Purification and Properties of Extracellular Amylase from the Hyperthermophilic Archaeon *Thermococcus profundus* DT5432

YOUNG CHUL CHUNG, TETSUO KOBAYASHI,* HARUHIKO KANAI,
TERUHIKO AKIBA, AND TOSHIKI KUDO

Laboratory of Microbiology, The Institute of Physical and Chemical Research (RIKEN),
2-1 Hirosawa, Wako, Saitama 351-01, Japan

Received 22 September 1994/Accepted 13 January 1995

A hyperthermophilic archaeon, *Thermococcus profundus* DT5432, produced extracellular thermostable amylases. One of the amylases (amylase S) was purified to homogeneity by ammonium sulfate precipitation, DEAE-Toyopearl chromatography, and gel filtration on Superdex 200HR. The molecular weight of the enzyme was estimated to be 42,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The amylase exhibited maximal activity at pH 5.5 to 6.0 and was stable in the range of pH 5.9 to 9.8. The optimum temperature for the activity was 80°C. Half-life of the enzyme was 3 h at 80°C and 15 min at 90°C. Thermostability of the enzyme was enhanced in the presence of 5 mM Ca^{2+} or 0.5% soluble starch at temperatures above 80°C. The enzyme activity was inhibited in the presence of 5 mM iodoacetic acid or 1 mM *N*-bromosuccinimide, suggesting that cysteine and tryptophan residues play an important role in the catalytic action. The amylase hydrolyzed soluble starch, amylose, amylopectin, and glycogen to produce maltose and maltotriose of α -configuration as the main products. Smaller amounts of larger maltooligosaccharides were also produced with a trace amount of glucose. Pullulan; α -, β -, and γ -cyclodextrins; maltose; and maltotriose were not hydrolyzed.

Hyperthermophilic bacteria and archaea which grow optimally above 80°C have generated considerable interest because of the hyperthermostability of their enzymes and their ancestral location in relation to the other extant organisms on the universal phylogenetic tree based on 16S-18S rRNA sequences (20). In industry, hyperthermophilic enzymes could be advantageous because of their resistance to denaturing agents, solvents, and proteolytic enzymes in addition to their extreme thermostability (2).

The best-characterized hyperthermophile is *Pyrococcus furiosus* (6) (order *Thermococcales*), which can grow above 100°C with a rapid growth rate and a high cell yield. Organisms of the order *Thermococcales* are anaerobic fermentative heterotrophs which commonly utilize peptides as energy and carbon sources (21). Some of them also exhibit growth on starch, which indicates the existence of extracellular amylolytic enzymes such as the α -amylase of *Pyrococcus woesei* (10) or the amylopullulanases of *P. furiosus* and *Thermococcus litoralis* (4). Maltooligosaccharides produced by these enzymes would be translocated into cytoplasm and further hydrolyzed to glucose by intracellular amylolytic enzymes such as the intracellular α -amylase (13) and the α -glucosidase (5) of *P. furiosus*.

α -Amylases from hyperthermophilic archaea are potential model enzymes to study hyperthermophily since a wealth of data are currently available for this enzyme family from among the eubacteria. The α -amylases purified from hyperthermophilic archaea, however, are limited to only two species, *P. furiosus* and *P. woesei* (10, 13). The *P. furiosus* enzyme is intracellular and does not share any sequence identity with the consensus α -amylase sequences (12). The *P. woesei* enzyme is extracellular, and its primary structure has yet to be determined.

Thermococcus profundus DT5432 is a hyperthermophilic archaeon isolated from the deep-sea hydrothermal vent system (9). The organism exhibited growth on starch and expressed extracellular amylolytic activity. Here we describe the purification and characterization of a major extracellular α -amylase which was isolated from this organism and which displayed enzymatic properties distinct from those of the *P. woesei* enzyme.

