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Purification and Characterization of a Maltase from the Extremely Thermophilic Crenarchaeote *Sulfolobus solfataricus*

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A soluble maltase (α-glucosidase) with an apparent subunit mass of 80 kDa was purified to homogeneity from *Sulfolobus solfataricus*. The enzyme liberates glucose from maltose and malto-oligomers. Maximal activity was observed at 105°C, with half-lives of 11 h (85°C), 3.0 h (95°C), and 2.75 h (100°C). The enzyme was generally resistant to proteolysis and denaturants including aliphatic alcohols. *n*-Propanol treatment at 85°C increased both K_m and V_{max} for maltose hydrolysis.

Sulfolobus solfataricus (5) is an aerobic, extremely thermophilic microorganism found in acidic hotsprings. It has been assigned to the subgroup Crenarchaeota within the domain Archaea by 16S rDNA sequence analysis (17). *S. solfataricus* has the ability to grow lithoautotrophically utilizing sulfur as an energy source or chemoheterotrophically on a variety of sugars and polysaccharides, including starch, as sole carbon and energy sources (2, 5, 7). Starch catabolism frequently depends upon a secreted α-amylase which generates linear maltodextrins from starch as well as a cell-associated α-glucosidase (maltase) which converts maltose and maltodextrins to glucose (11). Extremely thermophilic α-amylases have been identified in members of the obligately anaerobic sulfur-reducing genus *Pyrococcus* (3, 13, 15), which is classified within the euryarchaeotal branch of the domain Archaea. A monomeric 125-kDa α-glucosidase has also been identified in this group (4, 12). Several lines of evidence suggested that an α-glucosidase was similarly present in the crenarchaeote *S. solfactaricus*. These included the ability of the organism to utilize maltose as the sole carbon and energy source (7) and the presence of a *p*-nitrophenyl-α-D-glucopyranoside (PNPG) hydrolytic activity in crude cell extracts (1).

***S. solfataricus* 98/2.** *S. solfataricus* 98/2 was obtained from L. Hochstein (10). Cells were cultured at 80°C as described previously (2) at pH 3.0 with maltose at 0.2% (wt/vol) as the sole carbon and energy source. Cultures were grown in 500-ml volumes and were harvested in late exponential phase, which was equivalent to a cell density of 10^9 cells per ml and an A_{540} of 1.0. Cells were recovered by centrifugation at 4°C, and the resulting cell pellet was frozen at −20°C. Species identification was determined by rDNA sequence analysis (9). Such information was important in light of the confusion regarding *Sulfolobus* isolates (14, 18). DNA sequence analysis of a 487-bp 16S rDNA sequence indicated that strain 98/2 shared 100% homology with *S. solfataricus* P2, 98.8% homology with *S. shibatae* B12 (6), and 87.6% homology with *S. acidocaldarius* (9, 14, 18). In addition, *S. solfataricus* P2 and 98/2 utilize a range of sugars, including maltose, mannose, and trehalose, which cannot be utilized by *S. acidocaldarius*, as well as cellobiose, which is used either poorly or not at all by either *S. acidocaldarius* or *S. shibatae* (7).

Purification of the *S. solfataricus* maltase. The colorimetric maltose analog PNPG was used as a substrate to monitor maltase activity during the purification procedure. All manipulations were performed at 4°C. Protein concentrations were determined by the bicinchoninic acid assay (Pierce Inc.), and bovine serum albumin was used as a protein standard. Three grams of packed cells was resuspended in 30 mM morpholine propanesulfonic acid (MOPS; pH 8.0) (M buffer), lysed by sonication, and clarified by centrifugation at 200,000 × *g* for 30 min. Forty-milligram quantities of the resulting supernatant were then applied to a Mono Q column (HR5/5; Pharmacia), and the column was developed by using a linear gradient of 50 to 400 mM sodium chloride in M buffer. PNPG hydrolytic activity resolved into a single peak, and fractions containing PNPG hydrolytic activity were pooled and dialyzed into 50 mM sodium phosphate buffer (pH 7.0) containing 1 M ammonium sulfate (S buffer). This material was then concentrated by ultrafiltration and applied in 5-mg amounts to a phenyl-Superose (HR 5/5; Pharmacia) column. The column was developed with a decreasing linear gradient of 1 M to 100 mM ammonium sulfate in S buffer and an increasing linear gradient of 0 to 25% (vol/vol) ethylene glycol. PNPG hydrolytic activity resolved into two peaks, and the major peak (60% of the total activity) was collected, dialyzed into 100 mM sodium phosphate (pH 6.0) buffer (SP buffer), and concentrated by ultrafiltration. Three-milligram quantities of this material were applied to a set of gel filtration columns including a Superdex 75 HR 10/30 (Pharmacia) column and two Superose 12 10/30 (Pharmacia) columns connected in series and developed with SP buffer. The activity eluted as a single peak (Fig. 1). Gel filtration chromatography of the purified enzyme indicated that it had a native molecular mass of approximately 400 kDa. A summary of the purification procedure is presented in Table 1. The soluble PNPG hydrolytic activity was purified 268-fold over the level observed in crude cell extracts. All active fractions were then examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reducing conditions (16), using unstained molecular mass (Bio-Rad) markers (Fig. 2). Prior to electrophoresis, samples were adjusted to 2% (wt/vol) SDS and 3 mM β-mercaptoethanol and boiled for 30 min (Fig. 2). SDS-polyacrylamide gels were stained with Coomassie blue R250 to visualize protein. Twenty micrograms of protein was loaded in lanes 1 to 4, and 5 μg of protein was loaded in lanes 5 and 6. In the absence of the added denaturation step, the enzyme did not enter the 12.5% (wt/vol) acrylamide separating gel and instead remained in the 8% (wt/vol) acrylamide stacking gel (Fig. 2, lane 5, upper arrowhead). To achieve complete protein denaturation, samples of the purified protein were treated with

