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Purification and Characterization of an α-Glucosidase from a Hyperthermophilic Archaebacterium, *Pyrococcus furiosus*, Exhibiting a Temperature Optimum of 105 to 115°C

HENRY R. COSTANTINO, STEPHEN H. BROWN, AND ROBERT M. KELLY*

Department of Chemical Engineering, The Johns Hopkins University, Baltimore, Maryland 21218, and Center of Marine Biotechnology, University of Maryland, Baltimore, Maryland 21202

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Pyrococcus furiosus **is a strictly anaerobic hyperthermophilic archaebacterium with an optimal growth temperature of about 100°C. When this organism was grown in the presence of certain complex carbohydrates, the production of several amylolytic enzymes was noted. These enzymes included an α-glucosidase that was located in the cell cytoplasm. This α-glucosidase has been purified 310-fold and corresponded to a protein band of 125 kilodaltons as resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The enzyme exhibited optimum activity at pH 5.0 to 6.0 and over a temperature range of 105 to 115°C. Kinetic analysis conducted at 108°C revealed hydrolysis of the substrates *p*-nitrophenyl-α-D-glucopyranoside (PNPG), methyl-α-D-glucopyranoside, maltose, and isomaltose. Trace activity was detected towards *p*-nitrophenyl-β-D-glucopyranoside, and no activity could be detected towards starch or sucrose. Inhibition studies conducted at 108°C with PNPG as the substrate and maltose as the inhibitor yielded a K_i for maltose of 14.3 mM. Preincubation for 30 min at 98°C in 100 mM dithiothreitol and 1.0 M urea had little effect on enzyme activity, whereas preincubation in 1.0% sodium dodecyl sulfate and 1.0 M guanidine hydrochloride resulted in significant loss of enzyme activity. Purified α-glucosidase from *P. furiosus* exhibited remarkable thermostability; incubation of the enzyme at 98°C resulted in a half life of nearly 48 h.**

Enzymes from hyperthermophilic marine archaebacteria, including *Pyrococcus furiosus*, have attracted attention for several reasons. In addition to their evolutionary significance, enzymes from these organisms provide the opportunity to investigate the structure and function of proteins at very high temperatures. The study of constituent enzymes will also help to answer questions about the growth and metabolism of these organisms. Additionally, enzymes from hyperthermophiles have significant biotechnological potential, since they may be able to advantageously replace enzymes in existing applications and also create new opportunities.

P. furiosus is a member of a recently described genus of hyperthermophilic archaebacteria which grows optimally at about 100°C. Discovered by Fiala and Stetter (6), this organism is a strict anaerobe and grows heterotrophically by a fermentative-type metabolism, utilizing a range of carbohydrates. In the absence of elemental sulfur (S^0), the end product gases are CO_2 and H_2; H_2 has been shown to be inhibitory to the organism (6). When grown in the presence of S^0, *P. furiosus* also generates H_2S. When grown with S^0 on carbohydrates, such as starch, the CO_2 production rate relative to the H_2S production rate increases significantly, suggesting that *P. furiosus* is metabolizing the carbohydrate. At present, it has not been determined how this organism metabolizes carbohydrates and whether the enzymes involved are comparable to those found in mesophilic organisms that utilize carbohydrates.

α-Glucosidase (EC 3.2.1.20; α-D-glucoside glucohydrolase) catalyzes the hydrolysis of terminal, nonreducing D-glucose residues of a variety of substrates, including disaccharides, oligosaccharides, and other aryl- and alkyl-α-glucopyranosides. It has been discovered that many organisms that produce extracellular amylolytic enzymes also produce an intracellular α-glucosidase. In this instance, α-glucosidase is the final enzyme involved in the metabolism of starch, or perhaps other carbohydrates, to glucose.

α-Glucosidase has been isolated from a variety of bacteria, including some moderate thermophiles. For example, Suzuki et al. (18) purified an extracellular α-glucosidase from *Bacillus thermoglucosidus* which exhibited a temperature optimum of 75°C but was rapidly inactivated at higher temperatures. This enzyme has also been purified from other *Bacillus* species, including *Bacillus caldovelox* DSM411 (8) and *Bacillus stearothermophilus* ATCC 12016 (17). These enzymes were shown to be optimally active at 60 and 70°C, respectively.

