 mM to 500 mM potassium phosphate buffer, pH 7.0. β-Mannosidase activity eluted between 110 and 135 mM potassium phosphate buffer. Active fractions were pooled, concentrated, and equilibrated to 100 mM sodium phosphate buffer, pH 7.0.

Gel Filtration Chromatography—The concentrated pool from the previous column was applied to a Pharmacia HiLoad 16/60 Superdex 200 gel filtration column (V_o = 39.3 ml; V_t = 120.6 ml) pre-equilibrated to 100 mM sodium phosphate buffer, pH 7.0. The column was developed at 0.5 ml/min. β-Mannosidase activity eluted as a symmetrical peak at 75.0 ml.

Enzyme Assays

β-Mannosidase activity was assayed routinely using 1.0 mM *para*-nitrophenol (*p*Np)[1] substrate equilibrated to the desired temperature and pH. After equilibrating the sample to the desired temperature and pH in a heat block containing silicone oil (Dow Corning Corp., Midland, MI), the reaction was initiated by adding equilibrated substrate to the sample in an Eppendorf tube (U.S.A. Scientific Products, Milton Keynes, United Kingdom). The reaction was stopped at 5 min by chilling on ice. After cooling, the liquid in the Eppendorf tube was transferred to a microtiter plate (Corning, Corning, NY). The increase in absorbance at 405 nm as a result of *p*Np liberation was measured using an EL 340 Microplate Bio-Kinetics Reader (Bio-Tek™ Instruments, Winooski, VT). All activities were corrected for thermal degradation of the *p*Np substrate, which was below 0.5% of the enzyme hydrolysis rate. Absorbances were converted to concentrations of *p*Np using standards of known concentration. All assays were performed in triplicate. One unit of glycosidase activity was defined as that amount of enzyme required to catalyze the release of 1.0 μmol of *p*Np/min.

The purified enzyme was also tested for amylase activity. A standard reaction mixture contained 17 g/liter soluble starch equilibrated to the desired temperature and pH. The procedure was the same as above. The reaction was followed using the method of Laderman *et al.* (5). One unit of amylase activity was defined as that amount of enzyme hydrolyzing 1 mg of starch/min. All assays were performed in triplicate.

Total Protein Assays

Total protein concentration was determined using a BCA protein assay reagent kit (Pierce). Samples were diluted to the linear range (where A_{595} = 0.1–1.0) and incubated with reagent at 50 °C for 30 min in a sealed microtiter plate. The absorbance at 595 nm was determined using an EL 340 Microplate Bio-Kinetics Reader with albumin as the standard (Sigma).

Electrophoresis and Activity Staining

Isoelectric focusing was carried out on a Phast System (Pharmacia), according to manufacturer's protocols. Native- and SDS-PAGE were performed using standard procedures (38). High molecular mass (Boehringer Mannheim) and broad pI standards (Pharmacia) were used for PAGE and isoelectric focusing, respectively. For β-mannosidase activity staining, non-fixed gels were incubated at 95 °C for several minutes in 100 mM sodium phosphate buffer, pH 7.0, containing 1.0 mM Man*p*βNp. Upon the appearance of a yellow band, the gel was marked at that location.

Estimation of Molecular Mass

The *P. furiosus* β-mannosidase was treated with the bifunctional reagent dimethyl suberimidate (Sigma) according to Davies and Stark (39). The homogeneous enzyme (3.0 mg/ml) in 200 mM triethanolamine/HCl, pH 8.5, was mixed (in various proportions) with dimethyl suberimidate (1 mg/ml) in the same buffer and incubated for 3 h at 25 °C. The reaction was stopped, and the proteins were denatured by incubation at 90 °C for 60 min in the presence of 1% SDS and 1% β-mercaptoethanol as described by Pisani *et al.* (33) and subjected to 10% SDS-PAGE. Aldolase (Boehringer Mannheim) was used as a cross-linking control as described by Pisani *et al.* (33).

Kinetic Constants and Substrate Specificity

Kinetics parameters of *P. furiosus* β-mannosidase were determined using standard reaction mixtures, containing either Man*p*βNp or Glc*p*βNp. The reactions were performed at 95 °C. The release of *p*Np was measured as described above using different initial concentrations of substrate (0.05–10 mM). All assays were performed in triplicate. Values for the maximal reaction velocity (V_{max}) and the Michaelis-Menten constant (K_m) were determined from Lineweaver-Burk plots. Substrate specificity was determined using the standard reaction mixture, except that alternate substrates to Man*p*βNp were used. Depending on the substrate, either the amount of *p*Np released or the amount of starch degraded was measured after a 5-min incubation at 95 °C.

Thermostability

For thermostability determination, the homogeneous enzyme was incubated in Eppendorf tubes submersed in oil baths, at 90, 100, and 110 °C. The samples were covered with Ampliwax (Perkin-Elmer) to prevent evaporation. At appropriate time intervals, aliquots were withdrawn and tested for β-mannosidase activity at 95 °C in a standard reaction mixture.

