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Characterization of Amylolytic Enzyme Activities Associated with the Hyperthermophilic Archaebacterium *Pyrococcus furiosus*

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The hyperthermophilic archaebacterium *Pyrococcus furiosus* produces several amylolytic enzymes in response to the presence of complex carbohydrates in the growth medium. These enzyme activities, α-glucosidase, pullulanase, and α-amylase, were detected in both cell extracts and culture supernatants. All activities were characterized by temperature optima of at least 100°C as well as a high degree of thermostability. The existence of this collection of activities in *P. furiosus* suggests that polysaccharide availability in its growth environment is a significant aspect of the niche from which it was isolated.

As the collection of microorganisms isolated from high-temperature environments expands, the relationship between the metabolic characteristics of these organisms and the niches from which they were isolated should be examined. This should help establish a basis for understanding the ecological role of these microorganisms in a particular extreme environment and may also provide insight into the biological relationships among apparently dissimilar high-temperature systems. Questions concerning the significance of pressure or salinity or a number of other distinguishing features of high-temperature locations can be addressed as the metabolisms of the associated extremely thermophilic and hyperthermophilic microorganisms are better understood.

The term hyperthermophile has been used to refer to a small but growing number of archaebacteria with optimum growth temperatures at or above 100°C (13, 17, 41, 42). Although none of the organisms in this group has been studied in great detail, a survey of the literature suggests that one, *Pyrococcus furiosus*, has received the most attention. *P. furiosus*, isolated from shallow thermal waters near Vulcano Island, Italy (13), is an obligate anaerobe that grows heterotrophically on complex media with or without elemental sulfur. In the absence of sulfur, H_2 and CO_2 are produced by a fermentative-type metabolism, and cell growth is inhibited as the levels of H_2 increase. When sulfur is present, growth inhibition is relieved, with the production of H_2S instead of H_2. The optimal growth temperature of this organism is 98 to 100°C, and maximal cell densities are about 1×10^8 to 3×10^8 cells per ml. Some preliminary characterization of the metabolism of *P. furiosus* has been reported (8, 28; I. I. Blumentals, S. H. Brown, R. N. Schicho, A. K. Skaja, H. R. Costantino, and R. M. Kelly, Ann. N.Y. Acad. Sci., in press), and the purification of several proteins involved in electron transport has been described (3, 9).

Along with several other heterotrophs isolated from high-temperature environments, *P. furiosus* has been shown to grow in the presence of starch and other carbohydrates (18, 40, 42). This suggests that a variety of amylolytic enzymes may be important in the metabolism of the organism and that these substrates, their precursors, or related polysaccharides are available in the natural environment of the organism. In any case, by characterizing those amylolytic activities responsible for carbohydrate utilization by *P. furiosus* or other hyperthermophilic heterotrophs, a basis of comparison for these organisms should result. Also, by examining the distribution of potential amylolytic substrates in a given high-temperature niche, the chances of isolating a particular type of organism can be assessed.

MATERIALS AND METHODS

Culture protocols. *P. furiosus* DSM 3638 was obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Federal Republic of Germany. The organism was grown in artificial seawater supplemented with 0.1% yeast extract and 0.5% tryptone (Difco Laboratories, Detroit, Mich.). The artificial seawater was a modification of that described by Kester et al. (22) and was formulated as follows: solution A—23.9 g of NaCl, 10.8 g of $MgCl_2 \cdot 6H_2O$, 4.0 g of Na_2SO_4, 1.5 g of $CaCl_2 \cdot 2H_2O$, 0.7 g of KCl, 0.2 g of $NaHCO_3$, 0.1 g of KBr, 0.03 g of H_3BO_3, and 0.025 g of $SrCl_2 \cdot 6H_2O$ per liter; solution B—25.0 g of NH_4Cl and 14.0 g of K_2HPO_4. Yeast extract and tryptone were added to solution A, and both solutions were sterilized separately by autoclaving. After the solutions were cooled, 10 ml of solution B was added to 1 liter of solution A. Carbohydrates were added at a concentration of 0.5%. Starch, glycogen, and pullulan were added to solution A before autoclaving, whereas glucose and maltose were sterilized separately.