Madi et al. (15) have described amylolytic enzymes from *Clostridium* sp. strain EM1, including α-amylase, pullulanase, and α-glucosidase. The α-glucosidase, which was present both in cells and in the culture fluid, exhibited a temperature optimum of about 65°C in an unpurified form, and about 35% of the maximum activity could be measured at 80°C. Working with the same organism in continuous culture, Antranikian and co-workers (1) demonstrated that amylolytic enzyme production and excretion were greatly dependent upon the dilution rate and pH of the medium. Hyun et al. (11) investigated the amylosaccharide metabolism of two *Clostridium* species, *C. thermosulfurogenes* and *C. thermohydrosulfuricum*. The latter organism converted starch and maltose to glucose extracellularly, as demonstrated by the accumulation of glucose in the medium. Maltase activity was also detected, as measured at 60°C. The study concluded that conversion of starch or maltose into glucose was the rate-limiting step in cell growth.

Recent investigations on enzymes from hyperthermophiles have focused on metabolically important enzymes, including a hydrogenase (4) and a ferredoxin (2) from *P.*

* Corresponding author.

furiosus and a hydrogenase from the autotrophic hyperthermophilic archaebacterium *Pyrodictium brockii* (16). These enzymes exhibit considerable thermostability and activity at high temperatures. For example, *P. furiosus* ferredoxin (2) is unaffected by a 12-h incubation at 95°C. To date, there have been no reports published specifically on amylolytic enzymes from hyperthermophilic organisms. Several of these organisms, including *P. furiosus*, have been reported to grow in the presence of polysaccharides such as starch (6, 19). Preliminary studies in our laboratory demonstrate that *P. furiosus* exhibits amylase and pullulanase activity both intracellularly and extracellularly. In addition, an intracellular α-glucosidase has been observed and is the focus of this communication. Amylolytic enzymes from *P. furiosus* all have temperature optima of at least 100°C and exhibit remarkable thermostability (S. H. Brown, H. R. Costantino, and R. M. Kelly, unpublished data). As evidence accumulates, it is becoming clear that the inherent thermostability of these enzymes may allow them, in some instances, to take advantage of increased reaction rates at higher temperatures. Study of these enzymes will allow elucidation of the mechanisms of protein thermostability and provide insight into microbial growth and metabolism at temperatures up to and above 100°C.

MATERIALS AND METHODS

Chemicals. Soluble starch, pullulan, D-glucose, maltose, glycogen, *p*-nitrophenyl-α-D-glucopyranoside (PNPG), *p*-nitrophenol (PNP), hydroxyapatite, isomaltose, dextran, and methyl-D-glucopyranoside were obtained from Sigma Chemical Co. Tryptone and yeast extract were from Difco Laboratories. Dithiothreitol (DTT) was purchased from Bio-Rad Laboratories. Chromatography media and supplied as follows: Sephadex G-200, Pharmacia; DE52, Whatman. Protein molecular weight standards were obtained through Bio-Rad. All other chemicals were of reagent grade.

Purification of α-glucosidase. Unless otherwise stated, all steps were conducted at room temperature. Sodium phosphate buffers were prepared from monobasic and dibasic salts by the method of Gomori (9) to ensure proper pH. All buffers were titrated to the desired pH at room temperature.

(i) Cell cultivation and harvesting. *P. furiosus* DSM 3638 was obtained from Deutsche Sammlung von Mikroorganismen, Federal Republic of Germany. *P. furiosus* was grown in continuous culture with S^0 following the procedure described by Brown and Kelly (3), except that the culture medium was modified slightly and contained 1 g of yeast extract, 2 g of tryptone, and 3 g of soluble starch per liter. The fermentation flask had a total volume of 3 liters and a working volume of 1.5 liters and was kept at 98°C. Cells were grown in batch mode until mid-log phase and then switched to continuous mode with a dilution rate of about 0.5 h^{-1}. Cell densities were monitored by epifluorescence microscopy with acridine orange stain (10). A total of 80 liters of cells were grown and subsequently harvested by centrifugation at about 12,000 × *g* for 20 min. Cell pellets were frozen at −20°C until use.

