APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1997, p. 3569–3576 Vol. 63, No. 9
0099-2240/97/$04.00+0

Cloning, Sequencing, and Expression of the Gene Encoding Extracellular α-Amylase from *Pyrococcus furiosus* and Biochemical Characterization of the Recombinant Enzyme

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Received 24 March 1997/Accepted 23 June 1997

The gene encoding the hyperthermophilic extracellular α-amylase from *Pyrococcus furiosus* was cloned by activity screening in *Escherichia coli*. The gene encoded a single 460-residue polypeptide chain. The polypeptide contained a 26-residue signal peptide, indicating that this *Pyrococcus* α-amylase was an extracellular enzyme. Unlike the *P. furiosus* intracellular α-amylase, this extracellular enzyme showed 45 to 56% similarity and 20 to 35% identity to other amylolytic enzymes of the α-amylase family and contained the four consensus regions characteristic of that enzyme family. The recombinant protein was a homodimer with a molecular weight of 100,000, as estimated by gel filtration. Both the dimer and monomer retained starch-degrading activity after extensive denaturation and migration on sodium dodecyl sulfate-polyacrylamide gels. The *P. furiosus* α-amylase was a liquefying enzyme with a specific activity of 3,900 U mg^{-1} at 98°C. It was optimally active at 100°C and pH 5.5 to 6.0 and did not require Ca^{2+} for activity or thermostability. With a half-life of 13 h at 98°C, the *P. furiosus* enzyme was significantly more thermostable than the commercially available *Bacillus licheniformis* α-amylase (Taka-therm).

α-Amylases (EC 3.2.1.1) are endo-acting enzymes that hydrolyze starch by cleaving α-1,4-glucosidic linkages at random. They are among the most important commercial enzymes, having wide applications in starch-processing, brewing, alcohol production, textile, and other industries. Numerous α-amylases from eubacteria, fungi, plants, and animals have been characterized, and their genes have been cloned. With the exception of one eubacterial enzyme (18), they all belong to a same α-amylase family, having similar structures, similar catalytic sites, and the same catalytic mechanism (22). α-Amylases contain three domains: (i) domain A corresponds to an $(\alpha/\beta)_8$ barrel; (ii) domain B is the very long $\beta_3 \rightarrow \alpha_3$ loop of the $(\alpha/\beta)_8$ barrel; and (iii) domain C is a separate globular domain composed of β-strands arranged in a Greek key motif (10). Four highly conserved regions come together through the interaction of domains A and B to form the active center, the substrate binding site, and a Ca^{2+} binding site. The Ca^{2+} cation is essential for enzyme folding (10) and for optimal activity and stability (41).

Since starch starts being soluble only at 100°C and above, the majority of industrial applications of α-amylases require their use at temperatures of up to 110°C (5). The most thermostable α-amylase (Taka-therm) used in industry is produced from *Bacillus licheniformis*. It has an optimal temperature of 90°C and requires additional Ca^{2+} for its thermostability (41). Hyperthermophilic archaea are attracting increasing applied research attention, since their enzymes show extreme thermostability (2, 39). Many hyperthermophiles can grow on starch and other carbohydrates, suggesting that they express a variety of amylolytic enzymes that could be of industrial interest (1, 39). Recently, several hyperthermostable amylolytic enzymes from *Pyrococcus furiosus* (8, 16, 24, 26), *Pyrococcus woesei* (23), and *Thermococcus profundus* (12) have been described. Only the *P. furiosus* intracellular α-amylase gene was cloned and expressed in *Escherichia coli* (25).

We report here the characterization of a hyperthermostable *P. furiosus* extracellular α-amylase (PFA). Its gene was cloned and expressed in *E. coli*, and the sequence of the gene was determined. The purified recombinant enzyme was compared to the commercial *B. licheniformis* α-amylase (BLA).

MATERIALS AND METHODS

Growth conditions for *P. furiosus*. *P. furiosus* DSM 3638 was cultivated in artificial seawater (8) supplemented with 0.25% soluble starch, 2.5% tryptone, 2% yeast extract, and 0.1% elemental sulfur. The pH of the medium was adjusted to 7.0 with 1 M NaOH. The fermentation was performed in a 15-liter vessel with a 5% inoculum in a working volume of 10 liters (B. Braun Biotech, Bethlehem, Pa.) at 90°C for 20 h under constant gassing with N_2. Cells were harvested in the stationary growth phase and stored at −20°C before use.

Library construction and screening. Extraction of *P. furiosus* chromosomal DNA and genomic library construction were performed as described previously (3, 40). *E. coli* Sure (Stratagene, La Jolla, Calif.) transformed with the ligation mixture was plated on 1.5% Luria-Bertani (LB) agar–ampicillin (100 μg/ml) plates. After 16 to 20 h of incubation at 37°C, colonies were replicated onto a new set of LB-ampicillin plates containing 1% Phytagel (Sigma, St. Louis, Mo.) instead of agar and 0.2% soluble starch. After overnight growth, the plates were incubated at 80°C for 8 to 10 h. Amylase activity was detected by flooding the plates with I_2-KI.

Nucleotide sequence determination and site-directed mutagenesis. Restriction analysis and plasmid DNA purification were performed as described previously (3). Nested deletions for sequencing were generated on both sides of the insert as described previously (20). Sequences were analyzed in both directions by using the Sequenase version 2.0 T7 DNA polymerase (U.S. Biochemicals, Cleveland, Ohio) and the ThermoSequenase (Amersham Life Science, Arlington Heights, Ill.) sequencing kits. Sequencing data were analyzed by using the Genetics Computer Group Sequencing Analysis Software Package (version 7.0) (14). The PFA amino acid sequence was compared with the sequences of other amylolytic enzymes available through the GenBank/EMBL data bank (IntelliGenetics Inc., Mountain View, Calif.). Two oligonucleotides (5′-CAAATGTCACGTTGTAT GG-3′ and 5′-GAGAGTGGTGCAAAGGTC-3′) identical to different sequences of the pS4 insert were synthesized and used as primers for PCR with *P. furiosus* genomic DNA as the template. The PCR product was analyzed on an agarose gel and sequenced.

The initiation codon (GTG) of the PFA-encoding gene (*amyA*) was changed

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to ATG by using the QuikChange site-directed mutagenesis kit (Stratagene) with primers 5′-GAGGTGATCACATGAACATAAAGAAATTAACACC-3′ and 5′-GGTGTTAATTTCTTTATGTTCATGTGATCACCT-3′. Oligonucleotides were synthesized by the Michigan State University Macromolecular Facility.

Enzyme purification. All purification steps were performed at room temperature under aerobic conditions. When expressed in *E. coli*, PFA was not secreted into the medium. Cells carrying plasmid pS4 were grown in LB-ampicillin (100 μg/ml). Forty grams (wet weight) of cells was suspended in 120 ml of 50 mM Tris-HCl (pH 7.5). A cell homogenate was prepared by passing the cell suspension through a French press at 15,000 lb/in^2 twice. After heat treatment at 80°C for 15 min, the cell homogenate was centrifuged at 16,000 × *g* for 20 min. The enzyme was precipitated by adding 60% $(NH_4)_2SO_4$ to the supernatant, and the pellet was resuspended in 50 mM sodium acetate (pH 6.0).