MATERIALS AND METHODS

Bacterial strain and cultivation. *T. profundus* DT5432 (JCM 9378) was isolated from the deep-sea hydrothermal vent system (depth, 1,395 m) at the Middle Okinawa Trough (27°33'N, 126°58'E). The medium DT14 (9) enriched with the addition of 0.5% maltose was used as the growth medium. The organism was grown overnight anaerobically at 80°C in 2-liter screw-cap bottles.

Amylase purification. All enzyme purification steps were conducted at room temperature unless otherwise noted. Culture broth (100 liters) was filtered through Toyo no. 1 filter paper to remove elemental sulfur and centrifuged at $6,500 \times g$ for 30 min to remove the cells. Solid ammonium sulfate was slowly added to the supernatant fraction to yield 80% saturation, and the mixture was kept overnight at 4°C. The precipitate was collected by centrifugation at $7,000 \times g$ for 30 min, dissolved in 50 mM Tris-HCl buffer (pH 7.5), and dialyzed overnight against the same buffer (800 ml). Solid NaCl was added to the enzyme solution to a final concentration of 1 M prior to application of the solution to a DEAE-Toyopearl 650M column (4.5 by 25 cm) pre-equilibrated with a buffer containing 50 mM Tris-HCl (pH 7.5) and 1 M NaCl. The column was washed with 2 liters of the same buffer, and the enzyme was eluted with 3.5 M Tris-HCl (pH 7.5) at a flow rate of 0.67 ml/min. The active fractions (90 ml) were pooled and dialyzed against 50 mM Tris-HCl (pH 7.5) for 36 h at 4°C three times. The enzyme solution was concentrated to a 1.0-ml volume by Ficol 400 powder and freeze-drying and then loaded onto a gel filtration column of Superdex 200 HR 10/30 (Pharmacia AB) equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The enzyme was eluted at a flow rate of 0.4 ml/min.

Gel electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was carried out by using a premade gel system (TEF Co.) according to the supplier's instructions. Phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20 kDa) were used as molecular mass markers. Protein bands were visualized by staining with 0.2% Coomassie brilliant blue R-250. Active staining of the amylase in a sodium dodecyl sulfate (SDS)-PAGE gel was performed after washing the gel twice in 50 mM Tris-HCl buffer (pH 7.5) containing 25% isopropanol for 1 h and then in 50 mM Tris-HCl buffer (pH 7.5) for 1 h at room temperature. The gel was incubated in 50 mM Tris-HCl buffer (pH 7.5) containing 1% soluble starch at 80°C for 1 h. The

* Corresponding author. Mailing address: Laboratory of Microbiology, The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama 351-01, Japan. Phone: 048-462-1111, ext. 5724. Fax: 048-462-4672. Electronic mail address: koba@rkna50.riken.go.jp.

TABLE 1. Summary of purification of amylase S

Treatment	Total activity (U)	Protein (mg)	Sp act (U/mg)	Yield (%)	Purification (fold)
(NH ₄) ₂ SO ₄	608	434	1.4	100	1
DEAE	582	3.4	171	96	122
Superdex	160	0.14	1,143	26	816

amylase activity was visualized by staining the gel in a solution containing 0.15% (wt/vol) I₂ and 1.5% (wt/vol) KI (8).

Amylase assay. A 10- μ l aliquot of enzyme solution was mixed with 0.99 ml of 1.5% soluble starch in 50 mM sodium-potassium phosphate buffer (pH 5.5) and incubated at 70°C for 30 min. The amount of reducing sugar liberated was determined by Nelson's adaptation of the method of Somogyi (14). One unit of amylase activity was defined as the amount of the enzyme which released 1 μ mol of reducing sugar equivalent to glucose per minute.

Protein determination. Protein concentration was measured with a bicinchoninic acid protein assay kit (Pierce Chemical Co.) with bovine serum albumin as the standard.

Thin-layer chromatography. Products released through hydrolysis of various polysaccharides and oligosaccharides by the amylase were identified by thin-layer chromatography with precoated silica gel plates (Kiesel gel 60 F254; Merck). The thin-layer chromatography plate separation of products was developed by multiple ascents with a solvent system of 1-butanol-methanol-water (4:2:1 [vol/vol/vol]). The products were detected by the method described by Pastuska (16).