(ii) Cell disruption. Cell paste was suspended in about 50 ml of 0.1 M sodium phosphate (pH 7.3). After being vortexed, the suspended cells were sonicated in 10-ml portions for a total of 2 min in a Tekmar sonic disruptor (model TM300) working at 50% duty cycle. Following sonication, the suspension was centrifuged at 18,000 × *g* for 30 min. The supernatant was collected; the pellet, which consisted of sulfur and cell debris, had no significant α-glucosidase activity and was discarded.

(iii) Ammonium sulfate precipitation. Solid ammonium sulfate was added to the supernatant described above to a final concentration of 176 g/liter (30% saturation at room temperature) and stirred for 2 h. The solution was centrifuged at 15,000 × *g* for 30 min. The volume of the supernatant fluid was measured and brought to 70% saturation by an additional 273 g of ammonium sulfate per liter, stirred for 2 h, and then centrifuged at 15,000 × *g* for 30 min. The pellet was suspended in 20 mM Tris hydrochloride (Tris-HCl, pH 8.5) and dialyzed against this buffer for 4 h.

(iv) Anion-exchange chromatography. The sample was applied in about 25-ml portions in two separate runs to a DEAE-cellulose (DE52) column (2 by 30 cm), equilibrated with 20 mM Tris-HCl (pH 8.5). The α-glucosidase activity was eluted from the column with a linear gradient of 240 ml total volume from 20 mM Tris-HCl (pH 8.5) to 20 mM Tris-HCl (pH 8.5) plus 400 mM NaCl. Fractions of 60 drops per tube (about 2.4 ml per tube) were collected and monitored for absorbance at 280 nm, α-glucosidase activity, and conductivity (to determine the NaCl concentration). Fractions containing activity were pooled (total volume from both runs was 108 ml) and concentrated in a Filtron Technology Novacell stirred cell with a 10-kilodalton (kDa) molecular size cutoff (MSC) under 40 lb/in^2 pressure. The retentate (about 10 ml) was dialyzed overnight against 0.1 M sodium phosphate (pH 7.0) containing 0.1 g of sodium azide per liter.

(v) Chromatography on hydroxyapatite. The concentrated sample was applied to a hydroxyapatite column (2 by 20 cm) equilibrated with 0.1 M sodium phosphate (pH 7.0). The α-glucosidase activity was eluted with a linear gradient of sodium phosphate from 0.1 to 0.5 M (240 ml total volume). Fractions of about 2.4 ml per tube were collected and monitored for A_{280}, α-glucosidase activity, and conductivity. Fractions containing activity were pooled (total volume, 36 ml), concentrated to about 10 ml in the Novacell stirred cell, and further concentrated to about 3 ml by centrifugation at 4,000 × *g* for about 30 min in Amicon Microconcentrators with a 10-kDa MSC.

(vi) Gel filtration chromatography. The concentrated sample was applied to a Sephadex G-200 column (3 by 90 cm) equilibrated with 0.1 M sodium phosphate (pH 7.3) containing 0.1 g of sodium azide per liter. Fractions of about 2.4 ml per tube were collected and monitored for A_{280} and α-glucosidase activity. Fractions containing activity were pooled (36 ml total volume). The pool was concentrated in the Novacell stirred cell to about 10 ml and further concentrated to about 0.5 ml by successive centrifugation (total time, about 1 h) in the Amicon microconcentrators.

(vii) Electrophoresis and electroelution. Native (nondissociating, discontinuous) polyacrylamide gel electrophoresis of the concentrated pool containing α-glucosidase activity and detection of activity within the gel were performed as described below. The activity band was cut from the gel, and electroelution was accomplished in a horizontal electrophoresis system for submerged-gel electrophoresis. For electroelution, the running buffer was the same as that used for native polyacrylamide gel electrophoresis. The horizontal unit was run at 25 mA for about 12 h. Following electroelution, the sample was dialyzed against 0.1 M sodium phosphate (pH 6.8).