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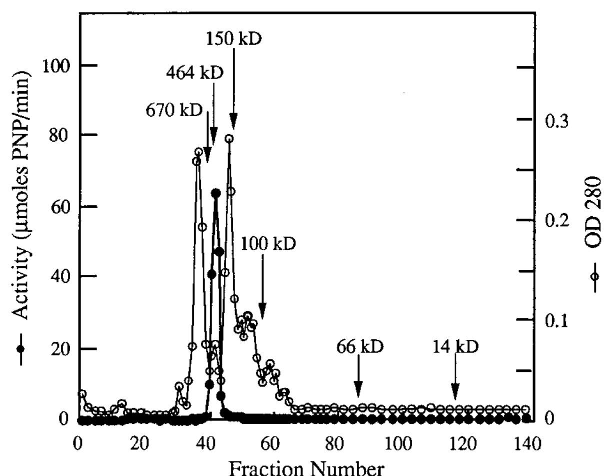

FIG. 1. Gel filtration of the *S. solfataricus* maltase. Shown is the elution profile of PNPG hydrolytic activity on a fast protein liquid chromatography gel filtration column series. Protein standards used to estimate molecular mass were porcine thyroglobulin (670 kDa), *Escherichia coli* β-galactosidase (464 kDa), dog immunoglobulin G (150 kDa), bovine serum albumin (66 kDa), porcine heart citrate synthase (100 kDa), and hen egg white lysozyme (14 kDa). PNP, *p*-nitrophenol; OD 280, optical density at 280 nm.

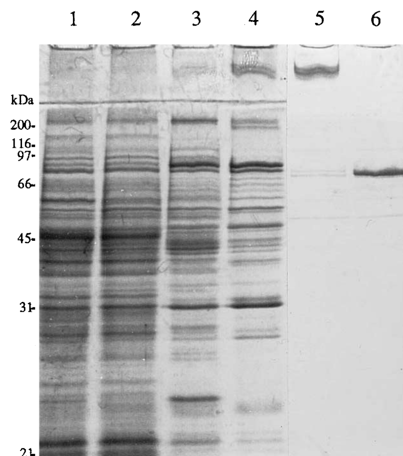

FIG. 2. SDS-PAGE of purified fractions. The samples were crude cell extract (lane 1), S-200 fraction (lane 2), Mono Q fraction (lane 3), phenyl-superose fraction (lane 4), gel filtration fraction (lane 5), and gel filtration fraction with guanidine hydrochloride pretreatment (lane 6).

6 M guanidine hydrochloride prior to electrophoresis as described previously (8) (Fig. 2, lane 6, lower arrowhead). The apparent subunit mass of the purified enzyme was 80 kDa, indicating that the native form consists of four to five identical subunits.