The concentrated crude enzyme was loaded onto a phenyl-Sepharose (Pharmacia Fine Chemica AB, Uppsala, Sweden) column (1.5 by 18 cm) equilibrated with 50 mM acetate (pH 6.0). The column was washed with the same buffer and then with 50 mM Tris-HCl (pH 8.0). The enzyme was eluted with 6 M urea in 20 mM Tris-HCl (pH 9.4). After concentration in an ultrafiltration cell equipped with a 30,000-molecular-weight-cutoff membrane (Amicon, Beverly, Mass.), the enzyme was dialyzed against 50 mM Tris-HCl (pH 6.0), and the hydrophobic interaction chromatography was repeated as described above. The fractions with α-amylase activity were pooled and concentrated by ultrafiltration (see above).

The concentrated enzyme was loaded onto a Sephacryl S200 (Pharmacia Fine Chemica AB) column (1.5 by 80 cm) equilibrated with 20 mM Tris-HCl (pH 9.4) containing 5% glycerol. The active fractions were concentrated by ultrafiltration (see above) and dialyzed against 50 mM sodium acetate (pH 5.6) (buffer A). The N terminus of the recombinant PFA was sequenced by the Michigan State University Macromolecular Facility.

Commercial Taka-therm L-340 BLA, a gift from Genencor International Inc. (Rochester, N.Y.), was dialyzed against 50 mM sodium acetate (pH 6.0).

Enzyme assays. PFA activity was determined by measuring the amount of reducing sugar released during enzymatic hydrolysis of 1% soluble starch in buffer A at 98°C for 15 min. A control without enzyme was used. The amount of reducing sugar was measured by a modified dinitrosalicylic acid method (6). One unit of amylase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar as glucose per min under the assay conditions. BLA was assayed at 90°C in 50 mM sodium acetate (pH 6.0) containing 0.5 mM Ca^{2+} (buffer B). Other conditions were the same as described above. The protein concentration was determined by using the Bio-Rad (Richmond, Calif.) protein assay kit with bovine serum albumin as a standard.

MW determination. A 0.5- by 45-cm column containing Sephacryl S200 was equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.2 M NaCl. The purified sample and marker proteins (i.e., carbonic anhydrase [molecular weight {MW}, 29,000], bovine serum albumin [66,000], alcohol dehydrogenase [150,000], and Blue Dextran [2,000,000]) were applied to the column at a 7-ml/h flow rate. Elutions of the marker proteins and the recombinant PFA were followed by UV detection at 280 nm and activity assay.

Gel electrophoresis. Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide) (27). Protein samples were denatured by incubation with denaturing buffer (2% SDS and 0.64 M mercaptoethanol [final concentrations]) at different temperatures in water or silicon oil baths for various periods. Low-MW protein markers (Bio-Rad) were used as standards. Native PAGE was performed under the same conditions as described above except for the absence of SDS in the gel and buffer system. Samples were heat treated in 0.1 M acetate (pH 5.6) at different temperatures before being loaded. Proteins were stained with Coomassie brilliant blue R-250. For activity staining, 0.66% soluble starch was added during preparation of the SDS-polyacrylamide gels. After electrophoresis, starch-containing gels were washed with buffer A and incubated at 90°C for 10 min in buffer A. Enzyme activity was visualized by flooding with I_2-KI solution. Gels containing BLA were washed with buffer B and incubated at 80°C for 10 min in buffer B. Other conditions were the same as described above.

Analysis of PFA and BLA hydrolysis products. The recombinant PFA (2.5 U/ml) was incubated at 90°C with 1% (wt/vol) soluble starch, pullulan, glycogen, amylose, amylopectin, or oligosaccharides. Samples were withdrawn after various periods. Hydrolysis products were analyzed by high-performance anion-exchange chromatography with pulse amperometric detection and a CarboPac PAI column (4 by 250 mm) (Dionex system). Hydrolysis products were identified and quantified by using the PEAK II computer software (SRI Instruments, Torrance, Calif.). Glucose (G_1), maltose (G_2), maltotriose (G_3), maltotetraose (G_4), maltopentaose (G_5), maltohexaose (G_6), and maltoheptaose (G_7) were the standards. Starch, pullulan, glycogen, amylose, amylopectin, and oligosaccharides were also incubated with BLA (2.5 U/ml) at 80°C in buffer B, and hydrolysis products were analyzed for comparison.

pH and temperature studies. The optimal pH for PFA activity was determined at 98°C in 50 mM sodium acetate (pH 3.5 to 6.0) and 50 mM Tris-HCl (pH 6 to 11). All pHs were adjusted at room temperature, and corresponding pHs at high temperatures were calculated by using the equation $\Delta pK_a/\Delta T°C = 0.000$ and -0.031 for sodium acetate and Tris, respectively (29). BLA was assayed at 90°C in the same buffers in the presence of 0.5 mM Ca^{2+}.

The temperatures for maximal activities of PFA and BLA were determined by performing standard enzyme assays at different temperatures.

For stability studies at high temperatures, both enzymes were EDTA treated. They were dialyzed extensively first against buffer A (for PFA) or buffer B without Ca^{2+} (for BLA) containing 2 mM EDTA and then twice against the same buffer without EDTA. Enzyme thermal inactivation studies were performed by incubating 1-ml gas chromatography tubes (Alltech Associates, Deefield, Ill.) that contained 800 μl of purified enzyme in 0.1 M sodium acetate (pH 5.6 for PFA and pH 6.0 for BLA) in the presence or absence of 5 mM Ca^{2+} at 90 or 98°C. After various incubation periods, samples were withdrawn and tested for residual α-amylase activity under the standard assay conditions for each enzyme.

PFA overexpression in *E. coli*. The *P. furiosus amyA* gene was amplified by PCR without its signal peptide. The oligonucleotide 5′-AGCTAGCTTG GAGCTTGAAGAGGGAG-3′ was used as the forward primer. The sequence AAATCA, encoding the two N-terminal residues Lys and Tyr, was replaced by GCTAGC, encoding Ala-Ser, to create an *Nhe*I site. The oligonucleotide 5′-ACTCGAGACCACAATAACTCCATACGGAG-3′ was used as the reverse primer. The sequence GTTGGG, encoding the C-terminal residues Val and Gly, was replaced by CTCGAG, encoding Leu-Glu, to create an *Xho*I site. The amplified gene was cloned in pCR2.1 (Invitrogen, Carlsbad, Calif.), and its sequence was verified. It was then subcloned in pET21 (Novagen, Madison, Wis.), yielding recombinant plasmid pET213. *E. coli* HMS174(DE3) (Novagen) was cotransformed with pET213 and the compatible plasmid pT-Trx, which expressed the *E. coli* thioredoxin gene under the control of the T7 promoter (46). HMS174(DE3)(pET213)(pT-Trx) was grown in SB (modified TB medium [3] containing 1.3% glycerol, 0.088 M K_2HPO_4, 0.012 M KH_2PO_4, and 1 mM $MgSO_4$)-ampicillin (100 μg/ml)-chloramphenicol (20 μg/ml) medium at 37°C to an optical density at 600 nm of 0.3. Ethanol (1% final concentration) was added to the culture, and growth was continued at 18°C for 1 h longer. PFA expression was then induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (0.2 mM final concentration) at 18°C. After 22 h of induction, cell extracts were prepared and analyzed by SDS-PAGE and activity assay.

Nucleotide sequence accession number. The sequence of the 2.6-kb insert of plasmid pS4 is available in GenBank under accession no. AF001268.