[1] The abbreviations used are: *p*Np, *para*-nitrophenol; Glc*p*βNp, *p*-nitrophenyl-β-D-glucopyranoside; Man*p*βNp, *p*-nitrophenyl-β-D-mannopyranoside; Gal*p*βNp, *p*-nitrophenyl-β-D-galactopyranoside; Gal*p*αNp, *p*-nitrophenyl-α-D-galactopyranoside; Glc*p*αNp, *p*-nitrophenyl-α-glucopyranoside; Xyl*p*βNp, *p*-nitrophenyl-β-D-xylopyranoside; PAGE, polyacrylamide gel electrophoresis; X-glu, 5-bromo-4-chloro-3-indolyl-β-D-glucoside; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; GlcNAc, *N*-acetylglucosamine.

Sequencing

All DNA sequencing reactions were performed using either the Perkin-Elmer Applied Biosystems dye primer or dye terminator cycle sequencing kits and a model 377 automated DNA sequencer (Perkin-Elmer). Sequences were aligned and edited using the program Sequencher (Genecodes, Ann Arbor, MI).

Purified β-mannosidase and β-glucosidase were denatured and run on 12.5% polyacrylamide using standard procedures (38). Protein was electroblotted to a polyvinylidene difluoride membrane and Ponceau S-stained (38). N-terminal Edman degradation was performed on single bands with approximate molecular masses of 60 and 58 kDa for the β-mannosidase and β-glucosidase, respectively, using a liquid phase sequencer (Applied Biosystems model 477).

Expression Screening

The F factor F′kan from *E. coli* strain CSH118 (40) was introduced into the pho$^-$phn$^-$lac$^-$ *E. coli* strain BW14893 (41). A library prepared from randomly sheared genomic *P. furiosus* DNA was obtained from M. Snead (Recombinant BioCatalysis, Inc., La Jolla, CA) and was introduced into BW14893 F′kan. Cells were plated on 100-mm LB plates containing 100 μg/ml ampicillin, 80 μg/ml methicillin, and 1 mM isopropyl β-D-thiogalactopyranoside at a density of greater than 1000 colonies/plate. Colony lifts were performed using Millipore HATF membrane filters. Transformation plates were returned to the 37 °C incubator after the filter-lift to regenerate colonies. The transferred colonies were lysed with chloroform vapor in 150-mm glass Petri dishes. The filters containing lysed colonies were transferred to 100-mm glass Petri dishes containing Whatman 3MM filter paper saturated with Z buffer (40) and either 1 mg/ml X-glu (Diagnostic Chemicals Ltd, Oxford, CT) or 1 mg/ml X-gal (ChemBridge Corp., Northbrook, IL). The dishes were incubated at 80–85 °C. "Positives" were observed as blue spots on the filter membranes. Approximately 20–30 positives/plate were observed. One positive clone from the X-gal assay was purified by restreaking cells from the original regenerated plate. Several other positives from both assays were recovered by transforming DNA isolated from the blue spots on the filter lifts into electrocompetent *E. coli* DH10B cells. The filter-lift assay was repeated on transformation plates to identify "positives." LB medium containing 100 μg/ml ampicillin was inoculated with repurified positives and incubated at 37 °C overnight. Plasmid DNA was isolated from these cultures,and the plasmid insert was sequenced. The partial sequences of three clones (two from X-glu, one from X-gal) that contained inserts revealed that two of the clones overlapped (one from X-gal, one from X-glu) and one was unique (X-glu).

RESULTS

Purification of β-Mannosidase—Fractions from DEAE-Sepharose Fast Flow anion-exchange chromatography were assayed for α-amylase, α-glucosidase, β-glucosidase, and β-mannosidase activities (Fig. 1). Three peaks of β-glycosidase activity eluted at 0, 0.26–0.28, and 0.33–0.36 M NaCl, respectively. The peaks at 0 and 0.33–0.36 M NaCl had an identical substrate specificity to that reported for the β-glucosidase (4). The peak of β-glycosidase activity that eluted between 0.26 and 0.28 M NaCl showed different relative specific activities on several aryl glycosides than the previously reported glycosyl hydrolases from *P. furiosus* (4–7). The purification procedure for this β-glycosidase activity is shown in Table I.

Substrate Specificity—The purified enzyme was tested for its substrate specificity. Table II shows the activity of the enzyme toward several aryl-glycosides. The substrate specificities of the *P. furiosus* β-glucosidase and α-glucosidase are reported for comparison. The new enzyme exhibited the highest specific activity with Man*p*βNp as substrate and, therefore, was characterized as a β-mannosidase. The β-mannosidase did not hydrolyze the α-glycosidic linkages of Glc*p*αNp or Gal*p*αNp, nor did it degrade starch.