Effect of different carbohydrates. *P. furiosus* was grown in batch culture in the presence of a number of carbohydrates to assess their effect on the production of α-glucosidase. Cultures (1 liter) were grown to late log phase (~12 h) at 98°C on a medium containing 5 g of the appropriate carbohydrate,

10 g of S^0, 5 g of tryptone, and 1 g of yeast extract per liter in the artificial sea water medium reported previously (3). Cells were harvested by filtering the cultures through Whatman no. 1 filter paper to remove sulfur and centrifuging the filtrate at 12,000 × *g* for 20 min. The sedimented cells were suspended in about 5 ml of 0.1 M sodium phosphate buffer (pH 7.3), and cell extracts were prepared as discussed above. These extracts were dialyzed overnight at 4°C against 0.1 M sodium phosphate buffer (pH 7.3) containing 0.1 g of sodium azide per liter and stored at 4°C until assay. The supernatant from each culture was concentrated to ~75 ml with a Minitan ultrafiltration cell with a 10-kDa MSC membrane and further concentrated to ~7 ml with the Novacell stirred cell. Supernatant samples were dialyzed overnight at 4°C against 0.05 M EPPS [*N*-(2-hydroxyethyl)piperazine-*N'*-3-propane sulfonic acid] buffer (pH 7.0) containing 0.01% sodium azide and stored at 4°C until assay. EPPS was used instead of phosphate to avoid precipitation of salts present in the supernatant.

Enzyme assays (α-glucosidase). (i) Standard assay (PNPG). Assays were conducted with PNPG as the substrate. Spectrophotometric readings were conducted with a Perkin Elmer Lambda 3 spectrophotometer with thermostatted cell holder and six-cell transport. The bath fluid consisted of a mixture of ethylene glycol-water in a ratio of about 1:1 and was maintained at a constant temperature and pumped through the cell holder by a circulating temperature bath. Temperature inside the cuvettes was monitored with a thermocouple. For temperatures over 100°C, cuvettes were sealed with rubber septa to keep the sample from boiling. These septa were successful up to temperatures of about 115°C. The six-cell transport was controlled and data were collected and analyzed by Perkin Elmer software (PECSS) run on a microcomputer.

Routine enzymatic assays were conducted as follows. For each assay, 1 ml of sample buffer consisting of 20 mM PNPG in 0.1 M sodium phosphate (pH 5.5) was pipetted into a quartz cuvette. The cuvettes were then inserted into the cell holder and incubated for 5 to 10 min to allow the cuvettes to reach the desired temperature. Following this incubation, sample was added to the cuvette, and the evolution of PNP was followed by monitoring absorbance at 405 nm. Activity units are expressed as micromoles of PNP released per minute per milligram of protein. V_{max} and K_m were determined by using Enzfitter software (Elsevier-BIOSOFT) run on a microcomputer. To perform calculations yielding glucosidase activity units, it was necessary to investigate the effect of pH and temperature on the molar absorbancy index (A_m) of PNP. All measurements were conducted in the presence of 0.1 M sodium phosphate buffer.

(ii) Alternative substrate assays. Alternative substrates were used to determine the specificity of enzymatic action. Assays were conducted at 108°C. A 1-ml amount of 0.1 M sodium phosphate (pH 5.5) and the desired amount of substrate were incubated for 10 min. Enzyme to be assayed was injected through the septum, and the sample was held at 108°C for an additional 5 min. Following incubation, the samples were cooled rapidly on ice for 5 min. Release of glucose was measured with a Sigma glucose diagnostic kit (Kit 510-A, colorimetric). Activity is expressed as micromoles of glucose liberated per minute per milligram of protein. Alternative substrates examined in this manner were starch, sucrose, maltose, isomaltose, and methyl-α-D-glucopyranoside. V_{max} and K_m were determined with Enzfitter software.

Effect of pH and temperature on α-glucosidase activity. Enzymatic activities were determined as described above (PNPG standard assay) except that temperature and pH were varied independently. For all calculations, activity is expressed as activity above background degradation of PNPG. This was particularly important at high pH, where PNPG becomes increasingly unstable. The A_m of PNP decreases with decreasing pH and temperature, which results in decreased sensitivity for the assay under these conditions.

Thermostability of α-glucosidase. Purified α-glucosidase was diluted 1:10 in 0.1 M sodium phosphate (pH 6.5) and incubated in screw-top microcentrifuge tubes for various lengths of time in an oil bath maintained at 98°C. Remaining α-glucosidase activity was measured as described above.

Protein assays. Protein concentrations were measured with the bicinchonic acid system (Sigma; procedure TPRO-562). Bovine serum albumin was used as the protein standard.