RESULTS AND DISCUSSION

Cloning and sequencing of the gene encoding PFA. Among about 10,000 clones screened on starch-containing plates, two colonies developed a clear halo. Both transformants expressed thermostable α-amylase activity but no pullulanase activity. They both constitutively expressed the α-amylase in a starch-free medium in the absence of IPTG, the inducer of the *lac* promoter. The two recombinant plasmids were shown by restriction analysis to contain overlapping inserts. The smallest, pS4, carried a 2.6-kb insert and was selected for further studies. Plasmid pS4's insert was sequenced entirely. To confirm that the insert corresponded to *P. furiosus* genomic DNA, two oligonucleotides corresponding to different pS4 insert sequences were used as primers in a PCR with *P. furiosus* genomic DNA as the template. The sequence of the PCR product was identical to the corresponding sequence in the pS4 insert, proving that the insert originated from *P. furiosus* chromosomal DNA. The pS4 insert contained a single complete open reading frame (ORF1) (nucleotides [nt] 676 to 2055). The 491-residue polypeptide encoded by ORF1 showed an overall 35.7% identity to BLA (GenBank accession no. M38570), indicating that ORF1 encoded the α-amylase expressed by pS4. The PFA N-terminal sequence was identical to residues KYLEL located at residues 58 to 62 downstream of ORF1's first ATG. Since most α-amylases, including BLA, are extracellular enzymes, we checked if the first 57 residues encoded by ORF1 could correspond to a signal peptide. The 26-residue stretch located just upstream of the KYLEL sequence showed all the characteristics of a prokaryotic signal peptide (45). It showed 66.7 and 57.7% similarity to *P. furiosus* amylopullulanase (16) and pyrolysin (43) signal peptides, respectively. This 26-residue sequence starts with a GTG-encoded formylmethionyl residue (Fig. 1). Several arguments confirm that this GTG is the translational start codon. (i) It is the best start codon downstream of a reasonable ribosome binding site (i.e., the sequence GGAGGT located 5 nt upstream of the starting GTG) in that region. (ii) PFA's similarity with BLA starts at the level of this

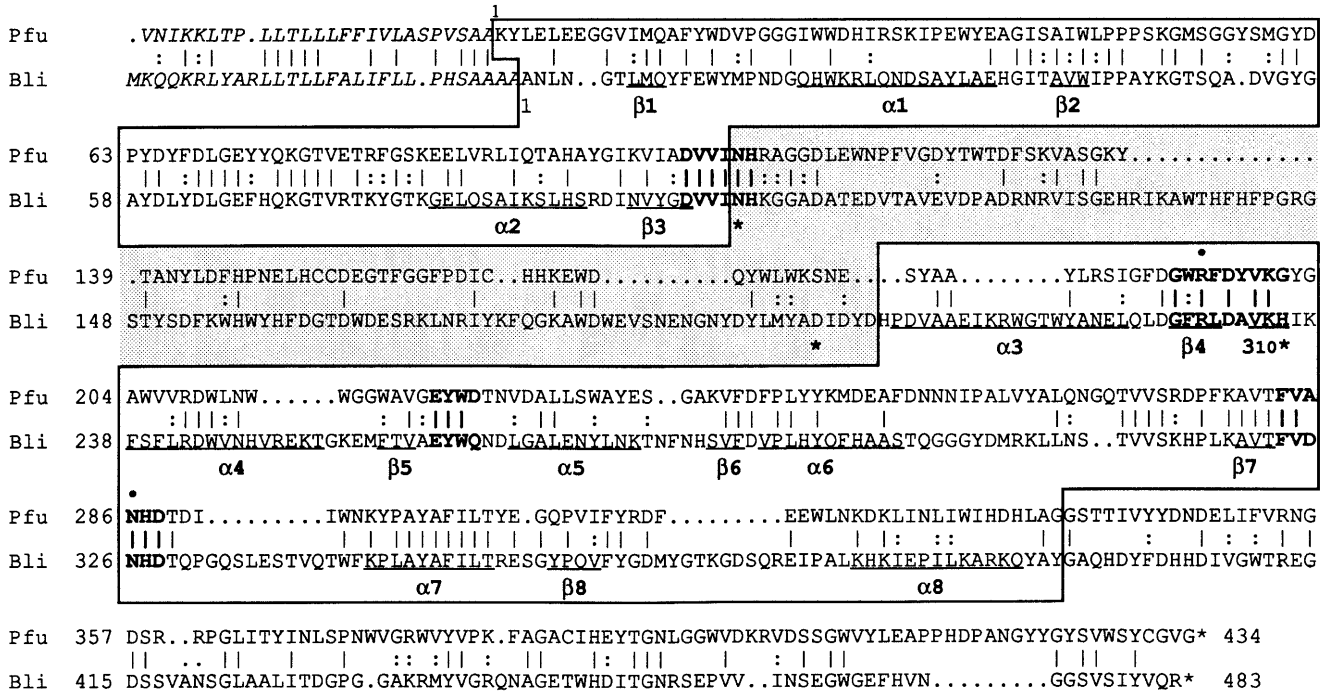

FIG. 1. Sequence alignment of PFA (Pfu) and BLA (Bli). Vertical lines and colons denote identical and similar residues, respectively. Numbering starts after the signal peptides. Signal peptides are in italics. The four active-site conserved regions are in boldface. The α/β barrel (domain A) and domain B are boxed and shaded, respectively. Underlined sequences correspond to the secondary structures in the BLA α/β barrel, as described by Machius et al. (28). Corresponding secondary structures are indicated below the sequence. BLA residues involved in Ca^{2+} (*) and Cl^- (●) binding (28) are indicated below and above the sequences, respectively.

residue (Fig. 1). (iii) Since GTG is rarely used as starting codon in *E. coli* genes, the starting GTG was mutagenized into ATG. Expression of the mutant enzyme in *E. coli* was eight times greater than that of the wild-type enzyme (not shown).

The ORF1 stop codon was immediately followed by a 19-residue stretch of pyrimidines containing the sequence TTTTTCT, which is typical of archaeal transcription termination signals (32). Two truncated ORFs (nt 1 to 524 and 2221 to 2627) were detected upstream and downstream of ORF1, in the opposite orientation. Neither of them showed significant homology to any sequence present in the GenBank/EMBL database.

The G+C content of *P. furiosus amyA* was 41.9%, which is slightly higher than the value (38%) reported for the total genome (17). As has been seen for other genes sequenced from hyperthermophiles, A and T were the preferred bases (62%) in the third positions of codons (47). Proline and threonine codons ending with G were rarely used. Like for other reported hyperthermophilic archaeal protein genes (36), AGG and AGA arginine codons were strongly preferred. Interestingly, PFA contained five cysteines. It is now well known that cysteine residues are among the residues most sensitive to degradation at high temperatures (42) and that they are usually rare in highly thermostable enzymes (15, 47). The presence of five cysteines in PFA is, therefore, surprising. Chung et al. (12) reported that an accessible cysteine may be involved in *T. profundus* α-amylase catalysis. A similar situation could happen with PFA if the two α-amylases are related.