Physical Properties—The purified enzyme displayed optimal activity at 105 °C (Fig. 2) and pH 7.4 (Fig. 3). An isoelectric point of 6.9 was determined from an activity-stained isoelectric focusing gel (data not shown). The molecular mass of denatured β-mannosidase was approximately 60 kDa as determined from SDS-PAGE. When the β-mannosidase was treated with dimethyl suberimidate (at the enzyme/bifunctional reagent molar ratio of 1:300), four protein bands were noted after SDS-PAGE, corresponding to molecular masses of 60, 140, 180, and 220 kDa (Fig. 4). These results indicate that the *P. furiosus* β-mannosidase in its native conformation is a tetramer consisting of four identical subunits similar to the *S. solfataricus* glycosidases (32, 33) and the *P. furiosus* β-glucosidase (4), which were also determined to be homotetramers.

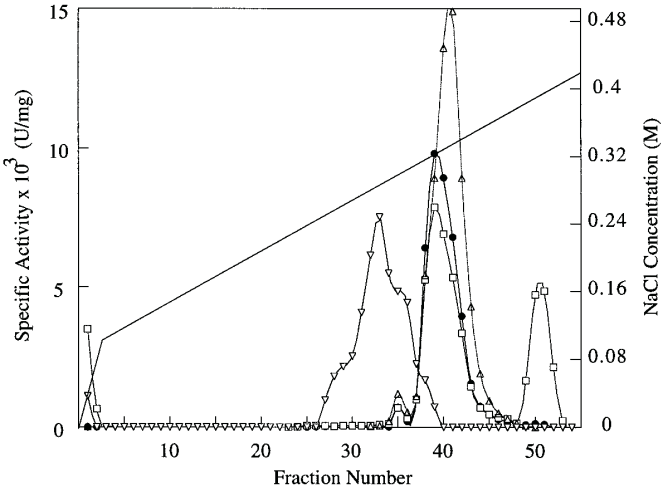

FIG. 1. **Chromatography of cell-free extract of *P. furiosus* on DEAE-Sepharose Fast Flow.** Two successive linear NaCl gradients were applied as indicated. First, one 4-liter and one 3-liter fraction were collected. Thereafter, 90-ml fractions were collected. Samples were analyzed for β-mannosidase activity (●), β-glucosidase activity (■), and α-glucosidase activity (△), using 1.0 mM *p*Np-sugars as substrate. One unit of β-mannosidase, β-glucosidase, and α-glucosidase activity was defined as that amount of enzyme catalyzing the release of 1 μmol of *p*Np/min. α-Amylase activity (▽) was measured using 1% soluble starch. One unit of α-amylase activity was defined as that amount of enzyme catalyzing the degradation of 1 mg of starch/min. The β-glucosidase activities of the first two fractions are shown as 1% of the actual values.

Kinetic Properties—The rate dependence on substrate concentration followed Michaelis-Menten kinetics. From Lineweaver-Burk plots, K_m and V_{max} values of 0.79 mM and 31.1 units/mg were determined with Man*p*βNp as substrate (Table III). In addition, the same analysis with Glc*p*βNp as substrate was used to determine K_m and V_{max} values of 2.9 mM and 14.8 units/mg. Assuming that the smallest catalytic unit of the β-mannosidase was 1 monomer unit with a molecular mass of 60 kDa, turnover numbers (k_{cat} values) of 40 and 5.3 s^{-1} were calculated for Man*p*βNp and Glc*p*βNp, respectively. Table III provides a comparison between the kinetic rate constants of the β-mannosidase and β-glucosidase from *P. furiosus*. The β-mannosidase had a significantly lower catalytic efficiency for the hydrolysis of β-glycosidic bonds than the β-glucosidase. The β-mannosidase had a higher K_m and lower V_{max} with Glc*p*βNp as substrate than with Man*p*βNp, indicating both a more specific binding and a more efficient cleavage of the glycosidic linkage when mannose is the terminal, non-reducing moiety.

Thermostability—The thermostability of purified β-mannosidase was measured at 90 °C and 100 °C (Fig. 5). At 90 °C, the β-mannosidase showed appreciable thermostability, with almost no loss of activity after 24 h and a half-life of greater than 60 h. At 100 °C, the half-life diminished to 77 min. At 110 °C, however, the enzyme had a half-life of less than 15 min (data not shown).

Sequences—The nucleotide and deduced amino acid sequences for the *P. furiosus* β-mannosidase and β-glucosidase were determined. The sequence for the β-glucosidase was identical to that reported previously (43). The NH_2 termini of the

TABLE I
Purification of the β-mannosidase from P. furiosus

β-Mannosidase activity was determined at 95 °C, using 1.0 mM Man*p*βNp as substrate.