The culture medium prepared as described above was dispensed into 1-liter Wheaton glass bottles, and 10 g of elemental sulfur per liter was added along with 1.0 mg of resazurin per liter. The bottles were placed in a temperature bath containing silicone oil (Dow Corning Corp., Midland, Mich.) at 98°C and heated for 1 h. Anaerobic conditions were achieved by sparging with helium for 5 min, followed by the addition of 0.5 g of Na_2S per liter. Approximately 20 ml of a late-log-phase culture (containing no carbohydrate) was added to each bottle, the caps were tightly closed, and the cultures were maintained under quiescent conditions at 98°C for ~12 h. Cells were harvested as described below.

Preparation of enzyme samples. Culture broth was filtered through Whatman no. 1 filter paper to remove elemental sulfur, and the cell pellet was recovered by centrifugation at about 12,000 × *g* for 30 min at 4°C. This pellet was suspended in 0.1 M sodium phosphate buffer (pH 7.0) at a ratio of 0.5 ml of buffer per liter of original culture and sonicated six times for 30 s each time at 50% duty cycle using

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a Tekmar sonic disruptor model TM300. The sonically treated cells were centrifuged at about 18,000 × *g* for 30 min at 4°C to remove the remaining sulfur and cell debris (which had no enzyme activity). The supernatant from this step was dialyzed for ~18 h at 4°C against 0.1 M sodium phosphate buffer (pH 7.0) containing 0.01% sodium azide. This material was used as a crude enzyme preparation and is referred to as cell extract.

Supernatant from the original centrifugation was concentrated from 1 liter to about 5 ml by use of, in succession, a Minitan ultrafiltration unit (Millipore Corp., Bedford, Mass.) with a 10,000-molecular-weight-cut-off polysulfone membrane and a Novacell stirred cell with a 10,000-molecular-weight-cut-off membrane. These samples were dialyzed for ~18 h at 4°C against 0.05 M *N*-(2-hydroxyethyl)-piperazine-*N'*-(3-propanesulfonic acid) (EPPS) buffer (pH 7.0) containing 0.01% sodium azide. EPPS buffer was used in this case instead of phosphate to prevent precipitation of salts from the media. This material was also examined for enzyme activity and is referred to as supernatant.

Detection of enzyme activity. Amylase and pullulanase activities were determined by measuring the amount of reducing sugar released during incubation with starch and pullulan, respectively. Soluble starch (2%) was autoclaved for 10 min prior to use in enzyme assays. A 0.5-ml volume of 2% soluble starch or 2% pullulan was added to 0.5 ml of 0.1 M sodium acetate-acetic acid buffer (pH 5.6). A portion (10 to 100 μl) of enzyme solution was added, and the samples were incubated at 98°C for 30 min. The reaction was stopped by cooling on ice, and the amount of reducing sugar released was determined by using the dinitrosalicylic acid method (5). Sample blanks were used to correct for nonenzymatic release of reducing sugar. One unit of enzyme activity was defined as the amount of enzyme that released 1 nmol of reducing sugar (as glucose standard) per min under the assay conditions specified. Enzyme samples were diluted appropriately to maintain linearity over the assay interval used.