Electrophoresis and electroelution. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was accomplished with the discontinuous system of Laemmli (14). Protein samples were prepared by boiling for 10 min in 2.5% SDS–20 mM DTT. For determination of molecular weight, protein standards were run in parallel lanes. Protein bands were visualized by staining with silver stain (Bio-Rad silver stain kit) or 0.1% Coomassie brilliant blue R. R_f values were calculated relative to the bromphenol-blue tracking dye. α-Glucosidase activity was measured within the gel by incubating the gel in 1% Triton X-100 for 30 min to remove SDS, followed by incubation in 10 mM PNPG for 30 min. The gel was then incubated at 70°C until a yellow band appeared.

Native polyacrylamide gel electrophoresis was conducted in the same manner as above except for the elimination of SDS. Proteins were visualized by silver staining as above, and α-glucosidase activity was detected similarly, except the Triton X-100 incubation was eliminated. For electroelution, the activity band was cut from a native polyacrylamide gel and eluted with a horizontal electrophoresis system (Bethesda Research Laboratories model H5). The running buffer was the same as that used in preparing the native gel. The horizontal unit was run at 25 mA for about 12 h. Following electroelution, the sample was dialyzed against 0.1 M sodium phosphate (pH 6.8).

RESULTS AND DISCUSSION

Growth of *P. furiosus* and carbohydrate metabolism. A total volume of 80 liters of *P. furiosus* culture was collected from growth in continuous mode. Cell densities averaged about 0.8×10^8 to 1.4×10^8 cells per ml, and the final cell yield was 37.6 g (wet weight) or 0.47 g (wet weight) per liter of culture. This material was used in purification of *P. furiosus* α-glucosidase. Additional growth studies were conducted to determine the effect of various carbon sources on production of α-glucosidase in this organism. Table 1 shows the intracellular and extracellular specific activities of α-glucosidase following growth in the presence of various simple and complex carbohydrates. Typically, over 90% of total α-glucosidase activity was present intracellularly. When grown in the absence of carbohydrate or in the presence of glucose, specific activity of α-glucosidase was lower than when grown in the presence of a carbohydrate containing α-1,4 linkages. The addition of 5 g of glucose per liter to medium containing starch did not repress the production of α-glucosidase to constitutive levels. Similar inductive effects have been observed for other amylolytic enzymes

TABLE 1. Effect of carbon source on α-glucosidase production by *P. furiosus*

Carbohydrate	α-Glucosidase activity (mU/mg of protein)	
	Cell extract	Supernatant
None	140	29
Glucose	178	26
Maltose	434	39
Starch	629	80
Starch + glucose	460	21
Glycogen	768	80
Pullulan	539	55
Dextran	262	5

from *P. furiosus*. Because glucose is not metabolized by *P. furiosus* and is not conducive to amylolytic enzyme production, it appears that maltose, or degradation products of the polysaccharides, is transported into the cell in a passive or active fashion (maltose permease) and acts as an inducer for the amylolytic enzymes. In many bacterial systems, an intracellular α-glucosidase often represents the final stage in the metabolism of starch to glucose. This is a possible model of amylosaccharide metabolism in *P. furiosus*.

Purification of α-glucosidase. The enzyme was purified from cell extracts as described above. The α-glucosidase was determined to be soluble because the material that sedimented following sonication had no significant activity. Initial purification work demonstrated that incubation at 4°C or on ice apparently resulted in loss of α-glucosidase activity. In the purification of hydrogenase from *P. furiosus*, Bryant and Adams (4) discovered anomalous results which resulted from prolonged incubation of their cell extracts at 4°C. They attributed this behavior to some form of aggregation or association of the protein. Perhaps some form of aggregation or cold denaturation explains the loss of α-glucosidase activity when the organism is exposed to low temperatures. Because of this phenomenon, all purification procedures were conducted at room temperature. It was also observed that when purified enzyme was incubated on ice for several hours, about half the α-glucosidase activity was lost (results not shown). Kept at room temperature, purified α-glucosidase retained full activity for at least a month.

The purification procedure is shown in Table 2. *P. furiosus* α-glucosidase was purified 310-fold over the activity in the cell extract, with a final yield of 8.5%. From a starting material of 37.6 g (wet weight) of cell paste, approximately 0.26 mg of α-glucosidase was obtained. SDS-polyacrylamide gel electrophoresis of the purified α-glucosidase is shown in Fig. 1. About 500 ng of total protein was loaded. Silver staining revealed a protein band at about 125 kDa. Gel activity staining following treatment with Triton X-100 to reconstitute the enzyme confirmed that this band was responsible for the α-glucosidase activity. Another faint protein band of about 83 kDa was present. This band exhibited no detectable α-glucosidase activity as determined by gel activity staining. No evidence of subunits was observed under the conditions used, although the existence of multiple subunits of equal size cannot be ruled out.