Purification step	Volume	Protein	Specific activity	Total activity	-Fold	Recovery
	ml	*mg/ml*	*units/mg*	*units*		%
Cell extract	134	15.1	0.044	89.0	1	100
DEAE	268	1.75	0.058	27.2	1.3	31
HIC	27	1.25	0.62	20.9	14	24
HAP	1.3	0.68	5.3	4.69	120	5.3
Superdex 200	1.22	0.07	31.1	2.66	700	3.0

TABLE II
Substrate specificities of purified β-mannosidase, β-glucosidase, and α-glucosidase from P. furiosus

Activity was determined by measuring the release of *p*Np by absorbance at 405 nm. *p*Np-sugars were used at 1 mM concentrations. Assays of β-mannosidase-specific activity were performed at 95 °C in 100 mM sodium phosphate buffer, pH 7.0. Assays of β-glucosidase-specific activity were performed at 90 °C in 100 mM sodium citrate buffer, pH 5.5 (4). Assays of α-glucosidase-specific activity with Glc*p*αNp were performed at 108 °C in 100 mM sodium phosphate buffer, pH 5.5 (7), and with Man*p*βNp, Glc*p*βNp, Gal*p*βNp, and Xyl*p*βNp at 95 °C in 100 mM sodium phosphate buffer, pH 7.0.

Substrate	β-Mannosidase		β-Glucosidase		α-Glucosidase	
	Specific activity	Relative activity	Specific activity	Relative activity	Specific activity	Relative activity
	units/mg	%	*units/mg*	%	*units/mg*	%
Man*p*βNp	31.1	100	16	3.6	0	0
Gal*p*βNp	19.2	61.8	153	34.3	0	0
Glc*p*βNp	1.4	4.7	446	100	0	0
Xyl*p*βNp	1.3	4.2	41	9.2	0	0
Glc*p*αNp	0	0	0	0	287	100

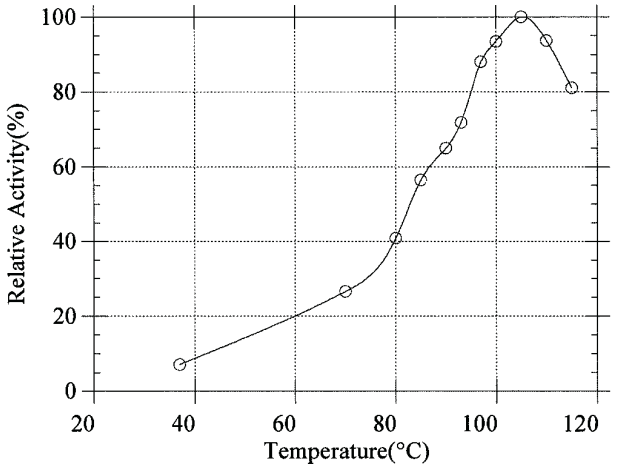

FIG. 2. **Effect of temperature on the activity of purified β-mannosidase from *P. furiosus*.** Activity was determined in 100 mM sodium phosphate buffer, pH 7.0, by measuring the amount of *p*Np released during a 5-min incubation at the desired temperature. For temperatures below 95 °C, assays were performed in triplicate. For temperatures above 95 °C, six assays were performed at each temperature. The data were fit with a cubic spline.

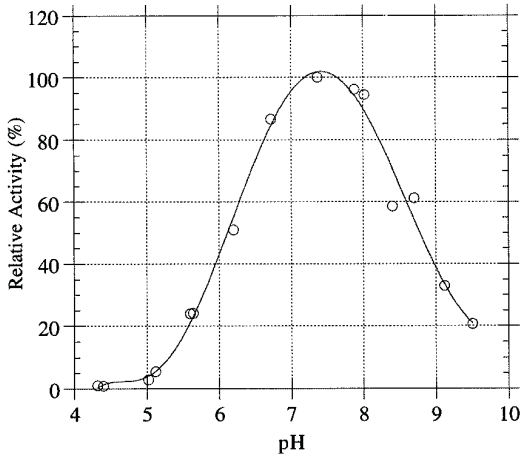

FIG. 3. **Effect of pH of the activity of purified β-mannosidase from *P. furiosus*.** Activity was determined in 100 mM sodium acetate/acetic acid (pH 4.2–5.6), 100 mM sodium phosphate (pH 5.6–8.0), and 100 mM glycine/NaOH (pH 7.9–9.3). Activities in sodium acetate/acetic acid and glycine/NaOH buffers were normalized to the activity in sodium phosphate buffer using the pH values that were common to both buffers. The data were fit with a fifth order polynomial.

β-mannosidase and the β-glucosidase purified from *P. furiosus* were determined by Edman degradation to be MFPEKFL*X*GVAQ*X*GFQ*X*EMGD and MKFPKNFMF, respectively, and were identical to the deduced amino acid sequences. The deduced amino acid sequences of the β-mannosidase and the β-glucosidase were 46.5% identical (Fig. 6). The β-mannosidase and β-glucosidase amino acid sequences were 19.0 and 17.9% identical to the predicted amino acid sequence of the α-amylase (42), respectively. In addition, the β-mannosidase and β-glucosidase NH_2-terminal amino acid sequences shared no homology with the 13 amino acid residues at the NH_2 terminus of the α-glucosidase (data not shown) from *P. furiosus*.