Enzyme substrate specificity. Routine assays for the hydrolysis of PNPG, maltose, or other glycosides were performed with 10 mM substrates in 100 mM sodium acetate (pH 4.5) buffer (SA buffer) at 85°C. Reactions were initiated by the addition of enzyme to prewarmed solutions and terminated by addition of 1 M sodium carbonate, resulting in a sample pH of 10.0 for PNPG, or, for maltose or other glycosides, by transfer to 4°C. The extent of PNPG hydrolysis was determined by the A_{420} of the sample, and the results were corrected for spontaneous substrate hydrolysis. The relationship between the amount of *p*-nitrophenol released and the absorbance of the sample was linear between absorbances of 0.25 to 1.0. Enzyme concentrations were adjusted to satisfy this requirement. The molar extinction coefficient, a_m, of *p*-nitrophenol at a pH of 10.0 was 20,000 M^{-1} cm^{-1}. All samples were assayed in duplicate, and the averages of the sample results are shown. The extent of hydrolysis of maltose or other glycosides was determined by measuring the levels of free glucose by using a glucose oxidase assay kit (model 510-A; Sigma) as described by the manufacturer. The concentration of free glucose was determined by the A_{450} of the sample and comparison with a glucose standard curve prepared in SA buffer. A glucose standard curve was performed with all assays and included standards ranging in amount from 0.5 to 20 μg of glucose per sample. A unit of maltase activity is defined as the amount of enzyme required to produce 1 μmol of glucose per min per mg of protein.

The purified enzyme had greatest preference for maltose (Table 2). K_m and V_{max} values for PNPG, which was used in the purification of the enzyme, were 3.2 mM and 2.3 μmol/min, respectively. In contrast, the K_m for maltose was 0.91 mM and the V_{max} for maltose was 29.8 μmol/min. Analysis of the activity of the enzyme toward a range of substrates indicated a preference for short maltosaccharides. Significant activity was detected toward dextrin but not against starch. No activity was observed against the β-linked glucoside *p*-nitrophenyl-β-D-glucopyranoside, indicating enzyme preference for α-linked glycosides. The apparent activity against PNPG indicates a lack of discrimination for glycosyl moieties in disaccharide substrates. Hydrolysis of the α-1,6-linked substrate, isomaltose, but not the α-1,6-linked polymer, dextran, was also observed and indicated the enzyme was tolerant of only small α-1,6-linked substrates. These experiments support the identification of the enzyme as a maltase (EC 3.2.1.20) with the accompanying substrate utilization profile of an α-glucosidase.

TABLE 1. Purification of the *S. solfataricus* maltase

Step	Protein (mg)	Activity (U)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Cell extract	634.9	28,600	45.0		
S-200	277.3	18,471	66.6	1.5	64.6
Mono Q	31.79	13,720	431.6	9.6	48.0
Phenyl-Superose	3.26	2,859	877.0	19.5	10.0
Gel filtration	0.102	1,233	12,088	268.6	4.3

TABLE 2. Substrate specificity of the *S. solfataricus* maltase

Substrate[a]	Sp act (μmol of glucose/min/mg)
Maltose	3.32 ± 0.46
Maltotriose	1.77 ± 0.26
Maltotetrose	1.91 ± 0.61
Maltoheptose	1.48 ± 0.00
Dextrin	0.85 ± 0.04
Starch	<0.01
PNPG	1.01 ± 0.21
Methyl-α-D-glucopyranoside	0.01 ± 0.00
Isomaltose	0.25 ± 0.01
Dextran	<0.01
p-Nitrophenyl-β-D-glucopyranoside	<0.01

[a] Substrate concentrations were 10 mM except for those of starch and dextran, which were 1.0% (wt/vol).