α-Glucosidase activity was determined by using *p*-nitrophenyl-α-D-glucopyranoside (PNPG) as the substrate. Spectrophotometric readings were taken with a Lambda 3 spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) with a thermostated six-cell transport. A water-circulating temperature bath (VWR Scientific model 1130) containing a 1:1 mixture of ethylene glycol and water was used to maintain the desired temperature in the cell holder. This temperature was monitored with a thermocouple mounted in a cuvette (containing the glycol-water mixture) that was placed in the cell transport. For temperatures over 100°C, cuvettes were sealed with rubber septa to prevent boiling and loss of sample. These septa could be used at temperatures up to 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 for α-glucosidase were conducted as follows. For each assay, 1-ml portions of substrate consisting of 10 mM PNPG in 0.1 M sodium phosphate buffer (pH 5.5) were pipetted into quartz cuvettes. The cuvettes were inserted into the cell holder, which was heated to 98°C, and preincubated for at least 10 min to allow the substrate to reach assay temperature. After the preincubation, 1 to 50 μl of sample was added to the cuvettes and the evolution of *p*-nitrophenol (PNP) was followed by monitoring the optical density at 405 nm. A blank containing no sample was also monitored and used to correct for nonenzymatic release of PNP. One unit of α-glucosidase activity was defined as the amount of enzyme releasing 1 nmol of PNP per min under the specified assay conditions. Since the molar absorbancy index of PNP varies with both temperature and pH, this value was determined experimentally for each reaction condition of pH and temperature (data not shown).

To determine pH optima, the protocols above were used with the substitution of the appropriate buffer. For pH values of 4.0 to 5.6, 0.1 M sodium acetate-acetic acid buffer was used, whereas, for pH values from 5.7 to 8.0, 0.1 M sodium phosphate buffer was used. The pH values of buffers were adjusted to the desired value at room temperature, and these values were found to be reasonably stable upon heating. Temperature optima were determined by performing the appropriate assay at the temperature indicated. The effect of temperature on the thermostability was observed in samples of cell extract (pH 7.0) incubated at 85, 98, and 105°C. The protein content of enzyme samples was determined by use of the bicinchoninic acid system (procedure no. TPRO-562; Sigma Chemical Co., St. Louis, Mo.), with bovine serum albumin as standard.

Characterization of hydrolysis products. The pattern of polysaccharide hydrolysis by amylolytic enzymes from *P. furiosus* was examined by using thin-layer chromatography. The initial products released from starch, pullulan, glycogen, and dextran were characterized by adding 200 μl of cell extract to 1 ml of solution containing 2% of each substrate in 0.1 M acetate buffer (pH 5.6). The mixtures were incubated at 98°C for 40 min and quenched on ice. The time course of starch hydrolysis was examined by adding 2 ml of cell extract to 5 ml of 2% soluble starch in 0.1 M acetate buffer (pH 5.6). This reaction mixture was incubated at 90°C, and samples were withdrawn periodically and quenched on ice. Samples were analyzed by using the method of Hansen (16).

Gel electrophoresis. Samples of supernatant from cultures grown on starch and maltose were pooled and concentrated fivefold by using Ultracent-10 ultrafilters (Bio-Rad Laboratories, Richmond, Calif.). Proteins were separated by native polyacrylamide gel electrophoresis by use of the discontinuous system of Laemmli (26) with the elimination of sodium dodecyl sulfate. Bands corresponding to amylase and pullulanase activities were visualized by use of substrate overlay gels containing starch azure and Remazol brilliant blue (RBB)-derivatized pullulan, respectively. RBB-derivatized pullulan was prepared by using the method of Rinderknecht et al. (36). Substrate gels were formulated with 10% acrylamide, plus either 1.5% starch azure or 2% RBB-derivatized pullulan, in 0.05 M acetate buffer (pH 5.6). After electrophoresis of protein samples, substrate gels were placed on top of the native gel and incubated in 0.05 M acetate buffer (pH 5.6) at 75°C until clear zones appeared. α-Glucosidase activity from a sample of cell extract was detected by incubating the native gel in 0.05 M phosphate buffer (pH 5.5) containing 20 mM PNPG at 75°C until a yellow band was visible.

Effect of inhibitors. The effect of EDTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), α-cyclodextrin, and β-cyclodextrin on enzyme activity was examined. Samples of cell extract containing the desired amount of inhibitor were preincubated at 75°C for 15 min. These samples were cooled on ice and assayed for enzyme activity as described above, with the addition of inhibitor at the specified level to the assay mixture.