The deduced amino acid sequences for the β-mannosidase and the β-glucosidase were similar to the sequences for other glycosyl hydrolases (Table IV). On this basis, both the β-mannosidase and the β-glucosidase were classified as family 1 glycosyl hydrolases. The β-mannosidase and the β-glucosidase sequences showed the greatest homology with the β-glycosidases from two *Sulfolobus solfataricus* strains (44, 45). The *P. furiosus* enzymes were more distantly related to the family 1 glycosyl hydrolases from bacteria and eucarya. Clustering of family 1 glycosyl hydrolases into three groups has previously been reported (31). One group is composed of bacterial and eucaryal β-glycosidases, a second group contains the bacterial phospho-β-glycosidases, and a third group contains the archaeal β-glycosidases. The *P. furiosus* β-mannosidase and β-glucosidase both contained the conserved active site residues found in other family 1 enzymes. Two conserved carboxylates (residues 210 and 414 of *P. furiosus* β-mannosidase) presumably act as the acid/base and nucleophile, respectively, in the mechanism of glycoside-linkage hydrolysis (31, 46, 47).

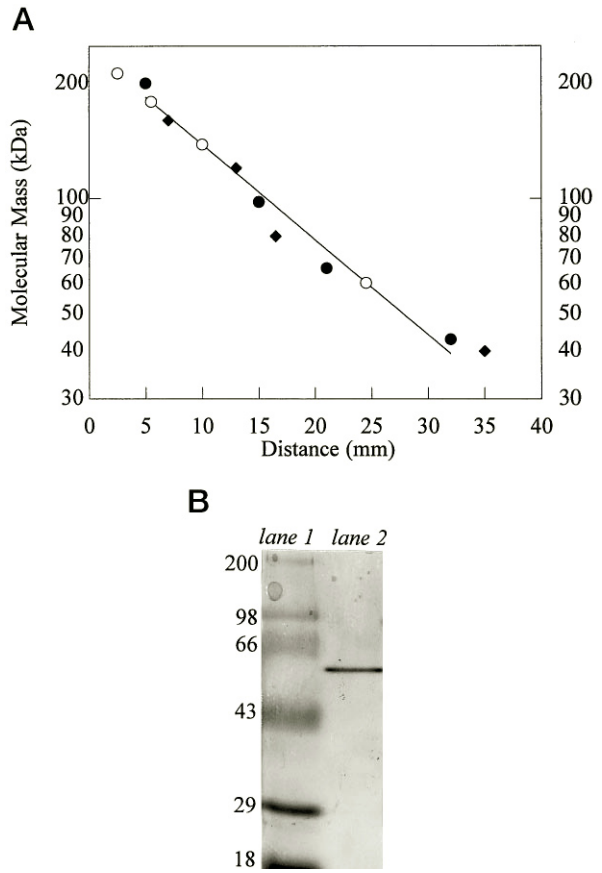

FIG. 4. *A*, determination of the native conformation of the β-mannosidase from *P. furiosus* after treatment with the cross-linker dimethyl suberimidate. Molecular mass markers (●) were used to determine the molecular mass of the monomeric (*1*), dimeric (*2*), trimeric (*3*), and tetrameric (*4*) forms of the cross-linked β-mannosidase (○). Aldolase (♦) was used as a positive control. *B*, SDS-PAGE showing the subunit of the β-mannosidase purified from cell extracts of *P. furiosus*. *Lane 1*, molecular mass markers used were carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), and phosphorylase (97 kDa). *Lane 2*, *P. furiosus* β-glucosidase.

TABLE III
Kinetic constants for the β-mannosidase and β-glucosidase from P. furiosus

Lineweaver-Burk plots were used to determine the kinetic constants. Activity of the β-mannosidase was determined using different initial concentrations of Man*p*βNp and Glc*p*βNp in 100 mM sodium phosphate, pH 7. The amount of *p*Np released was measured following a 5-min incubation at 95 °C. Activity of β-mannosidase was determined at 95 °C, and activity of the β-glucosidase was determined using Glc*p*βNp in 100 mM sodium citrate, pH 5.5, at 90 °C (4). ND, kinetic analysis with this substrate was not performed.

Kinetic parameter	Substrate	β-Mannosidase	β-Glucosidase
K_m (mM)	Glc*p*βNp	2.93	0.15
	Man*p*βNp	0.79	ND
V_{max} (units/mg)	Glc*p*βNp	14.8	700
	Man*p*βNp	31.1	25.2[a]
k_{cat}/K_m (s^{-1} mM^{-1})	Glc*p*βNp	5.3	4,500
	Man*p*βNp	40	ND

[a] Calculated as 3.6% of V_{max} for Glc*p*βNp (4).