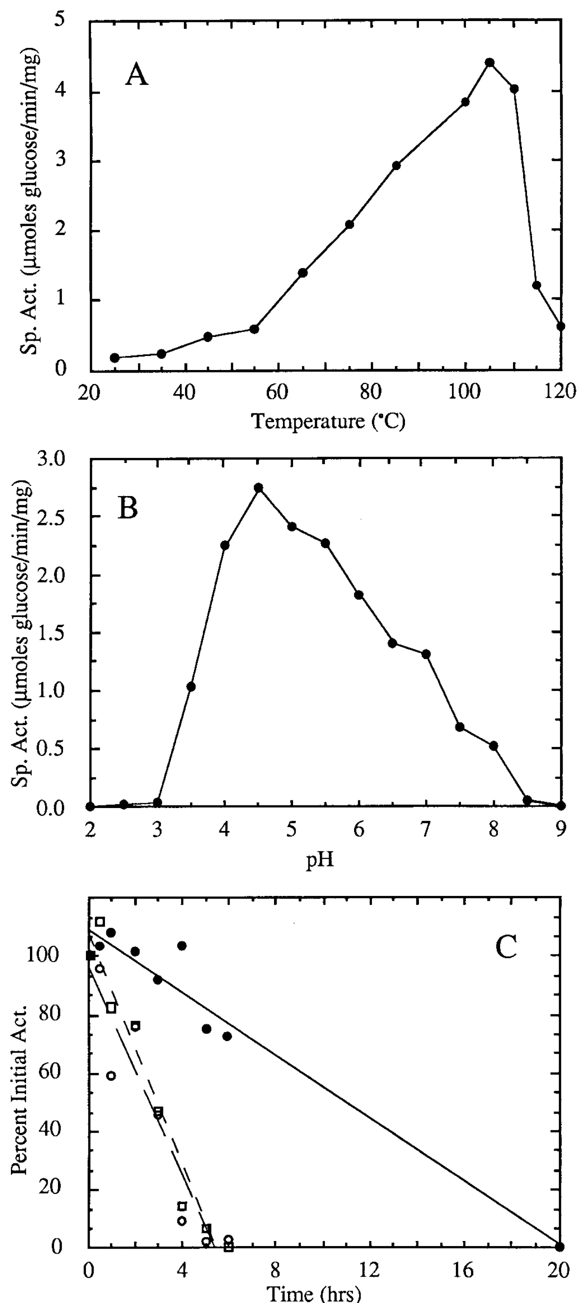

FIG. 3. Physical characterization of the purified maltase. (A) Temperature optimum for maltose hydrolysis. (B) pH optimum for maltose hydrolysis at 85°C. One hundred millimolar sodium acetate was used for buffers ranging from pH 2.0 to 5.0, and 100 mM sodium phosphate was used for buffers ranging from pH 5.5 to 9.0. (C) Thermostability of the maltase. The temperatures examined were 85°C (closed circles), 95°C (open squares), and 100°C (open circles).

Thermotolerance and proteolytic insensitivity. The temperature optimum, pH optimum, and thermostability were determined by assaying the extent of maltose hydrolysis at enzyme concentrations of 2.1, 5, and 52 µg/ml, respectively, in sealed tubes. The enzyme exhibited a temperature optimum of 105°C (Fig. 3A). The pH optimum of the purified enzyme at 85°C was 4.5, with 50% activity remaining over the ranges of pH 3.5 to 6.5 (Fig. 3B). The half-life of the purified enzyme was examined at several temperatures. The half-lives at 100, 95, and 85°C were found to be 2.75, 3.0, and 11 h, respectively (Fig. 3C).

The stability of the enzyme toward proteolytic digestion was also examined. Analysis of the native enzyme by SDS-PAGE produced a single band which migrated only partially through the 8% (wt/vol) acrylamide stacking gel (Fig. 2). Proteolytic sensitivity was monitored by the response of this material to protease pretreatment. Treatment of the native enzyme with trypsin (12.5 U) or chymotrypsin (1.12 U) at 30°C or proteinase K (0.5 U) at 30 or 55°C for 20 min in 40 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.6)–8 mM magnesium acetate–0.3 mM EDTA–2 mM dithiothreitol–10 mM sodium chloride–20 mM potassium chloride had no effect on the enzyme. However, proteolytic treatment of chemically denatured maltase generated by guanidine hydrochloride pretreatment resulted in complete maltase degradation by all three of the proteases examined.

Aliphatic alcohol treatment. Aliphatic alcohols were used as model solvents to examine how increased solvent hydrophobicity might influence enzyme stability. The maltase was suspended in SA buffer and treated with either methanol, ethanol, or *n*-propanol at a concentration of 25 or 50% (vol/vol). Samples were removed after 1, 5, 10, and 20 min and assayed for glucose content. Following 20 min of exposure, no significant reduction in maltase activity was observed relative to an untreated control under any conditions tested. Surprisingly, several of the conditions studied led to enhanced maltase activity. After 20 min, both *n*-propanol (50%) and ethanol (25%) increased maltase activity by over 50%. To better understand the mechanism of this stimulatory effect, the kinetic consequences of *n*-propanol exposure were examined at 85°C. Maximal *n*-propanol stimulation was observed at a concentration of 7.5% (vol/vol), and kinetic parameters were determined by incubating samples for 1, 3, 5, 10, 20, and 30 min at substrate concentrations of 1.5, 2, 5, 10, 12, and 15 mM maltose in duplicate. *n*-Propanol treatment resulted in a 3-fold increase in K_m, from 911 to 2,770 µM, and a 1.6-fold increase in V_{max}, from 29.8 to 47.6 µmol/min.

Summary. The purified soluble *S. solfataricus* maltase shares the substrate specificity of analogous enzymes from both eubacterial and eukaryotic sources but appears to be structurally unrelated to these enzymes (11). In addition, the *S. solfataricus* enzyme is physically distinct from several euryarchaeotal enzymes with similar specificities (4, 12). The *S. solfataricus* maltase exhibited a temperature optimum of 105°C, 25°C above the cultivation temperature of the organism. This is the highest temperature optimum yet reported for an enzyme from the family *Sulfolobaceae*. Several additional characteristics observed for the *S. solfataricus* enzyme indicate an unusual degree of stability.

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