Chemicals. Soluble starch, starch azure, pullulan, D-glucose, maltose, glycogen, RBB, PNPG, PNP, α- and β-cyclodextrin, and dextran were obtained from Sigma. Elemental sulfur was a product of J. T. Baker Chemical Co., Phillipsburg, N.J. Tryptone and yeast extract were Difco products. All other chemicals were of reagent grade.

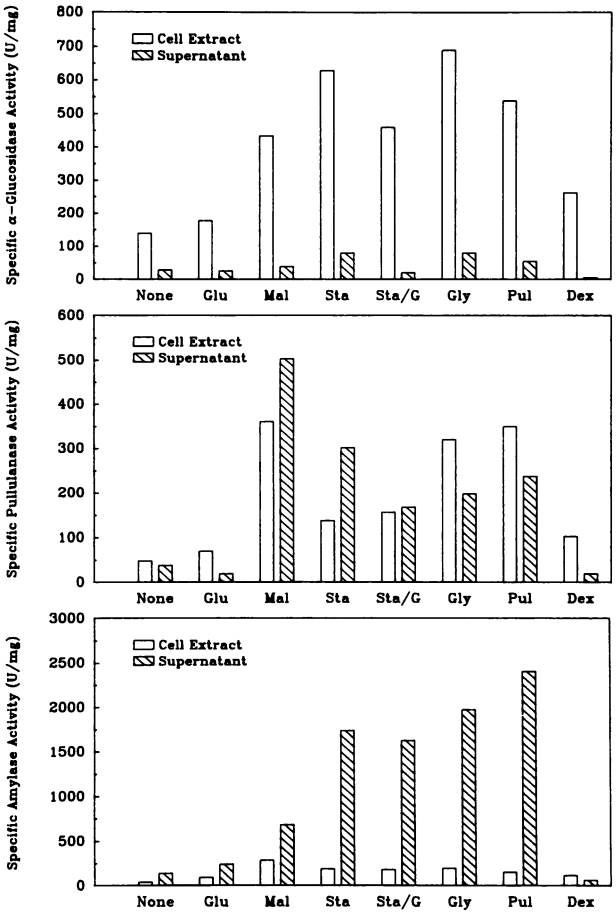

FIG. 1. Effect of added carbohydrate on amylolytic enzyme production by *P. furiosus*. Activities were determined by using crude enzyme preparations. None, No added carbohydrate; Glu, glucose; Mal, maltose; Sta, starch; Sta/G, starch plus glucose; Gly, glycogen; Pul, pullulan; Dex, dextran.

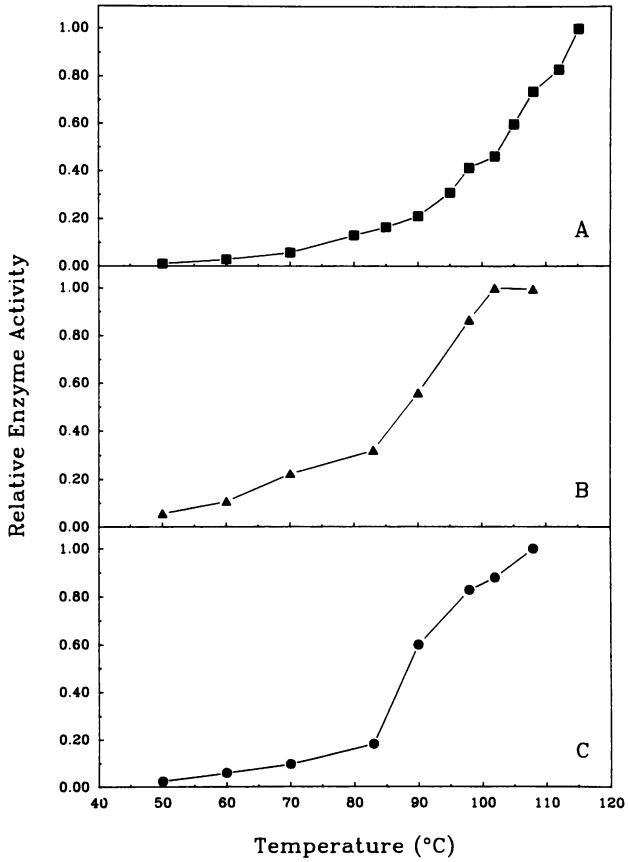

FIG. 2. Activity versus temperature for amylolytic enzymes from *P. furiosus* (cell extract). A, α-Glucosidase; B, pullulanase; C, α-amylase.