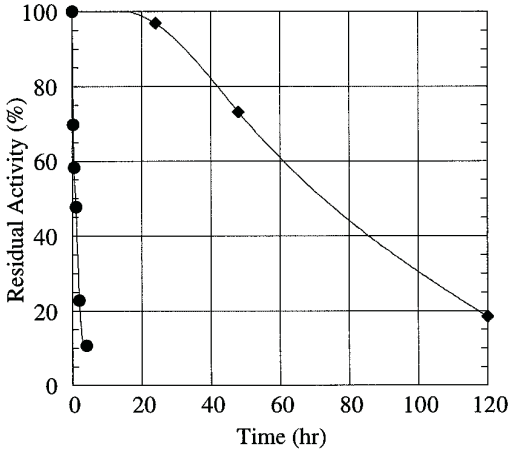

FIG. 5. **Thermostability of *P. furiosus* β-mannosidase at 90 and 100 °C.** Purified enzyme was incubated at 90 (♦) and 100 °C (●) for various time intervals. The remaining activity was determined by measuring the amount of *p*Np released during a 5-min incubation at 95 °C. The data were fit with a cubic spline.

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  1 .MFPEKFLWGVAQSGFQFEMGDKLRRNIDTNTDWWHWVRDKTNIEKGLVS 49
    .||..|::| . ||||||||    .. :.:.||| ||:||.||..||||
  1 MKFPKNFMFGYSWSGFQFEMG...LPGSEVESDWWVWVHDKENIASGLVS 47
          .         .         .         .         .
 50 GDLPEEGINNYELYEKDHEIARKLGLNAYRIGIEWSRIFPWPTTFIDVDY 99
    |||||:| . :.||..||:|| |||::..| ||||.||||.||  :.||
 48 GDLPENGPAYWHLYKQDHDIAEKLGMDCIRGGIEWARIFPKPTFDVKVDV 97
          .         .         .         .         .
100 SYNESYNLIEDVKITKDTLEELDEIANKREVAYYRSVINSLRSKGFKVIV 149
    . :|. |:| .|.:...|:.||:.|||. .:..||.:...::.:| ..|:
 98 EKDEEGNII.SVDVPESTIKELEKIANMEALEHYRKIYSDWKERGKTFIL 146
          .         .         .         .         .
150 NLNHFTLPYWLHDPIEARERALTNKRNGWVNPRTVIEFAKYAAYIAYKFG 199
    || |:.||.|:||||..|. : .. ..||:|.:||:||.|:||::||.::
147 NLYHWPLPLWIHDPIAVRKLGPDRAPAGWLDEKTVVEFVKFAAFVAYHLD 196
          .⇓        .         .         .         .
200 DIVDMWSTFNEPMVVVELGYLAPYSGFPPGVLNPEAAKLAILHMINAHAL 249
    |:|||||:||| || : ||:. ||||| |. |||. | :::|.||
197 DLVDMWSTMNEPNVVYNQGYINLRSGFPPGYLSFEAAEKAKFNLIQAHIG 246
          .⇑        .         .         .         .
250 AYRQIKKFDTEKADKDSKEPAEVGIIY.....NNIGVAYPKDPNDSKDVK 294
    || .||.:...          .||:||     : :: .|..:.:: :. |
247 AYDAIKEYSEK..........SVGVIYAFAWHDPLAEEYKDEVEEIRK.K 285
          .         .         .         .         .
295 AAENDNFFHSGLFFEAIHKGKLNIEFDGETFIDAPYLKGNDWIGVNYYTR 344
    . |  .::||        ||||                  ||||||||.|
286 DYEFVTILHS........KGKL..................DWIGVNYYSR 309
          .         .         .         .         .
345 EVVTYQEPMFPSIPLITFKGVQGYGYACRPGTLSKDDRPVSDIGWELYPE 394
     | . .:. :..:|        |||: : .|.:.|.:||.||:|||:|||
310 LVYGAKDGHLVPLP........GYGFMSERGGFAKSGRPASDFGWEMYPE 351
          .         .⇓        .         .         .
395 GMYDSI.VEAHKYGVPVYVTENGIADSKDILRPYYIASHIKMIEKAFEDG 443
    |: : :   .: |::|:.:||||:||. | .||.|:.||:| : .|:.:|
352 GLENLLKYLNNAYELPMIITENGMADAADRYRPHYLVSHLKAVYNAMKEG 401
          .         .⇑        .         .         .
444 YEVKGYFHWALTDNFEWALGFRMRFGLYEVNLITKERIPREKSVSIFREI 493
     :|:||:||.||||:||| |||||||| |:: ||.|. |...: :||||
402 ADVRGYLHWSLTDNYEWAQGFRMRFGLVYVDFETKKRYLRPSAL.VFREI 450
          .         .
494 VANNGVTKKIEEELLRG......... 510
    ...    |.|.||| :
451 ATQ....KEIPEELAHLADLKFVTRK 472
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FIG. 6. **Comparison of the amino acid sequences of the β-mannosidase and β-glucosidase from *P. furiosus*.** The amino acid sequences of the β-mannosidase (*upper*) and β-glucosidase (*lower*) were aligned with the GAP program of GCG (Genetics Computer Group, Inc., Madison, WI) using a gap weight of 3.0 and a gap length weight of 0.1. The first set of *arrows* (pointing to residue 210 in β-mannosidase sequence) indicate the putative active site acid/base, and the second set of *arrows* (pointing to residue 414 in β-mannosidase sequence) indicate the putative active site nucleophile.