Determination of the anomeric form of the products. Mutarotation of the products produced by the amylase action was determined by the method of Robyt and French with a digital polarimeter (JASCO DIP-370) (17).

Chemicals. Soluble starch was purchased from Kanto Chemical Co., Inc. Amylose A (short-chain amylose with degree of polymerization [DP] of 17), amylose B (long-chain amylose with a molecular weight of 16,000), amylopectin, oyster glycogen, and pullulan were the products of Nacalai Tesque Inc. Cyclodextrins and maltooligosaccharides were obtained from Nihon Shokuhin Kako Co.

RESULTS

Purification of amylase. *T. profundus* DT5432 produced extracellular amylase, and the amylase production was enhanced from 3.5 to 6.0 mU/ml (after 15 h of cultivation) by the addition of 0.5% maltose to the medium. The addition of 0.5% glucose did not affect amylase production (3.3 mU/ml). The culture supernatant fraction, concentrated by ammonium sulfate precipitation, revealed two amylase bands (designated amylases L and S) by activity staining on native PAGE (data not shown). Preliminary experiments in order to determine the conditions for amylase purification revealed that amylase S, which migrated faster on native PAGE gels than amylase L, tightly binds to the DEAE-Toyopearl column. Unlike amylase S, amylase L and most other proteins from this isolate were eluted under 1 M NaCl. Amylase S, however, was not eluted even at 5 M NaCl, but it could be eluted at 3.5 M Tris-HCl (pH 7.5).

A summary of purification is shown in Table 1. Amylases L and S were separated by DEAE-Toyopearl chromatography. Amylase activity of the fraction unadsorbed to DEAE-Toyopearl (amylase L) was 62 U, while the amylase S fraction gave 582 U, indicating that amylase S contributes 90% of the total amylase activity. Values in Table 1 were calculated on the basis of this distribution. Amylase S was purified 816-fold with a yield of 26%. The purified enzyme (0.14 mg) exhibited a specific activity of 1,143 U/mg per protein and was electrophoretically homogeneous (Fig. 1). The mobility of the active amylase band as determined by activity staining coincided with that of the single protein band stained with Coomassie brilliant blue.

Molecular mass. The molecular mass of the amylase was estimated to be 42 kDa by SDS-PAGE (Fig. 1).

Effect of pH. The enzyme exhibited a pH optimum at 5.5 to 6.0 with a rapid decline of the activity at the higher and lower pH ranges (Fig. 2). The amylase was stable in a pH range of 5.5

FIG. 1. SDS-PAGE of the purified amylase. The amylase was visualized by Coomassie brilliant blue staining (lane 1) and by activity staining (lane 2). Lane M, molecular mass markers.

to 9.8 at 60°C. The activity sharply declined in acidic conditions (72% inactivation at pH 4.3), but in an alkaline range, the enzyme was relatively stable with only 31% loss of the activity at pH 10.5 (Fig. 3). In the presence of 0.5% soluble starch, the enzyme was slightly stabilized with 45 and 19% loss of the activity at pH 4.3 and 10.5, respectively.

Effect of temperature. The amylase displayed optimal activity at 80°C, which is the optimal temperature for growth of *T. profundus* (Fig. 4). No loss of the activity was observed at 50, 60, and 70°C for 4 h. The half-life of the enzyme was 3 h at

FIG. 2. Effect of pH on the amylase activity. The following buffers were used at 50 mM: sodium acetate buffer between pH 3.8 and 5.7 (circles), sodium-potassium phosphate buffer between pH 5.6 and 7.3 (squares), and glycine-NaOH buffer between pH 7.7 and 9.0 (triangles). All the pH values were adjusted at 70°C.