RESULTS

Dependence of enzyme production on carbohydrate. Figure 1 shows the intracellular and extracellular levels of α-glucosidase, pullulanase, and α-amylase produced by *P. furiosus* grown in the presence of several simple and complex carbohydrates. Low constitutive levels of these enzymes were observed in the absence of added carbohydrate. Saccharides with α-1,4 linkages stimulated production of all three enzymes, and enzyme expression was not fully repressed when glucose was present along with starch. The α-glucosidase was primarily an intracellular enzyme activity, and the α-amylase was primarily extracellular, while the distribution of the pullulanase was somewhat more even. The maximum yield of each activity was as follows: α-glucosidase, 1.1×10^4 U per liter (glycogen); pullulanase, 1.2×10^4 U per liter (maltose); and α-amylase, 1.0×10^5 U per liter (pullulan). Further work will be necessary to determine whether each enzyme is synthesized (and secreted) during the growth or stationary phase as well as the effect of culture conditions (pH, substrate level, etc.) on enzyme production. The presence or absence of sulfur did not appear to affect enzyme synthesis (data not shown).

Enzyme properties. The effect of temperature on the amylolytic enzyme activities is shown in Fig. 2. Each of these enzyme activities had a temperature optimum of over 100°C, representing the highest temperature optimum reported for any enzyme. The pH optimum for α-glucosidase activity was about 5.0, while both the pullulanase and α-amylase had broad optima around pH 5.6 (data not shown).

The influence of incubation temperature on the thermal stability of amylolytic enzyme activities from *P. furiosus* is illustrated in Fig. 3. All three activities exhibited remarkable thermal stability. For example, the α-glucosidase had a half-life ($t_{1/2}$) of more than 24 h at 85°C, about 10 h at 98°C, and approximately 1 h at 105°C. The α-amylase, which appears to be the least thermostable of the three enzymes, was still able to maintain approximately 30% of its activity after 8 h at 98°C.

Two points should be noted here. First, the purified α-glucosidase from *P. furiosus* has been shown to exhibit a $t_{1/2}$ of more than 40 h at 98°C (12). Second, cell extracts of *P. furiosus* contain significant levels of thermally stable protease activity (6). Therefore, it appears that a significant portion of the activity lost from these three enzymes at elevated temperatures is due to proteolysis. Presumably, increased thermostability would be observed in preparations with reduced protease activity. Since all of these incubations were carried out in the absence of substrate, it is also likely that increased thermostability would result from the addition of substrate.