DISCUSSION

Relative to β-glucosidases, there are few reports on the purification and characterization of β-mannosidases. The β-mannosidases (EC 3.2.1.25) from bacteria, including *Enterococcus casseliflavus* (48) and *Bacillus* sp. AM001 (49), and lower eucarya, including *Aspergillus niger* (50) and *Aspergillus awamori* (51), degrade the terminal, non-reducing β-mannopyranoside linkages of mannan. The physiological role of the β-mannosidases from higher eucarya is different. These enzymes degrade the terminal, non-reducing β-mannopyranoside

TABLE IV
Comparison of amino acid sequences among family 1 glycosyl hydrolases

Identity is determined as the number of identical amino acid residues in each pairwise comparison, expressed as percentage of the total number of amino acid residues compared. The GAP program of GCG (Genetics Computer Group, Inc., Madison, WI) was used with a gap weight of 3.0 and a gap length weight of 0.1. The following abbreviations are used: Pfuβm, *P. furiosus* β-mannosidase; Pfuβg, *P. furiosus* β-glucosidase; Sso1, *Sulfolobus solfataricus* DSM 1616 β-galactosidase (44); Sso2, *S. solfataricus* MT-4 β-galactosidase (45); Cs, *Caldocellum saccharolyticum* β-glucosidase A (81); Tm, *Thermotoga maritima* β-glucosidase A (28); Ct, *Clostridium thermocellum* β-glucosidase A (31); Bpa, *Bacillus polymyxa* β-glucosidase A (11); Bpb, *B. polymyxa* β-glucosidase B (11); Lb, *Lactobacillus casei* phospho-β-galactosidase (14); Sr, *Streptococcus rochei* phospho-β-galactosidase (21); Bn, *Brassica napus* myrosinase (20); Sa, *Sinapsis alba* myrosinase (24); Hs, *Homo sapiens* lactase-phlorizin hydrolase (17); Oc, *Oryctolagus cuniculus* lactase-phlorizin hydrolase (17); Ec, *Escherichia coli* phospho-β-glucosidase (26).

Enzyme	% Identity															
	Pfuβm	Pfuβg	Sso1	Sso2	Cs	Tm	Ct	Bpa	Bpb	Bn	Sa	Hs	Oc	Lb	Sr	Ec
Pfuβm		46.5	46.3	45.0	33.7	34.4	31.3	33.6	32.2	29.1	29.8	30.0	31.3	32.4	32.2	25.6
Pfuβg			53.5	53.9	33.7	34.3	29.6	30.6	30.2	27.7	32.1	26.2	27.0	29.7	32.0	25.0
Sso1				72.0	28.8	32.2	30.9	26.1	22.1	27.1	22.7	29.2	27.7	23.4	30.8	22.7
Sso2					30.1	33.8	31.3	27.2	29.7	29.3	30.0	29.0	27.6	22.6	34.4	33.5
Cs						49.8	51.6	43.3	41.5	36.0	36.4	38.7	36.6	39.5	35.5	35.7
Tm							53.2	46.7	42.9	39.7	38.8	47.4	44.2	37.7	31.7	32.5
Ct								49.6	43.9	36.9	36.3	40.2	37.6	38.4	35.0	34.2
Bpa									44.4	35.4	35.0	37.2	34.8	36.2	31.9	33.9
Bpb										33.9	34.7	36.7	37.6	34.4	33.2	30.1
Bn											91.2	40.3	38.6	32.9	29.6	24.3
Sa												40.1	39.2	32.4	29.7	25.7
Hs													85.5	29.9	30.5	28.1
Oc														28.9	30.3	27.4
Lb															29.3	33.5
Sr																33.9

linkages of glycoproteins (52–54). Genetic disorders associated with β-mannosidase deficiency have been described in a number of mammals (55–58). In humans, the absence of β-mannosidase results in the deleterious storage of the disaccharide Manβ1–4GlcNAc (57–59). The gene for the bovine β-mannosidase has been sequenced (60) and the deduced amino acid sequence is similar to sequences of family 2 glycosyl hydrolases.

The *P. furiosus* β-mannosidase is distinct from the other glycosyl hydrolases from *P. furiosus*. The substrate specificity and predicted amino acid sequence of the β-mannosidase are significantly different from those of the β-glucosidase, α-glucosidase, and α-amylase previously purified from *P. furiosus*. Based on both substrate specificity and amino acid sequence, the β-mannosidase and β-glucosidase are the most closely related. Both of these enzymes are exo-acting, β-specific glycosyl hydrolases that release the terminal, non-reducing sugars from β-glycosidic bonds. The β-mannosidase is most active with mannose as the terminal non-reducing sugar, while the β-glucosidase has its highest specific activity with glucose in this location (4). This suggests a difference in the way that the two enzymes interact with the hydroxyl on C-2 of the terminal, non-reducing sugar.