TABLE 2. Purification of α-glucosidase from *P. furiosus*

Fraction	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude extract	50	15.1	19.3	0.782	1.0	100
Ammonium sulfate	50	12.6	13.3	0.950	1.2	84
Anion exchange	108	2.83	0.508	5.58	7.2	41
Hydroxyapatite	36	5.78	0.270	21.4	27	28
Gel filtration	36	4.61	0.0772	59.9	77	22
Electroelution	4.1	15.6	0.0638	245	310	8.5

FIG. 1. SDS-polyacrylamide gel electrophoresis of *P. furiosus* α-glucosidase. Purified enzyme (0.5 μg) was run in lane 1. Lane 2 contains protein molecular size standards (top to bottom): myosin (206 kDa), β-galactosidase (116.25 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), and ovalbumin (42.7 kDa). SDS-polyacrylamide gel electrophoresis and protein silver staining were performed as described under Materials and Methods.

As discussed below, the enzyme may retain measurable activity in the presence of SDS. When boiled for 2 min in 1.0% SDS–100 mM DTT and assayed in the presence of 0.1% SDS, the loss in α-glucosidase activity was about 90 to 95%. Protein samples for denaturing polyacrylamide gel electrophoresis were prepared by boiling for 10 min in 2.5% SDS–20 mM DTT. Under these conditions, a molecular mass of 125 kDa was reproducibly observed. However, since measurable residual activity may be retained, the molecular mass as determined by gel electrophoresis may be somewhat inaccurate. Anomalous behavior by proteins from *P. furiosus* during SDS-polyacrylamide gel electrophoresis has been observed previously (2, 4). These results indicate the need for the development of new techniques suitable for the analysis of highly stable proteins produced by this class of organisms.

Effect of pH and temperature on α-glucosidase activity. The effect of pH on α-glucosidase activity was determined at 98°C (Fig. 2). At this temperature, the α-glucosidase exhibited optimum activity at pH 5.0 to 6.0. This result is typical of many α-glucosidases (12). Figure 3 shows the effect of assay temperature on the α-glucosidase activity. *P. furiosus* α-glucosidase exhibited a broad temperature optimum at about 105 to 115°C, representing the highest reported temperature optimum for a purified enzyme.

Other enzymes from hyperthermophiles thus far studied also demonstrate remarkable temperature optima. Bryant

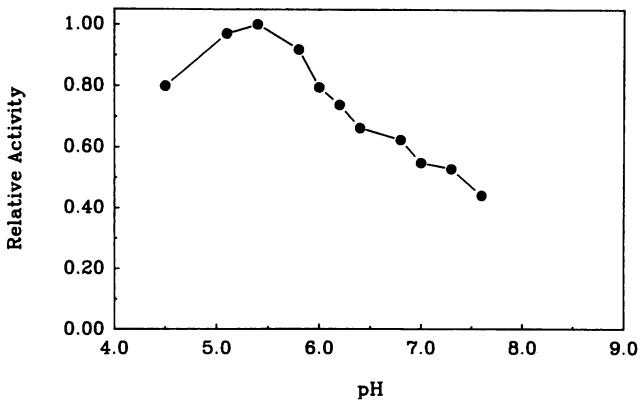

FIG. 2. Effect of pH on *P. furiosus* α-glucosidase activity. The relative α-glucosidase activity of the purified enzyme was determined at various pHs. Experimental details are described under Materials and Methods.

and Adams (4) reported a temperature optimum for the *P. furiosus* hydrogenase of >95°C, and Pihl et al. (16) have determined the temperature optimum for the hydrogenase from *Pyrodictium brockii* to be at least 90°C. The optimal temperature for amylase and pullulanase activities from cell extracts of *P. furiosus* has been found to be near 100°C. Limitations in instrumentation and substrate degradation often hinder the determination of temperature optima for hyperthermophilic enzymes.