Hydrolysis products. The hydrolysis products resulting

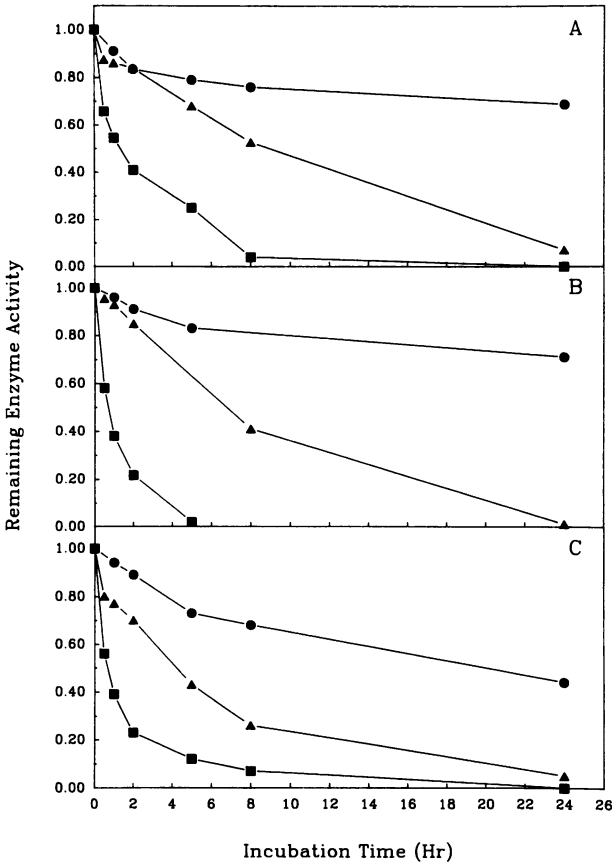

FIG. 3. Thermostability of amylolytic enzymes from *P. furiosus*. Samples of cell extract were incubated in 0.1 M sodium phosphate buffer (pH 7.0) and assayed for activity at the indicated intervals. A, α-Glucosidase; B, pullulanase; C, α-amylase. Symbols: ●, 85°C; ▲, 98°C; ■, 105°C.

from the action of a crude enzyme mixture on various polysaccharides are shown in Fig. 4. Polysaccharides down to the monosaccharide glucose, as well as unresolved dextrins, were produced from starch, pullulan, and glycogen. The presence of maltose and glucose in these three samples revealed that one of these enzymes, most likely α-glucosidase, was able to hydrolyze maltotriose. The relatively low amount of maltose visible in the starch sample and the appearance of maltotriose implied that the amylase activity was of the α type. A trace amount of glucose was detected in the dextran sample; this may be the result of abiotic hydrolysis. The crude enzyme mixture also readily hydrolyzed amylose azure and starch azure (data not shown).

Figure 5 illustrates the products formed during prolonged hydrolysis of starch. The shift to products of lower molecular weight as the reaction progressed was readily apparent, and starch degradation approached 100% after 48 h for the enzyme loadings used. Similar results were obtained when the hydrolysis was carried out with samples of supernatant instead of cell extract (data not shown).

Overlay gel electrophoresis. One band with amylase activity was detected (band A) whereas three bands of pullulanase activity were visible (P_1, P_2, and P_3) by overlay gel electrophoresis (Fig. 6). The correspondence between bands A and P_2 may indicate that the amylase has hydrolytic activity towards pullulan. It is not clear if band P_1 represents another enzyme with pullulanase activity or possibly some

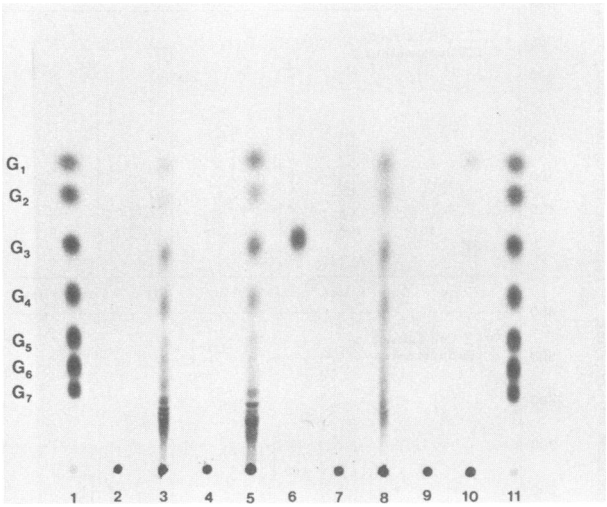

FIG. 4. Products resulting from the action of a crude *P. furiosus* amylolytic enzyme mixture on various polysaccharides at 98°C. Lanes: 2 and 3, starch (without and with enzyme, respectively); 4 and 5, pullulan; 6, maltotriose standard; 7 and 8, glycogen; 9 and 10, dextran; 1 and 11, 0.1% sugar standards (glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose [G_1 to G_7, respectively]). Cell extract was added to give (per ml) 356 U of amylase, 474 U of pullulanase, and 924 U of α-glucosidase in 2% substrate.

sort of aggregate involving band P_2 and/or P_3. Aggregation phenomena associated with enzymes from hyperthermophiles have been reported previously (3). Although the α-glucosidase activity as visualized by activity stain could not be photographed, its relative location was determined and is marked as band G (Fig. 6).