FIG. 3. Effect of pH on the amylase stability. The amylase was incubated for 30 min at 60°C in various buffers (50 mM) in either the absence (closed symbols) or the presence (open symbols) of 0.5% starch, and the remaining activity was determined. The buffers were sodium acetate from pH 4.3 to 5.7 (circles), imidazole-HCl from pH 5.7 to 7.1 (squares), and glycine-NaOH from pH 7.7 to 10.5 (triangles).

80°C and 15 min at 90°C. The activity was rapidly lost at 100°C (Fig. 5). The enzyme was stabilized by the addition of 0.5% soluble starch or 5 mM Ca^{2+} . The half-lives of the enzyme were 4 h at 80°C and 1 h at 90°C in the presence of soluble starch and 4 h at 80 and 90°C in the presence of Ca^{2+} .

Effect of metal ions and chemical reagents. The amylase activity was measured at pH 5.5 and 70°C in the presence of various metal ions at 0.1 and 1 mM or in the presence of various chemical reagents (Table 2). The amylase had been dialyzed against 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA before the experiments. The enzyme did not lose any activity by this dialysis. None of the metal ions was required for catalytic activity. Hg^{2+} completely inhibited enzyme activity at 1 mM. Ba^{2+} , Cu^{2+} , Cd^{2+} , and Zn^{2+} at 1 mM showed 33, 56, 31, and 36% inhibition, respectively.

Dithiothreitol, 2-mercaptoethanol, and phenylmethylsulfonyl fluoride had little effect on the enzyme activity up to 5 mM. The activity was totally lost in the presence of either 5 mM iodoacetic acid or 1 mM *N*-bromosuccinimide, suggesting that accessible cysteine and tryptophan residues play an important role in the catalytic action. Addition of SDS resulted in a significant (78% inhibition at 2% concentration) but not com-

FIG. 4. Effect of temperature on the amylase activity. The incubation at temperatures above 90°C was conducted in a silicon oil bath.

FIG. 5. Effect of temperature on the thermostability of the amylase. The amylase was incubated at each temperature for various time intervals, and the remaining activity was determined. The results at 90°C in the presence of 5 mM Ca^{2+} or 0.5% starch are also shown.

plete loss of activity. A concentration of 2.0 M guanidine hydrochloride resulted in 85% inhibition, but the same concentration of urea did not inhibit the enzyme.

Mode of action of the amylase. Amylase S hydrolyzed soluble starch, amylose, amylopectin, and glycogen to form maltose and maltotriose as the major products. Minor amounts of longer maltooligosaccharides (DP ≥ 4) with a trace amount of glucose were also produced. The enzyme did not attack pullulan or cyclodextrins (Fig. 6). Maltooligosaccharides of DP 3 to DP 7 were hydrolyzed at an increasing rate concomitant with the number of glucose units, such that the amylase had the highest activity for maltoheptaose (DP 7) (Fig. 6). Maltose was

TABLE 2. Effect of metal ions and chemical reagents on the amylase activity

Metal ion or chemical reagent ^a	Relative activity (%)
Metal ions	
Ba^{2+}	67
Ca^{2+}	115
Co^{2+}	106
Cs^{2+}	114
Cu^{2+}	44
Cd^{2+}	69
Fe^{2+}	102
Hg^{2+}	0
Mg^{2+}	113
Mn^{2+}	82
Ni^{2+}	80
Sr^{2+}	101
Zn^{2+}	66
Chemical reagents	
Dithiothreitol	83
2-Mercaptoethanol	93
Deoxyxojirimycin	102
PMSF	109
Iodoacetic acid	48
NBS	0
Guanidine-HCl	15
Urea	107
SDS	27

^a Concentrations of metal ions (as chloride salts) and chemical reagents were 1 mM except for guanidine-HCl (2 M), urea (2 M), and SDS (2%). PMSF and NBS represent phenylmethylsulfonyl fluoride and *N*-bromosuccinimide, respectively.