At 52 nt upstream of the start codon, the sequence TTTATA (nt 518 to 523) is identical to the consensus defined as box A in archaeal promoters (19). Generally, genes from hyperthermophilic archaea are not spontaneously expressed in *E. coli*: only the *P. furiosus* and *P. woesei apu* genes (16, 33) and the *P. furiosus* β-glucosidase (44) and β-mannosidase (4) genes were directly expressed in *E. coli* without using an *E. coli* promoter. While the sequences of the *P. woesei apu* gene and of the region upstream of the *P. furiosus* β-mannosidase gene are not available for analysis, both the *P. furiosus apu* and β-glucosidase genes are preceded by nucleotide sequences (TTACT AN_{17}TATAAT and TTTATAN_{16}TATAAA, respectively) highly similar to the *E. coli* consensus promoter sequences. One can expect that archaeal genes spontaneously expressed in *E. coli* are expressed from a sequence reminiscent of *E. coli* promoters rather than from their own promoters. Here, one of the two sequences TTCACAN_{17}TTATAT and TTTAT AN_{17}TACATT, located 80 to 52 and 58 to 29 nt upstream of the *P. furiosus amyA* start codon, respectively, and reminiscent of the *E. coli* consensus promoter sequence, is probably responsible for PFA expression in *E. coli.*

Comparison of PFA and BLA sequences. PFA showed 45 to 56% similarity and 20 to 35% identity to eubacterial α-amylases and other enzymes of the α-amylase family (e.g., neopullulanase, pullulanase, isoamylase, and amylopullulanase) (data not shown). The most similar enzyme was BLA (55.7% similarity and 35.7% identity). Since PFA was significantly more thermostable than the commercial BLA enzyme, the two enzyme sequences were carefully compared (Fig. 1) to look for potentially stabilizing elements in PFA. Conservation was not uniform along the whole sequence: the sequences corresponding to the α/β barrel, B, and C domains showed 40, 22, and 30% identity, respectively. With the exception of helices α_3 and α_4 and loops $\beta_7 \rightarrow \alpha_7$ and $\beta_8 \rightarrow \alpha_8$, which were shorter, the secondary structures of the PFA α/β barrel (Fig. 1) were well conserved. The four active-site consensus regions characteristic of the α-amylase family were present in PFA (Fig. 1). Of the three BLA residues (Asn104, Asp200, and His235) involved in

Ca^{2+} binding (28), only Asn104 was conserved in PFA (Fig. 1). This absence is not surprising, since PFA does not require Ca^{2+} for either its activity or its stability (see below). Interestingly, the two strictly conserved BLA residues involved in chloride binding (Arg229 and Asn326) are present in PFA (Fig. 1).

The amino acid compositions of the two enzymes (not shown) differed in two ways. (i) PFA was more negatively charged (net charge of −21) than BLA (net charge of −8). This difference is mainly due to a lower number of Lys-plus-Arg residues in PFA (35, compared to 54 in BLA) and is probably responsible for the two-unit difference between the isoelectric points of the enzymes (pIs of 4.78 and 6.83 for PFA and BLA, respectively) and for the two-unit difference between their optimum pHs for activity (pH 5.5 and 7.5 for PFA and BLA, respectively) (see below). It is not clear, though, if this charge difference affects PFA's stability. (ii) PFA contained 5% more aromatic residues than BLA (18.5 versus 13.7%). Aromatic residues have been shown to form networks of potentially stabilizing aromatic interactions in some thermostable enzymes (35). Knowledge of the three-dimensional structure of PFA will be required to determine if the additional aromatic residues participate in its stabilization.

PFA is 10% shorter than BLA. The areas affected by deletions in PFA are mostly regions with little secondary structure or higher flexibility, such as loops $\beta_7 \rightarrow \alpha_7$ and $\beta_8 \rightarrow \alpha_8$ in the α/β barrel, or the B domain (28) (29% shorter in PFA than in BLA). Loop shortening has been suggested as a protein-thermostabilizing factor (11). Here, however, the role of a shorter B domain in PFA stabilization is questionable, since the B domain of the mesophilic barley Amy2 α-amylase (not shown) is also much shorter than the BLA B domain. More experiments (e.g., circular dichroism and chemical denaturation studies) are required to address this point.

PFA was also compared to the two other *P. furiosus* amylolytic enzymes sequenced so far, the *P. furiosus* intracellular α-amylase (25) and amylopullulanase (16). No significant similarity with either of these enzymes was found. This absence of similarity is not surprising, since, together with the *Dictyoglomus thermophilum* AmyA α-amylase, *P. furiosus* intracellular α-amylase and amylopullulanase (16) belong to a second α-amylase family.

Purification of the recombinant PFA. PFA was very thermostable. It did not lose any activity when the *E. coli* cell homogenate was treated at 100°C for 20 min. However, more than 50% of its activity was lost after centrifugation, due to coprecipitation of the enzyme with cell debris and other denatured proteins. The precipitated enzyme remained active and was detected after resuspension of the precipitate. Triton X-100 did not significantly prevent the protein from coprecipitating. To reduce coprecipitation, the cell homogenate was heated at 80°C for 15 min. PFA was so hydrophobic that it was directly absorbed onto the phenyl-Sepharose column in the absence of any salt. The hydrophobic interaction was weakened by raising the buffer pH. Since glycerol and ethylene glycol could elute only part of the enzyme, the enzyme was totally eluted with 6 M urea at pH 9.4. The purified PFA displayed one protein band on a native polyacrylamide gel (Fig. 2A) and had a specific activity of 3,900 U/mg at 98°C.

Comparison of the properties of PFA and BLA. The approximate MW of the recombinant PFA was 100,000 as estimated by gel filtration. This value was just twice that calculated according to the deduced polypeptide sequence, indicating that the protein was a homodimer. Figure 2 shows the behavior of the enzyme on native and SDS-polyacrylamide gels. The native gel showed one protein band (Fig. 2A). In SDS-PAGE, when denaturing temperatures were under 60°C, the protein remained dimeric with an apparent MW of 66,000. When denaturation was performed at 90°C or above, a 44,000-MW protein band appeared. At 110°C, all of the dimeric enzyme had dissociated into 44,000-MW monomers along with protein degradation products. Both the dimer and monomer showed sizes in SDS-PAGE that were smaller than the sizes expected from gel filtration and sequencing (100,000 and 52,000, respectively), suggesting that PFA monomers remained partially folded even after denaturation at 110°C in the presence of 2% SDS and 0.64 M mercaptoethanol. This behavior was also observed with other proteins from *Pyrococcus* (16, 33). The fact that α-amylase activity was detected at the level of PFA dimer and monomer bands in starch-containing SDS-PAGE after extensive denaturation (Fig. 2C) confirms this hypothesis. It is not clear, though, if PFA monomers are active or if they reassociate into active dimers after migration in the starch-containing SDS-PAGE. Due to the affinity of the dimeric enzyme for starch, the dimer migrated more slowly on a starch-containing SDS gel than in the absence of starch. A low-MW protein band appeared at the bottom of SDS gels as a result of protein degradation. Protein degradation in SDS buffers also has been observed with other proteins (26).