Effect of inhibitors. The effects of several compounds on the amylase and pullulanase activities from *P. furiosus* are listed in Table 1. EDTA and EGTA appear to have a

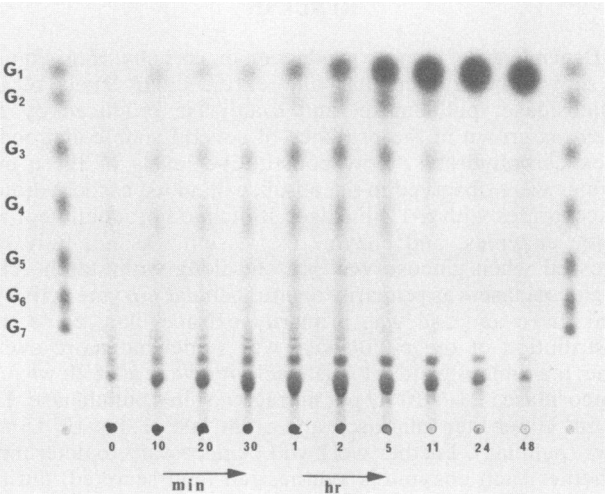

FIG. 5. Time course of starch hydrolysis by a cell extract of *P. furiosus*. Samples were incubated at 90°C for the time indicated. G_1 through G_7 are standards (see legend to Fig. 4). Enzyme concentrations were (per ml) 611 U of amylase, 813 U of pullulanase, and 1,584 U of α-glucosidase in 2% soluble starch.

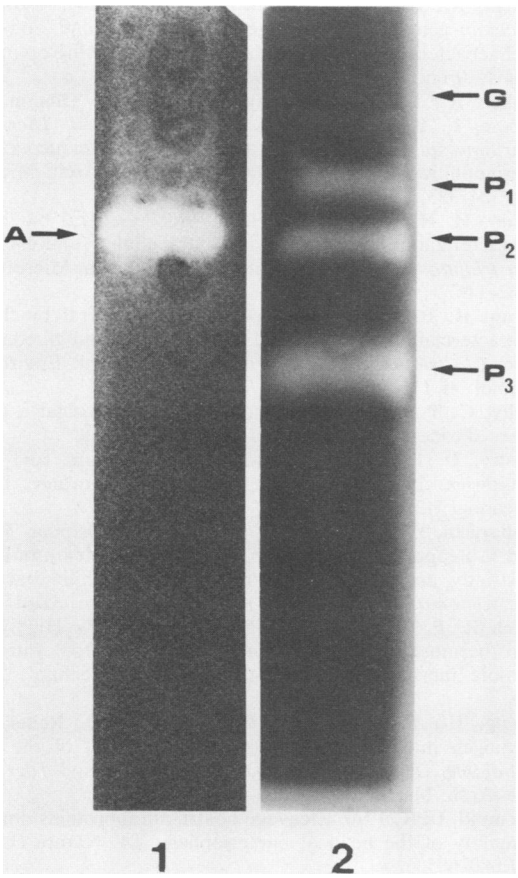

FIG. 6. Substrate overlay gel for detection of amylase and pullulanase activity. Substrate overlay gels were prepared with 10% polyacrylamide containing either 1.5% starch azure (lane 1) or 2% RBB-derivatized pullulan (lane 2). A, Amylase activity; P_1, P_2, and P_3, pullulanase activities. G shows the relative location of the α-glucosidase activity as visualized by activity stain by using PNPG. Lane 1 contained 4,307 U of amylase activity and 619 U of pullulanase activity, while lane 2 contained 5,743 U of amylase activity and 825 U of pullulanase activity.

significant negative effect on the amylase and pullulanase activities, and this effect was apparent at levels as low as 1 mM. Both α- and β-cyclodextrins have a strong inhibiting effect on the pullulanase activity. An increase in amylase activity was noted with an increase in the concentration of β-cyclodextrin. The reason for this increase is not clear. It is possible that β-cyclodextrin was hydrolyzed somewhat faster by the enzyme mixture than by soluble starch, leading to increased release of reducing sugar over the assay interval.