FIG. 6. Action patterns of the amylase on various substrates. Lanes 1 to 6 are the products from maltooligosaccharides ranging from DP 2 to DP 7. Lanes 7 to 11 represent the product from starch, amylose A (DP 17), amylose B (average molecular weight, 16,000), amylopectin, and glycogen, respectively. Lanes M represent a standard mixture of maltooligosaccharides ranging from DP 1 to DP 7.

not hydrolyzed by the enzyme. The enzyme possessed slight transferase activity, which was found by detection of trace amounts of maltotetraose and maltopentaose with maltotriose as the substrate. The main products, maltose and maltotriose, were readily apparent even during the early stages of the reaction and increased in concentration along with the time course of the reaction (Fig. 7). The enzyme may, therefore,

FIG. 7. Time course of maltooligosaccharide production by amylase activity with amylopectin as the substrate. Lanes 1 to 8 represent the products at 0, 0.5, 1, 2, 4, 6, 8, and 12 h. Lane M represents a standard mixture of maltooligosaccharides ranging from DP 1 to DP 7 (G1 to G7).

TABLE 3. K_m and V_{max} values for various substrates^a

Substrate	K_m (%)	V_{max} (mU)
Soluble starch	0.23 ± 0.12	9.4 ± 2.1
Amylose A (DP 17)	0.47 ± 0.27	9.4 ± 1.4
Amylopectin	0.13 ± 0.07	12.2 ± 1.7
Glycogen	0.05 ± 0.02	9.6 ± 1.6

^a The amount of enzyme used was 5.8 mU for each substrate. Substrate range of 0.063 to 1.0% was tested. The values are shown as the mean value $\pm$ standard deviation of four experiments.

preferentially cleave at the α -1,4-linkage adjacent to nonreducing ends, releasing maltose and maltotriose.

The amylase displayed Michaelis-Menten-type kinetics. The K_m values were determined to be 0.23% for soluble starch, 0.47% for amylose A, 0.13% for amylopectin, and 0.05% for glycogen, indicating that the enzyme prefers branched polysaccharides as substrates (Table 3). The anomeric form of the products had an α -configuration.

DISCUSSION

Hyperthermophiles have attracted growing attention because of the hyperthermophily of both the organisms themselves and their enzymes. Amylases are among the best-characterized enzymes and have been used to study hyperthermophily (10–13, 19). However, only a few amylases have been purified and characterized from the hyperthermophiles (10, 13). Despite this interest, the primary structure of these enzymes has been determined only for the intracellular amylase of *P. furiosus* (12). This enzyme does not share the conserved sequences observed among α -amylases, cyclomaltodextrin glucanotransferases, and pullulanases from a variety of sources including the α -amylase of the haloalkaliphilic archaeon *Natronococcus* sp. (7, 18). Therefore, it seems that this intracellular amylase and the other extracellular amylases may have diverged from different ancestral genes.

There have been only two extracellular amylases purified from hyperthermophilic archaea, one from *P. woesei* (10) and the amylase from *T. profundus* described here. Both of these enzymes are extremely thermophilic (optimum activity observed at 100 and 80°C, respectively), reflecting the optimum temperatures for growth of these organisms (9, 22). The half-lives of the enzymes are 4 and 3 h, respectively, at each optimum temperature for the activity. In addition to their thermophily, these enzymes exhibit similar properties including pH optima of 5.5 to 6.0 and higher specific activities compared with the enzymes from moderate thermophilic anaerobes (1). These enzymes do, however, exhibit other, very different features. The molecular masses are 70 and 42 kDa for the *P. woesei* and *T. profundus* enzymes, respectively, and the *P. woesei* enzyme is not able to attack maltooligosaccharides between DP 2 and DP 6, while the *T. profundus* amylase S hydrolyzes the maltooligosaccharides other than maltose (DP 2). The activity does decrease with the shorter length of the maltooligosaccharide. The main products of amylase S action were maltose (DP 2) and maltotriose (DP 3) for all substrates tested, but the *P. woesei* enzyme produces exclusively maltose and maltopentaose (DP 5) from maltoheptaose (DP 7). A comparison of amino acid sequences for these enzymes will provide fundamental information about thermophily as well as about the substrate specificity of amylases from other hyperthermophilic archaea.