Kinetic analysis of α-glucosidase. The substrate specificity of the purified enzyme was examined by kinetic analysis on various glucosides at 108°C (Table 3). *P. furiosus* α-glucosidase had a strong affinity for the α-enantiomer of PNPG, exhibiting a reaction velocity over 30-fold that of the β-form (data not shown). No activity could be detected with either starch or sucrose as the substrate. The K_ms obtained for the alkyl- and aryl-glucopyranosides, PNPG, and methyl-α-D-glucopyranoside were relatively low compared with the values determined for the disaccharides. This result has been seen for other α-glucosidases (for example, reference 17). The reaction velocities for the physiological substrates maltose and isomaltose were higher.

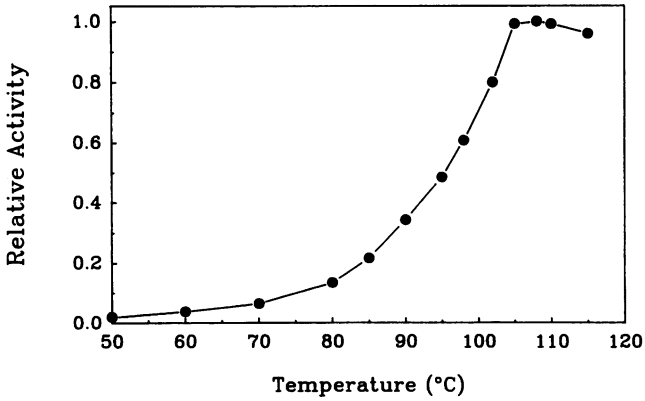

FIG. 3. Effect of temperature on *P. furiosus* α-glucosidase activity. The relative α-glucosidase activity of the purified enzyme was determined at various temperatures. Experimental procedures are described under Materials and Methods.

TABLE 3. Hydrolysis of various glucosides by *P. furiosus* α-glucosidase

Substrate	K_m (mM)	V_{max} (U/mg)
PNPG	0.26	287
Methyl-α-D-glucopyranoside	0.77	185
Maltose	5.2	1,760
Isomaltose	8.2	951

Microbial α-glucosidases exhibit significant diversity in their glucoside substrate specificity. Additionally, these enzymes can be either intracellular, extracellular, or membrane bound. Thus, classification of and enzymatic comparisons between α-glucosidases are difficult. Recently, Kelly and Fogarty (13) proposed a reclassification of bacterial α-glucosidase enzymes into those with highest specific activity toward maltose and those with highest activity toward aryl-D-glucosides. The *P. furiosus* α-glucosidase had highest activity towards maltose and isomaltose but also exhibited significant activity towards the PNPG substrate.

Inhibition of activity towards PNPG by maltose was also investigated and was consistent with the model of competitive enzyme inhibition (5). The data from this experiment, shown as a Dixon-Webb plot in Fig. 4, yielded a K_i of 14.3 mM for maltose.

The kinetic data for *P. furiosus* α-glucosidase were obtained at the highest temperature at which any such analysis has been conducted. Results for all substrates tested demonstrated typical enzyme catalysis, as described by the Michaelis-Menten model, and inhibition in the presence of maltose was also typical of competitive inhibition as described by Dixon and Webb (5).

Effect of various denaturing agents and chelators on α-glucosidase. The effect of incubation for 30 min at 98°C with a variety of agents is illustrated in Table 4. Because only a small fraction of activity was lost in the presence of the reducing agent DTT, it is likely that accessible cystinyl residues do not play an important role in the active-site conformation of this enzyme. A concentration of 1.0 M of

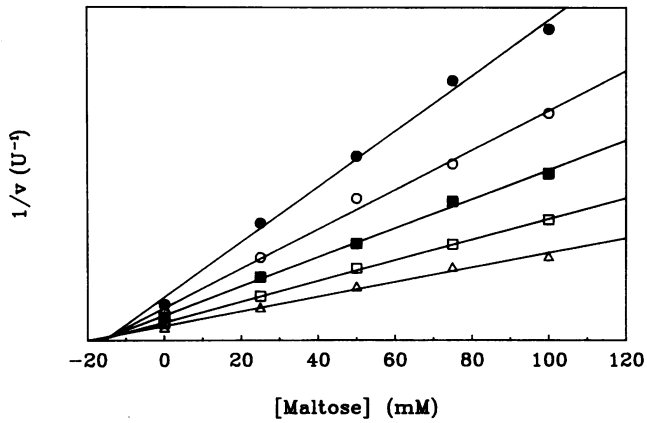

FIG. 4. Dixon-Webb plot. The α-glucosidase activity towards PNPG was measured in the presence of maltose. All assays were conducted in 0.1 M sodium phosphate (pH 5.5) at 108°C. PNPG was monitored as described under Materials and Methods. PNPG concentrations tested were (●) 0.1 mM, (○) 0.2 mM, (■), 0.3 mM, (□) 0.4 mM, and (△) 0.5 mM.