DISCUSSION

Numerous studies concerning the production and characteristics of thermostable amylolytic enzymes can be found in the literature (1, 2, 4, 7, 10, 14, 19, 20, 23, 24, 27, 29–32, 34, 35, 37–39). Comparisons of analogous enzymes with respect to temperature optima, and in particular thermostability, are difficult, since variations in assay conditions (presence of substrates and metal ions) and the use of crude rather than purified enzyme preparations are encountered. Within these limitations, some conclusions can be drawn. The most thermostable pullulanase previously reported appears to be

TABLE 1. Effect of EDTA, EGTA, and cyclodextrins on amylase and pullulanase activities from *P. furiosus*

Reagent added	Concn of reagent (mM)	% Activity remaining of:	
		Amylase	Pullulanase
None		100	100
EDTA	1	40	31
	5	38	33
	10	36	33
EGTA	1	52	36
	5	51	35
	10	51	32
α-Cyclodextrin	1	76	16
	5	67	13
	10	67	15
β-Cyclodextrin	1	73	16
	5	83	14
	10	92	18

that produced by *Thermus aquaticus* YT-1, with a temperature optimum of about 95°C in purified form and a $t_{1/2}$ of 4.5 h at this temperature (35). The amylase complex produced by *Dictyoglomus thermophilum* (23) contains at least one amylase with a $t_{1/2}$ of about 2 h at its optimum temperature of 90°C (14). α-Glucosidases are generally only moderately thermostable (21), and the most thermostable one reported to date appears to be that from *Bacillus caldovelox*, with a $t_{1/2}$ of about 1 h at 70°C (15).

The amylolytic enzymes produced by *P. furiosus* all have temperature optima of over 100°C and exhibit remarkable thermostability, even in crude extracts containing significant levels of thermostable protease activity. High levels of thermostability have also been observed with other enzymes from hyperthermophiles (3, 9, 33). It is probable that increased optimal reaction temperatures and increased thermostability will be observed in purified enzyme preparations and samples incubated in the presence of substrate, for example under conditions commonly encountered in the starch processing industry. Further evaluation with purified enzymes will be necessary to determine their substrate specificities and kinetic behavior, and the effect of metal ions and inhibitors on them.

Initial results indicate that these enzymes could be used to improve existing starch hydrolysis processes. The suitability of these enzymes for this application will likely depend on the potential for improving the cell and enzyme yields of *P. furiosus* through growth optimization studies. Another approach might involve cloning the genes for the amylolytic enzymes from *P. furiosus* into a host which is able to grow to higher densities and which therefore has the potential to produce higher amounts of enzyme. The success of this approach would be contingent on the resolution of issues relating to the expression of archaebacterial genes coding for thermophilic proteins in mesophilic or thermophilic eubacterial hosts.

This study points to the probable significance of polysaccharides in ecological systems such as the one *P. furiosus* was isolated from. Other microorganisms found in marine hydrothermal vent areas, such as *Thermotoga maritima* (18), have been shown to possess amylolytic enzyme activities (7). It is likely that the degradation of polysaccharides and the subsequent metabolism of maltose or other simple sugars play important roles in heterotrophy in high-temperature

niches. Potential sources of these compounds include other archaebacteria (25) as well as marine animals (11). However, it is less clear what precise function this nutritional characteristic represents, i.e., energy generation, carbon source, etc. Further studies in our laboratory of *P. furiosus* and other heterotrophs from high-temperature environments will address this issue.

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