Amylase S produces maltose (DP 2) and maltotriose (DP 3) as the main products from the first stages of the reaction and

in increasing amounts as the reaction proceeds, but the enzyme does not attack cyclodextrins. These results suggest that the enzyme preferentially cleaves substrates from their nonreducing ends. However, the enzyme does not seem to be an exolytic α -amylase since such enzymes generally release only a single maltooligosaccharide as the product. Further data have to be obtained in order to determine the mode of action for this amylase.

The amylases of *Thermotoga maritima*, a hyperthermophilic eubacterium, exhibit a significant catalytic activity only at elevated temperatures that is attributable to the exceedingly high activation energies (60 and 98 kJ/mol) relative to that of the *Bacillus licheniformis* amylase (14 kJ/mol) (19). *T. profundus* amylase S also exhibited a high activation energy (45 kJ/mol). We roughly calculated the activation energies of *Bacillus subtilis*, *Bacillus stearothermophilus*, and *P. woesei* amylases from the reported data (10, 15). Each enzyme gave 8, 22, and 74 kJ/mol (below 70°C), respectively. Thus, hyperthermophilic enzymes tend to have higher activation energies than mesophilic or moderately thermophilic enzymes, which may result in a high temperature requirement for significant activity.

In *P. furiosus*, saccharides with α -1,4-linkages stimulated amylase production (3), while production of *P. woesei* amylase is constitutive (10). Production of the extracellular amylases in *T. profundus* was slightly enhanced by the addition of maltose to the medium. However, further work will be necessary to determine whether the enhancement is due to induction or another unknown effect.

Amylase S from *T. profundus* may find an application in starch conversion biotechnologies because of its high specific activity, high temperature optimum, extreme thermostability, independence from metal ions, and unique substrate specificity and product pattern. We are currently working on isolation of the amylase gene by using an oligonucleotide mixture as a probe that corresponds to the NH₂-terminal amino acid sequence of the amylase.

ACKNOWLEDGMENTS

We gratefully thank Koki Horikoshi for his encouragement on this work. We thank Masao Chijimatsu for his help in determination of the NH₂-terminal sequence. We also thank Michael Travisano for careful reading of the manuscript.

This work was partially supported by a grant for the Bidesign Program to T. Kudo and a special grant for Promotion of Research to T. Kobayashi from the Institute of Physical and Chemical Research (RIKEN).