TABLE 4. Effect of various denaturing and chelating agents on *P. furiosus* α-glucosidase[a]

Agent	Concn	Activity remaining (%)
DTT	100 mM	83
Urea	1.0 M	84
Guanidine hydrochloride	1.0 M	<1.0
SDS	1%	22
EDTA	20 mM	50
Ethylene glycol bis(2-aminoethyl ether) tetraacetic acid	20 mM	85

[a] Activity remaining after incubation for 30 min at 98°C with the indicated agent.

the protein denaturant guanidine hydrochloride resulted in almost total loss of enzymatic activity, whereas the same amount of urea inhibited activity by only 16%. The stability of *P. furiosus* α-glucosidase under the latter conditions is intriguing. SDS incubation resulted in significant but not complete loss of activity. It was observed during preparation of electrophoresis samples that boiling for 10 min in 2.5% SDS–20 mM DTT resulted in about 90 to 95% loss of enzyme activity. Because DTT alone had little effect on enzyme activity, we conclude that under the conditions described, SDS almost totally denatures the enzyme. The significant loss of α-glucosidase activity after incubation with the chelator EDTA suggests that metal ions may be important in enzyme stability and/or activity.

Thermostability of α-glucosidase. Purified α-glucosidase from *P. furiosus* was incubated at 98°C, pH 6.5, portions were taken at various time points, and remaining activity was determined (Fig. 5). This enzyme exhibited extreme thermostability, with a half-life of about 46 to 48 h under these conditions. Thus, as well as exhibiting an extremely high temperature optimum, the *P. furiosus* α-glucosidase is the most thermostable α-glucosidase reported to date.

In general, α-glucosidases are only moderately thermostable (12). For example, the extracellular α-glucosidase from *Bacillus thermoglucosidus* KP 1006 (18), although stable at 60°C for 2 h with no loss of activity, was highly unstable at temperatures above 72°C. At temperatures above this, the enzyme exhibited a half-life of only 10 min or less. Similarly, the exo-α-1,4-glucosidase enzyme from *B. stearothermophilus* ATCC 12016 (17), which was optimally active at 70°C, retained only 7% of initial activity when incubated for 10 min at this temperature. The α-glucosidase from *B. caldovelox* DSM411 (8) demonstrated greater thermostability. This enzyme demonstrated a half-life of about 1 h at 70°C.

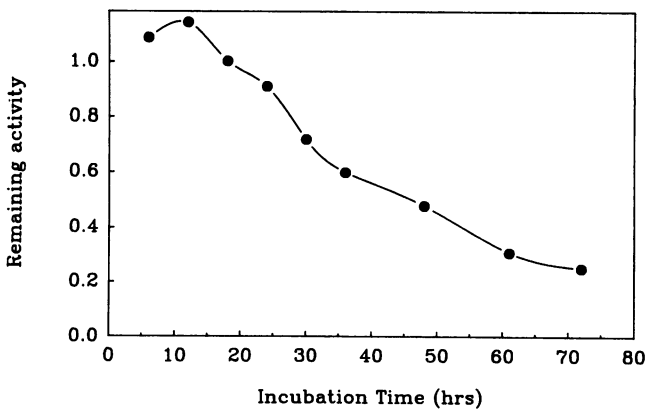

FIG. 5. Thermostability of *P. furiosus* α-glucosidase. Purified enzyme was incubated at 98°C for various time intervals and then assayed at 108°C as described in Materials and Methods.

P. furiosus α-glucosidase exhibits remarkable thermostability and considerable stability in the presence of various denaturing agents compared with other enzymes in this class. It is clear that *P. furiosus* α-glucosidase has some unique properties responsible for its remarkable thermostability. These factors remain unknown and will be the focus of future studies of this enzyme and other enzymes from hyperthermophilic organisms.

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