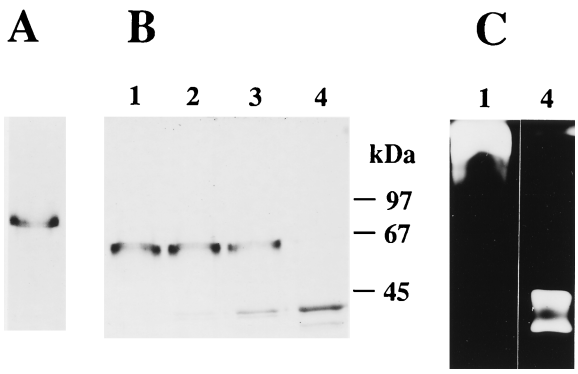

FIG. 2. Behavior of PFA in native PAGE (A), SDS-PAGE (B), and starch-containing SDS-PAGE (C). (A) The protein sample was not denatured before being loaded. (B and C) The protein samples were treated in denaturing buffer at 60°C (lanes 1), 90°C (lane 2), 100°C (lane 3), or 110°C (lanes 4) for 10 min before being loaded. (A and B) Coomassie blue staining; (C) staining for α-amylase activity.

BLA was more sensitive to denaturation than PFA. During denaturation at or below 60°C, the protein retained its dimeric form with an apparent MW of 122,000. Denaturation at 90°C led to complete dissociation of BLA dimers into monomers that migrated at 59,000 (Fig. 3A), as expected from the MW of 58,500 predicted from the BLA sequence. Once dissociated

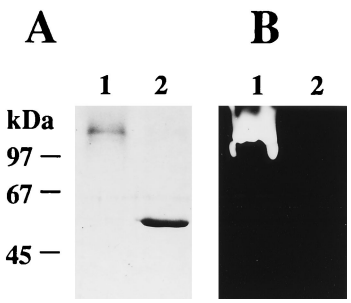

FIG. 3. Behavior of BLA in SDS-PAGE (A) and starch-containing SDS-PAGE (B). Protein samples were treated in denaturing buffer at 60°C (lanes 1) or 90°C (lanes 2) for 10 min before being loaded. (A) Coomassie blue staining; (B) staining for α-amylase activity.

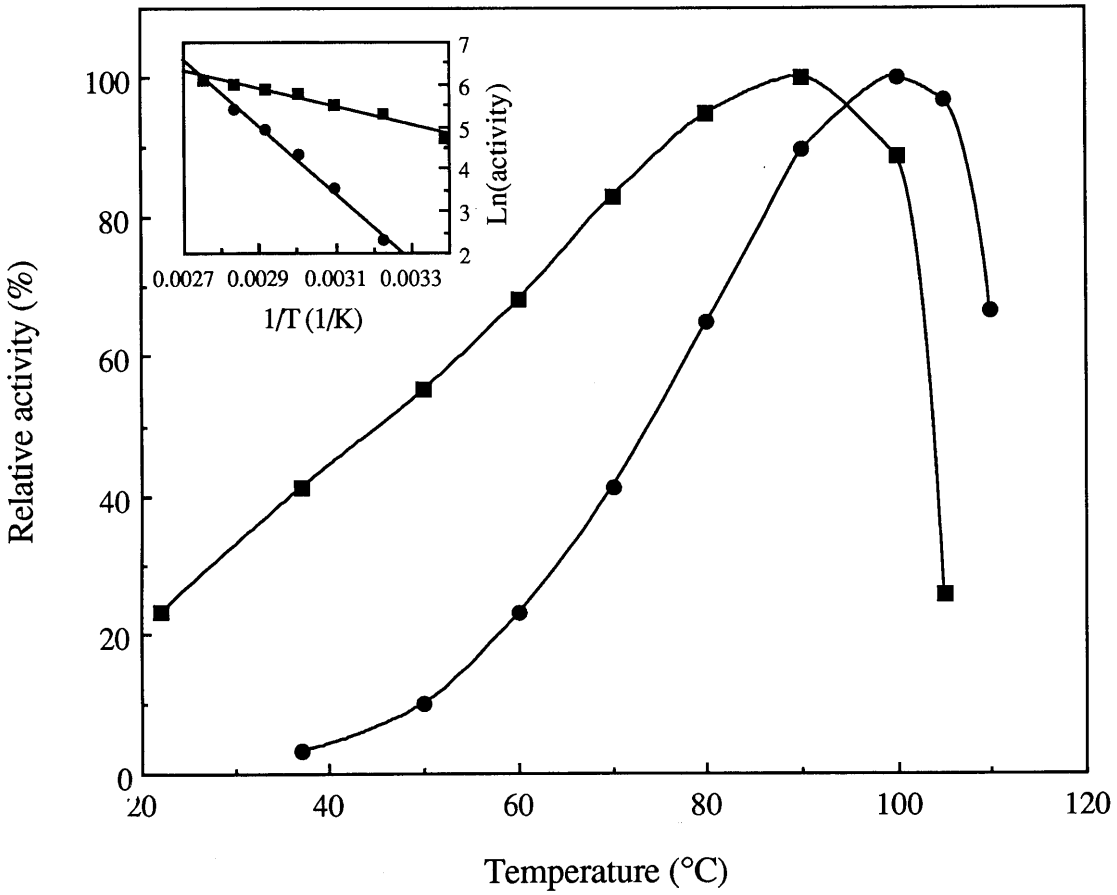

FIG. 4. Effect of temperature on PFA (●) and BLA (■) activities. BLA was assayed in the presence of 0.5 mM Ca^{2+}.

into its monomeric form, BLA was completely inactive (Fig. 3B).

Figure 4 shows the temperature-activity profiles for PFA and BLA. PFA displayed no activity at room temperature. Its activity increased with temperature up to an optimum at 100°C. BLA showed about 22% activity at room temperature and reached its highest activity at 90°C. Both Arrhenius plots were linear (Fig. 4, inset). Activation energies were 70 and 17 kJ mol^{-1} for PFA and BLA, respectively, as calculated from the Arrhenius equation $\ln k = B - E_{act}/RT$ (where k is the rate constant, B is a constant, E_{act} is the activation energy, R is the molar gas constant [8.314 J mol^{-1} K^{-1}], and T is the absolute temperature). Unlike BLA, whose activity increased by approximately 10% in the presence of 0.5 mM Ca^{2+}, PFA did not require Ca^{2+} for activity (data not shown). Figure 5 compares the thermostabilities of PFA and BLA. The time courses of inactivation of PFA in the presence (data not shown) and absence of Ca^{2+} were identical at the two temperatures tested (90 and 98°C). However, Ca^{2+} strongly stabilized BLA. At 90°C, the half-life of BLA was increased more than 20-fold in the presence of 5 mM Ca^{2+}. One hour of incubation at 98°C completely inactivated BLA, even in the presence of 5 mM Ca^{2+}. PFA had a lower optimal pH than BLA: it showed 80% activity or more between pH 4.7 and 7.0, with an optimal pH of around 5.5 to 6.0, whereas BLA was optimally active at around pH 7.0 to 8.0 (data not shown).

PFA hydrolyzed a wide variety of substrates, such as soluble starch, amylose, amylopectin, glycogen, and oligosaccharides. The enzyme did not hydrolyze pullulan, cyclodextrins, sucrose, or G_2 (data not shown). α-Amylases can be classified as liquefying- or saccharifying-type enzymes. Liquefying α-amylases have much wider commercial applications. Table 1 compares the products of hydrolysis of different substrates by PFA and BLA. Like BLA, PFA was a liquefying enzyme. The main products of polysaccharide hydrolysis were G_2 to G_7. A small amount of G_1 was formed after long hydrolysis periods. PFA hydrolyzed long-chain oligosaccharides faster than shorter-chain oligosaccharides, as interpreted from the quantitation of products formed after short versus long incubation times (Table 2).