REFERENCES

1. Antranikian, G. 1990. Physiology and enzymology of thermophilic anaerobic bacteria degrading starch. *FEMS Microbiol. Rev.* **75**:201–218.
2. Bragger, J. M., R. M. Daniel, T. Coolbear, and H. W. Morgan. 1989. Very stable enzymes from extremely thermophilic archaeobacteria and eubacteria. *Appl. Microbiol. Biotechnol.* **31**:556–561.
3. Brown, S. H., H. R. Costantino, and R. M. Kelly. 1990. Characterization of amylolytic enzyme activities associated with the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. *Appl. Environ. Microbiol.* **56**:1985–1991.
4. Brown, S. H., and R. M. Kelly. 1993. Characterization of amylolytic enzymes, having both α -1,4 and α -1,6 hydrolytic activity, from the thermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis*. *Appl. Environ. Microbiol.* **59**:2614–2621.
5. Costantino, H. R., S. H. Brown, and R. M. Kelly. 1990. Purification and characterization of an α -glucosidase from a hyperthermophilic archaeobacterium, *Pyrococcus furiosus*, exhibiting a temperature optimum of 105 to 115°C. *J. Bacteriol.* **172**:3654–3660.
6. Fiala, G., and K. O. Stetter. 1986. *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaeobacteria growing optimally at 100°C. *Arch. Microbiol.* **145**:56–61.
7. Kobayashi, T., H. Kanai, R. Aono, K. Horikoshi, and T. Kudo. 1994. Cloning, expression, and nucleotide sequence of the α -amylase gene from the haloalkaliphilic archaeon *Natronococcus* sp. strain Ah-36. *J. Bacteriol.* **176**:5131–5134.
8. Kobayashi, T., H. Kanai, T. Hayashi, T. Akiba, R. Akaboshi, and K. Horikoshi. 1992. Haloalkaliphilic maltotriose-forming α -amylase from the archaeobacterium *Natronococcus* sp. strain Ah-36. *J. Bacteriol.* **174**:3439–3444.
9. Kobayashi, T., Y. S. Kwak, T. Akiba, T. Kudo, and K. Horikoshi. 1994. *Thermococcus profundus* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *Syst. Appl. Microbiol.* **17**:232–236.
10. Koch, R., A. Spreinat, K. Lemke, and G. Antranikian. 1991. Purification and properties of a hyperthermoactive α -amylase from the archaeobacterium *Pyrococcus woesei*. *Arch. Microbiol.* **155**:572–578.
11. Koch, R., P. Zabłowski, A. Spreinat, and G. Antranikian. 1990. Extremely thermophilic amylolytic enzyme from the archaeobacterium *Pyrococcus furiosus*. *FEMS Microbiol. Lett.* **71**:21–26.
12. Laderman, K. A., K. Asada, T. Uemori, H. Mukai, Y. Taguchi, I. Kato, and C. B. Anfinsen. 1993. α -Amylase from the hyperthermophilic archaeobacterium *Pyrococcus furiosus*; cloning and sequencing of the gene and expression in *Escherichia coli*. *J. Biol. Chem.* **268**:24402–24407.
13. Laderman, K. A., B. R. Davis, H. C. Krutzsch, M. S. Lewis, Y. V. Griko, P. L. Privalov, and C. B. Anfinsen. 1993. The purification and characterization of an extremely thermostable α -amylase from the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. *J. Biol. Chem.* **268**:24394–24401.
14. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* **153**:375–380.
15. Ogasawara, K., A. Imanishi, and T. Isemura. 1970. Studies on thermophilic α -amylase from *Bacillus stearothermophilus*. *J. Biochem.* **67**:65–75.
16. Pastuska, G. 1961. Untersuchungen über die qualitative und quantitative Bestimmung der Zucker mit Hilfe der Kieselgelschicht-Chromatographie. *Z. Anal. Chem.* **179**:427–429.
17. Robyt, J., and D. French. 1964. Purification and action pattern of an amylase from *Bacillus polymixa*. *Arch. Biochem. Biophys.* **104**:338–345.
18. Sashihara, N., N. Nakamura, and K. Horikoshi. 1993. Subcloning and nucleotide sequencing of the *pul* gene of *Thermus* sp. AMD-33. *Starch Staerke* **45**:144–150.
19. Schumann, J., A. Wrba, R. Jaenicke, and K. O. Stetter. 1991. Topographical and enzymatic characterization of amylases from the extremely thermophilic eubacterium *Thermotoga maritima*. *FEBS Lett.* **282**:122–126.
20. Stetter, K. O. 1992. Life at the upper temperature border, p. 195–219. In J. Van, K. T. T. Van, J. C. Mounolou, J. Schneider, and C. McKay (ed.), *Colloque interdisciplinaire du comité national de la recherche scientifique, frontiers of life*. Editions Frontières, Gif-sur-Yvette, France.
21. Zillig, W. 1991. The order Thermococcales, p. 702–706. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), *The prokaryotes*—1991. Springer-Verlag, New York.
22. Zillig, W., I. Holz, H. P. Klenk, J. Trent, S. Wunderl, D. Janekovic, E. Imself, and B. Haas. 1987. *Pyrococcus woesei* sp. nov., an ultrathermophilic marine archaeobacterium, representing a novel order, *Thermococcales*. *Syst. Appl. Microbiol.* **9**:62–70.