Although the presence of two similar enzymes within *P. furiosus* might appear to be an unnecessary metabolic burden, several other organisms, including some thermophiles, contain the genes for two or more exo-acting, β-specific glycosyl hydrolases. For example, *Bacillus polymyxa* contains two genes, *bglA* and *bglB*, encoding different family 1 β-glucosidases (11). The deduced amino acid sequences are 44.7% identical, but the enzymes have distinctly different biochemical characteristics. β-Glucosidase A is intracellular and cleaves cellobiose through pyrophosphate-mediated hydrolysis, while β-glucosidase B is extracellular and cleaves cellobiose without cofactors (11). The thermophilic bacterium *Clostridium thermocellum* also has two β-glucosidase genes, *bglA* and *bglB* (31). The proteins encoded by these genes are only 21.7% identical. The sequence for β-glucosidase A is similar to family 1 glycosyl hydrolases (31), while β-glucosidase B is similar to family 3 glycosyl hydrolases, which include β-glucosidases from fungi and rumen bacteria (61). The thermophilic bacterium *Thermotoga maritima* may have as many as four different exo-acting, β-specific glycosyl hydrolases, including a β-xylosidase (27), a β-galactosidase (28), and possibly two β-glucosidases (28). The gene for one β-glucosidase (*bglA*) has been sequenced, and the deduced amino acid sequence is similar to family 1 glycosyl hydrolases. Two β-glucosidase-encoding genes have been sequenced from the thermoacidophilic archaeon *S. solfataricus*. Although these genes may have come from different strains of *S. solfataricus* (32), they are both homologous to family 1 glycosyl hydrolases.

The high degree of homology between the sequences of the β-mannosidase and β-glucosidase from *P. furiosus* suggests that the enzymes may be evolutionarily related. Gene duplication is frequently observed among glycosyl hydrolases (10). It has been proposed that the enzyme produced from the original gene copy would continue hydrolyzing the original substrate, while duplicate gene copies could constitute templates for constructing enzymes with activity directed to a new, but stereochemically similar, substrate (10). The divergence of glycosyl hydrolases to acquire new specificities is not unexpected, given the stereochemical resemblance among pyranoside substrates. It is not clear whether the two enzymes from *P. furiosus* represent a case of gene duplication, and, if so, which one was the predecessor.

All glycosyl hydrolases are believed to act by a general acid catalysis mechanism in which two amino acid residues participate in the hydrolysis of the glycosidic bond (62). For family 1 glycosyl hydrolases, the two catalytic residues are both glutamic acid residues (positions 210 and 414, *P. furiosus* β-mannosidase numbering) that are strictly conserved (47). The glutamic acid closer to the N terminus functions as the acid/base (*i.e.* proton donor) (31, 63) and the other glutamic acid acts as the nucleophile (45, 64). It has been suggested that all family 1 glycosyl hydrolases have an 8-fold β/α-barrel structure (48, 64). Structural and sequence comparisons of family 1 glycosyl hydrolases indicate that the two conserved carboxylates in these enzymes occur at the C-terminal ends of β-strands 4 and 7 (47). Similar structure and catalytic residues have been observed for glycosyl hydrolase families 2, 5, 10, 17, 30, 35, 39, and 42 and are the basis for the classification of these families into a superfamily (47, 65).

Some family 1 glycosyl hydrolases also have glycosyl transferase activities (4, 13–15, 32). The *S. solfataricus* β-glucosidase has been implicated in the glycosylation of membrane

lipid components (32). The β-glucosidases from both *P. furiosus* and *S. solfataricus* have been used for a variety of synthetic glycosyl transferase reactions (68, 69). Similarly, the *P. furiosus* β-mannosidase may be involved in the biosynthesis of intracellular components including proteins, membrane components, or other compounds. Mannose-conjugated glycolipids have been identified in a number of archaea (70–72). In addition, the major glycolipids of closely related halophilic euryarchaeota are built from a basic diglycoside, Manβ1–4Glc (72–78). A novel osmoprotectant, 2-*O*-β-D-mannosylglycerate, was recently discovered in both thermophilic bacteria (79) and archaea, including *P. furiosus* (80). When *P. furiosus* is grown at supraoptimal salt concentrations, 2-*O*-β-D-mannosylglycerate becomes the predominant intracellular solute (80). 2-*O*-β-D-Mannosylglycerate may play a role in osmoprotection (80), or it may be an activated precursor in the synthesis of certain membrane glycolipids in response to salt stress. We have begun experiments to determine if the β-mannosidase has glycosyl transferase activities and if it is involved in the synthesis of this novel compound.

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