Table 3 summarizes the biochemical differences between PFA and BLA. Liquefaction of starch requires the use of α-amylase at high temperatures (up to 110°C). BLA has a wide application in industry today. PFA showed properties promising advantages over BLA, however, as follows. (i) It displayed a higher optimal temperature and thermostability than BLA. With PFA, starch liquefaction can be performed at very high temperatures without the risk of losing activity, and the starch concentration can be increased during starch gelatinization and liquefaction. (ii) PFA had a low optimal pH (pH 5.5, versus pH 7.5 for BLA). Thus, starch liquefaction can be performed under pH conditions that reduce by-product formation. (iii) Unlike the case for BLA, Ca^{2+} was not needed for PFA activity and thermostability. Since Ca^{2+} is a strong inhibitor of glucose isomerase, performing starch liquefaction and saccharification in the absence of Ca^{2+} would be a significant

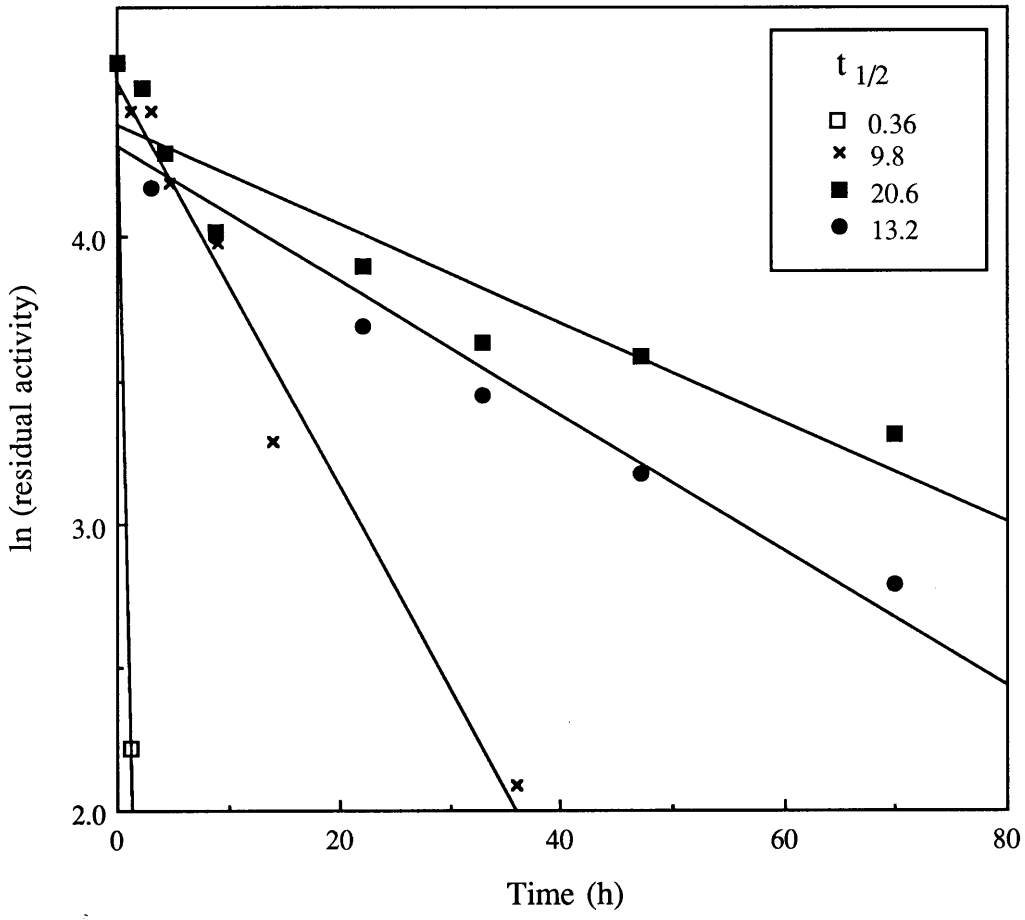

FIG. 5. Thermostabilities of the recombinant PFA (■ and ●) and BLA (× and □) in the absence (■, ●, and □) or presence (×) of 5 mM Ca^{2+} at 90°C (×, □, and ■) or 98°C (●). $t_{1/2}$, half-life.

improvement for high-fructose syrup production. (iv) Finally, PFA is about twice as active at 98°C as BLA is at 90°C.

Overexpression of PFA in *E. coli*. Since very little α-amylase was produced from plasmid pS4 (about 1 mg/liter of culture), we developed a construct that expressed more enzyme. In plasmid pET213, the *P. furiosus* α-amylase gene was cloned under the control of the T7 promoter, without the sequence encoding its signal peptide. A band corresponding to a 45-kDa protein was observed in SDS-PAGE of crude extracts of HMS174(DE3)(pET213) cultures after IPTG induction (data not shown). This band was absent in crude extracts of uninduced HMS174(DE3)(pET213) cultures. Although high levels of α-amylase activity (38,000 U per liter of induced culture) were obtained after induction under standard conditions (LB medium at 37°C), most of the overexpressed protein was found in the insoluble fraction (data not shown). To increase PFA solubility, the *E. coli* thioredoxin gene was overexpressed together with PFA in HMS174(DE3)(pET213)(pT-Trx). Induc-

TABLE 1. Products of hydrolysis of different substrates by PFA and BLA

Substrate	Enzyme source	Product (%)[a]							
		G_1	G_2	G_3	G_4	G_5	G_6	G_7	$>G_7$
Soluble starch	PFA	2.0	27.5	18.2	12.1	16.4	16.0	11.4	5.0
	BLA	13.5	31.9	18.2	11.5	16.5	6.1	2.4	1.1
Glycogen	PFA	1.0	15.7	14.9	14.7	15.4	15.1	7.8	15.3
	BLA	9.9	26.4	13.7	12.6	14.6	5.8	4.9	12.9
Amylose	PFA	2.4	41.7	23.2	11.4	11.9	9.5	0.0	0.0
	BLA	15.4	37.5	22.1	10.3	14.6	0.0	0.0	0.0

[a] The incubation period was 46 h.

TABLE 2. PFA product specificity: comparison of the hydrolysis products of different oligosaccharides

Substrate	Time (h)	Product/residual substrate (%)						
		G_1	G_2	G_3	G_4	G_5	G_6	G_7
G_4	0.8	0.0	2.3	13.0	84.8			
	46.0	0.2	25.2	24.7	49.5			
G_5	0.8	0.0	2.0	3.0	11.3	83.7		
	46.0	0.6	19.2	24.4	21.1	35.0		
G_6	0.8	0.1	1.5	1.8	2.0	14.2	80.4	
	46.0	1.6	18.3	18.2	14.5	19.2	28.1	
G_7	0.8	1.2	4.2	0.9	1.7	9.1	15.6	67.4
	46.0	3.64	32.6	12.4	14.0	23.0	13.5	0.9

TABLE 3. Comparison of general biochemical properties of PFA and BLA

Property	Value for:	
	PFA	BLA
Mol wt	100,000	122,000
Sp act (U/mg)	3,900 at 98°C	2,000 at 90°C
Subunit activity	Yes	No
Optimal pH	5.5–6.0	7.0–8.0
Optimal temp (°C)	100	90
Ca^{2+} requirement	No	Yes
Half-life (h) at 98°C	13	<1
End product	G_2–G_7	G_1–G_6

tion was carried out at 18°C in the presence of 1% ethanol. Twenty-eight milligrams of soluble PFA (109,000 U of α-amylase activity) could be recovered from a 1-liter induced culture.

PFA represents an excellent model enzyme for studying protein hyperthermostability, since it is highly similar to eubacterial α-amylases for which a wealth of data, including thermostability (37, 38, 41) and structural (7, 10, 28, 30, 31) data, are already available. Our overexpression system is still not optimal for commercial purposes, since the system requires IPTG and enzyme levels could be increased. It allows us, though, to make gram quantities of enzyme that will be tested for utility under practical industrial jet-cooking conditions or crystallized for structural studies.

PFA and other pyrococcal amylolytic enzymes. Koch et al. (24) described an extracellular α-amylase activity present in the supernatants of *P. furiosus* cultures. The activity they described corresponded to two starch-degrading protein bands of 96 and 136 kDa in native PAGE. It is not clear from their work if these two bands correspond to one or two separate enzymes and if the 96-kDa band is similar to the enzyme described here. It is unlikely that these bands are the *P. furiosus* amylopullulanase (9, 16), since no pullulanase activity was detected in their enzyme preparation (24). Another member of the order *Thermococcales*, *T. profundus*, optimally growing at 80°C, produced two extracellular amylases, S and L (12). With a molecular mass of 42 kDa in SDS-PAGE, amylase S is optimally active at pH 5.5 to 6.0 and 80°C and does not require Ca^{2+} for its activity. While no sequence is available for amylase S, its catalytic properties suggest that it is the *T. profundus* counterpart of PFA. Amylase L, a larger enzyme, could correspond to the *P. furiosus* 136-kDa amylolytic enzyme detected by Koch et al. (24).

A *P. woesei* extracellular α-amylase (PWA) has been purified and characterized by Koch et al. (23). PFA and PWA are optimally active under the same conditions of pH and temperature and have similar resistance to thermal inactivation. Although PWA was described as a 70,000-MW enzyme, as indicated from migration in SDS-PAGE, it could correspond to a dimeric enzyme showing aberrant behavior under these electrophoresis conditions, a behavior similar to that observed with PFA. PFA and PWA seem to differ in two aspects: (i) PWA shows almost six-times-lower specific activity than PFA (667 versus 3,900 U mg^{-1}), and (ii) their amino acid compositions seem to be different (in particular, PFA contains half the threonine residues present in PWA). *P. furiosus* and *P. woesei* amylopullulanases were also shown to be significantly different in a few aspects (16). Although these two organisms are considered to be very closely related, they still contain quite different enzymes.

PFA is the first archaeal amylolytic enzyme described that belongs to the α-amylase family. The characterization of extracellular α-amylases with similar properties from other hyperthermophilic archaea (12, 23) suggests that these enzymes also belong to the α-amylase family and that this enzyme family is widespread among the three kingdoms.

With the cloning and characterization of PFA, three *P. furiosus* amylolytic enzymes—intracellular α-amylase (25, 26), extracellular α-amylase, and amylopullulanase (16)—have now been characterized. We propose that *P. furiosus* amylopullulanase and PFA are involved in starch degradation. A putative integral membrane protein encoded by an ORF located upstream of the amylopullulanase gene (16) might participate in transporting the starch hydrolysis products inside the cells, where α-glucosidase hydrolyses them to G_1 (13). Because starch is typically an extracellular compound, the function of *P. furiosus* intracellular α-amylase is not clear. Several other starch-degrading hyperthermophilic eubacteria and archaea also contain two or more amylases. So far, though, all of them are extracellular (12, 18, 21, 24) or exposed on the cell surface (34).

ACKNOWLEDGMENTS

We gratefully acknowledge Maris Laivenieks for his technical assistance and helpful discussions. Plasmid pT-Trx was kindly provided by Shunsuke Ishii (University of Tsukuba, Ibaraki, Japan).

This research was supported by a grant from the Cooperative State Research Service, U.S. Department of Agriculture, under agreement 90-34189-5014 and by a grant from the National Research Initiative Competitive Grants Program, U.S. Department of Agriculture, under agreement 96-35500-3195.

REFERENCES

1. **Adams, M. W. W.** 1993. Enzymes and proteins from organisms that grow near and above 100°C. Annu. Rev. Microbiol. **47:**627–658.
2. **Adams, M. W. W., F. B. Perler, and R. M. Kelly.** 1995. Extremozymes: expanding the limits of biocatalysis. Bio/Technology **13:**662–668.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1993. Current protocols in molecular biology. Greene Publishing, New York, N.Y.
4. **Bauer, M. W., E. J. Bylina, R. V. Swanson, and R. M. Kelly.** 1996. Comparison of a β-glucosidase and a β-mannosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*: purification, characterization, gene cloning and sequence analysis. J. Biol. Chem. **271:**23749–23755.
5. **Bentley, I. S., and E. C. Williams.** 1996. Starch conversion, p. 339–357. *In* T. Godfrey and S. I. West (ed.), Industrial enzymology, 2nd ed. Stockton Press, New York, N.Y.
6. **Bernfeld, P.** 1955. Amylases α- and β-. Methods Enzymol. **1:**149–158.
7. **Boel, E., L. Brady, A. M. Brzozowsk, Z. Derewenda, G. G. Dogson, V. J. Jensen, S. B. Petersen, H. Swift, L. Thim, and H. F. Woldike.** 1990. Calcium binding in α-amylases: an X-ray diffraction study at 2.1-Å resolution of two enzymes from *Aspergillus*. Biochemistry **29:**6244–6249.
8. **Brown, S. H., H. R. Costantino, and R. M. Kelly.** 1990. Characterization of amylolytic activities associated with the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Appl. Environ. Microbiol. **56:**1985–1991.
9. **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.
10. **Buisson, G., E. Duee, R. Haser, and F. Payan.** 1987. Three dimensional structure of porcine pancreatic α-amylase at 2.9 Å resolution. Role of calcium in structure and activity. EMBO J. **6:**3909–3916.
11. **Chan, M. K., S. Mukund, A. Kletzin, M. W. W. Adams, and D. C. Rees.** 1995. Structure of a hyperthermophilic tungstopterin enzyme, aldehyde ferredoxin oxidoreductase. Science **267:**1463–1469.
12. **Chung, Y. C., T. Kobayashi, H. Kanai, T. Akiba, and T. Kudo.** 1995. Purification and properties of extracellular amylase from the hyperthermophilic archaeon *Thermococcus profundus* DT5432. Appl. Environ. Microbiol. **61:** 1502–1506.
13. **Costantino, H. R., S. H. Brown, and R. M. Kelly.** 1990. Purification and characterization of an α-glucosidase from a hyperthermophilic archaebacterium, *Pyrococcus furiosus*, exhibiting a temperature optimum of 105 to 115°C. J. Bacteriol. **172:**3654–3660.
14. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
15. **DiRuggiero, J., F. T. Robb, R. Jagus, H. H. Klump, K. M. Borgest, M. Kessel,**

X. Mai, and M. W. W. Adams. 1993. Characterization, cloning, and in vitro expression of the extremely thermostable glutamate dehydrogenase from the hyperthermophilic archaeon, ES4. J. Biol. Chem. **268:**17767–17774.
16. **Dong, G., C. Vieille, and J. G. Zeikus.** 1997. Cloning, sequencing, and expression of the gene encoding amylopullulanase from *Pyrococcus furiosus* and biochemical characterization of the recombinant enzyme. Appl. Environ. Microbiol. **63:**3577–3584.
17. **Fiala, G., and K. O. Stetter.** 1986. *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. Arch. Microbiol. **145:**56–61.
18. **Fukusumi, S., A. Kamizono, S. Horinouchi, and T. Beppu.** 1988. Cloning and nucleotide sequence of a heat-stable amylase gene from an anaerobic thermophile, *Dictyoglomus thermophilum*. Eur. J. Biochem. **174:**15–21.
19. **Hain, J., W.-D. Reiter, U. Hudepohl, and W. Zillig.** 1992. Elements of an archaeal promoter defined by mutational analysis. Nucleic Acids Res. **20:** 5423–5428.
20. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
21. **Horinouchi, S., S. Fukusumi, T. Ohshima, and T. Beppu.** 1988. Cloning and expression in *Escherichia coli* of two additional amylase genes of a strictly anaerobic thermophile, *Dictyoglomus thermophilum*, and their nucleotide sequences with extremely low guanine-plus-cytosine contents. Eur. J. Biochem. **176:**243–253.
22. **Jespersen, H. M., E. A. MacGregor, B. Henrissat, M. R. Sieks, and B. Svensson.** 1993. Starch- and glycogen-debranching and branching enzymes: prediction of structural features of the catalytic $(\beta/\alpha)_8$-barrel domain and evolutionary relationship of other amylolytic enzymes. J. Protein Chem. **12:**791–805.
23. **Koch, R., A. Spreinat, K. Lemke, and G. Antranikian.** 1991. Purification and properties of a hyperthermoactive α-amylase from archaebacterium *Pyrococcus woesei*. Arch. Microbiol. **155:**572–578.
24. **Koch, R., P. Zablowski, A. Spreinat, and G. Antranikian.** 1990. Extremely thermostable amylolytic enzyme from archaebacterium *Pyrococcus furiosus*. FEMS Microbiol. Lett. **71:**21–26.
25. **Laderman, K. A., K. Asada, T. Uemori, H. Mukai, Y. Taguchi, I. Kato, and C. B. Anfinsen.** 1993. α-Amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*, cloning and sequencing of the gene and expression in *Escherichia coli*. J. Biol. Chem. **268:**24402–24407.
26. **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 archaebacterium *Pyrococcus furiosus*. J. Biol. Chem. **268:**24394–24401.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
28. **Machius, M., G. Wiegand, and R. Huber.** 1995. Crystal structure of calcium-depleted *Bacillus licheniformis* α-amylase at 2.2 Å resolution. J. Mol. Biol. **246:**545–559.
29. **Perrin, D. D., and B. Dempsey (ed.).** 1979. Buffers for pH and metal ion control. Chapman and Hall, Ltd., London, United Kingdom.
30. **Qian, M., R. Haser, G. Buisson, E. Duée, and F. Payan.** 1994. The active center of a mammalian α-amylase. Structure of the complex of a pancreatic α-amylase with a carbohydrate inhibitor refined to 2.2-Å. Biochemistry **33:** 6284–6294.
31. **Qian, M., R. Haser, and F. Payan.** 1993. Structure and molecular model refinement of pig pancreatic α-amylase at 2.1 Å resolution. J. Mol. Biol. **231:**785–799.
32. **Reiter, W.-D., P. Palm, and W. Zillig.** 1988. Transcription termination in the archeabacterium *Sulfolobus*: signal structures and linkage to transcription initiation. Nucleic Acids Res. **16:**2445–2459.
33. **Rüdiger, A., P. L. Jorgensen, and G. Antranikian.** 1995. Isolation and characterization of a heat-stable pullulanase from the hyperthermophilic archaeon *Pyrococcus woesei* after cloning and expression of its gene in *Escherichia coli*. Appl. Environ. Microbiol. **61:**567–575.
34. **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.
35. **Teplyakov, A. V., I. P. Kuranova, E. H. Harutyunyan, B. K. Vainshtein, C. Frömmel, W. E. Höhne, and K. S. Wilson.** 1990. Crystal structure of thermitase at 1.4 Å resolution. J. Mol. Biol. **214:**261–279.
36. **Tiboni, O., P. Cammarano, and A. M. Sanangelantoni.** 1993. Cloning and sequencing of the gene encoding glutamine synthetase I from the archaeum *Pyrococcus woesei*: anomalous phylogenies inferred from analysis of archaeal and bacterial glutamine synthetase I sequences. J. Bacteriol. **175:**2961–2969.
37. **Tomazic, S. J., and A. M. Klibanov.** 1988. Mechanisms of irreversible thermal inactivation of *Bacillus* α-amylases. J. Biol. Chem. **263:**3086–3091.
38. **Tomazic, S. J., and A. M. Klibanov.** 1988. Why is one *Bacillus* α-amylase more resistant against irreversible thermoinactivation than another? J. Biol. Chem. **263:**3092–3096.
39. **Vieille, C., D. S. Burdette, and J. G. Zeikus.** 1996. Thermozymes. Biotechnol. Annu. Rev. **2:**1–83.
40. **Vieille, C., J. M. Hess, R. M. Kelly, and J. G. Zeikus.** 1995. *xylA* cloning and sequencing and biochemical characterization of xylose isomerase from *Thermotoga neapolitana*. Appl. Environ. Microbiol. **61:**1867–1875.
41. **Violet, M., and J.-C. Meunier.** 1989. Kinetic study of the irreversible thermal denaturation of *Bacillus licheniformis* α-amylase. Biochem. J. **263:**665–670.
42. **Volkin, D. B., and C. R. Middaugh.** 1992. The effect of temperature on protein structure, p. 215–247. *In* T. J. Ahern and M. C. Manning (ed.), Stability of protein pharmaceuticals, part A. Chemical and physical pathways of protein degradation. Plenum Press, New York, N.Y.
43. **Voorhorst, W. G. B., R. I. L. Eggen, A. C. M. Geerling, C. Platteeuw, R. J. Siezen, and W. M. de Vos.** 1996. Isolation and characterization of the hyperthermostable serine protease, pyrolysin, and its gene from the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Biol. Chem. **271:**20426–20431.
44. **Voorhorst, W. G. B., R. I. L. Eggen, E. J. Luesink, and W. M. de Vos.** 1995. Characterization of the *celB* gene coding for β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* and its expression and site-directed mutation in *Escherichia coli*. J. Bacteriol. **177:**7105–7111.
45. **Watson, M. E. E.** 1984. Compilation of published signal sequences. Nucleic Acids Res. **12:**5145–5264.
46. **Yasukawa, T., C. Kanei-Ishii, T. Maekawa, J. Fujimoto, T. Yamamoto, and S. Ishii.** 1995. Increase of solubility of foreign proteins in *Escherichia coli* by coproduction of the bacterial thioredoxin. J. Biol. Chem. **270:**25328–25331.
47. **Zwickl, P., S. Fabry, C. Bogedain, A. Haas, and R. Hensel.** 1990. Glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic archaebacterium *Pyrococcus woesei*: characterization of the enzyme, cloning and sequencing of the gene, and expression in *Escherichia coli*. J. Bacteriol. **172:** 4329–4338.