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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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-glycosidic 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 (α/β)\(_{8}\) barrel; (ii) domain B is the very long (α/β)\(_{8}\) 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 interest. These bacteria have been extensively characterized and their genes have been cloned. With the exception from eubacteria, fungi, plants, and animals have been characterized, and their genes have been cloned. With the exception from eubacteria, fungi, plants, and animals have been characterized, and their genes have been cloned. With the exception from eubacteria, fungi, plants, and animals have been characterized, and their genes have been cloned.

**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% Phytage (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'-CAGATTCACCGTGTGATGGG3' 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 (amyl) was changed using an in vitro mutagenesis approach. The nucleotide sequence of the reconstructed pFA DNA was analyzed by using the Sequenase method as described above.
to ATG by using the QuikChange site-directed mutagenesis kit (Stratagene) with primers 5′-GGAGTGATCACGATGACAAAGAATTAAACACC-3′ and 5′-GGGTTAATTTTGTATTTGTATGATTAGCT-3′. Oligonucleotides were synthesized by the Michigan State University Macromolecular Facility. 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/in² 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% (NH₄)₂SO₄ to the supernatant, and the pellet was resuspended in 50 mM sodium acetate (pH 5.6).

The concentrated crude enzyme was loaded onto a phenyl-Sepharose (Pharmacia Fine Chemica AB) column (1.5 by 8 cm) equilibrated with 50 mM sodium 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 sodium acetate (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 0.2 M NaCl. The active fractions were then 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.

Commercially Thermo-1 form 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. At 90°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 minute under the assay conditions.

**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* genomic DNA.

**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, pullullan, glycogen, amylase, 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 PA1 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), maltose (G₂), maltotriose (G₃), maltnolotetraose (G₄), maltopentaose (G₅), maltnohexaose (G₆), and maltotetraose (G₅) were the standards. Starch, pullulan, glycogen, amylase, 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) buffers at 80°C. Two other pairs of buffers were used for temperature studies (Table 2). Temperatures were calculated by using the equation ΔpK/ΔΤ cac = −0.006 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 Ca²⁺.

The temperature and maximal activities of PFA and BLA were determined by using 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 Ca²⁺ (for BLA) containing 2 mM EDTA and then twice against the same buffer without EDTA. Enzyme thermal inactivation studies were performed at temperatures of 50°C, 60°C, and 70°C. Two other pairs of buffers were used for temperature studies (Table 2). Activity remaining was measured at 90°C 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. AF016268.
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 TTTATA, 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 +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 genes, the starting GTG was mutagenized into ATG.

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 corresponded to the α/β barrel, B, and C domains showed 40, 22, and 30% identity, respectively, and reminiscent of the *E. coli* consensus promoter sequences. One can expect that archaeal genes spontaneously expressed in *E. coli* are expressed from a sequence reminiscent of the *E. coli* starts codon, respectively, and reminiscent of the *E. coli* consensus promoter sequence, is probably responsible for PFA expression in *E. coli*.

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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 \(\beta_2 \rightarrow \alpha_7\) and \(\beta_3 \rightarrow \alpha_8\) in the \(\alpha/\beta\) 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 \(\alpha\)-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 \(\alpha\)-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 \(Dicyoglomus thermophilum\) AmyA \(\alpha\)-amylase, \(P. furiosus\) intracellular \(\alpha\)-amylase and amylopullulanase (16) belong to a second \(\alpha\)-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 glyceral 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 polyacylamide 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).

**Purification of the recombinant BLA.** 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
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 \( \ln k = \frac{E_{\text{act}}}{R} \left( \frac{1}{T} \right) \) (where \( k \) is the rate constant, \( E_{\text{act}} \) 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 Ca²⁺, PFA did not require Ca²⁺ 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 Ca²⁺ were identical at the two temperatures tested (90 and 98°C). However, Ca²⁺ strongly stabilized BLA. At 90°C, the half-life of BLA was increased more than 20-fold in the presence of 5 mM Ca²⁺. One hour of incubation at 98°C completely inactivated BLA, even in the presence of 5 mM Ca²⁺. 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). \( \alpha \)-Amylases can be classified as liquefying- or saccharifying-type enzymes. Liquefying \( \alpha \)-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 \( \alpha \)-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, Ca²⁺ was not needed for PFA activity and thermostability. Since Ca²⁺ is a strong inhibitor of glucose isomerase, performing starch liquefaction and saccharification in the absence of Ca²⁺ would be a significant advantage for PFA.
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-

![Graph showing thermostabilities of recombinant PFA and BLA](https://journals.asm.org/journal/aem)

**FIG. 5.** Thermostabilities of the recombinant PFA (■ and ●) and BLA (× and □) in the absence (■, ●, and □) or presence (×) of 5 mM CaCl₂ at 90°C (×, □, and ■) or 98°C (●). t₁/₂, half-life.

<table>
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<tr>
<th>Substrate</th>
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<th>Product (%)</th>
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<td>Soluble starch</td>
<td>PFA</td>
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</tr>
<tr>
<td>Glycogen</td>
<td>PFA</td>
<td>1.0 15.7 14.9 14.7 15.4 15.1 7.8 15.3</td>
</tr>
<tr>
<td>Amylose</td>
<td>PFA</td>
<td>2.4 41.7 23.2 11.4 11.9 9.5 0.0 0.0</td>
</tr>
<tr>
<td>Glycogen</td>
<td>BLA</td>
<td>9.9 26.4 13.7 12.6 14.6 5.8 4.9 12.9</td>
</tr>
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<tr>
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<th>Time (h)</th>
<th>Product/residual substrate (%)</th>
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<tr>
<td>46.0 0.2 25.2 24.7 49.5</td>
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<tr>
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<td>0.8 0.0 2.0 3.0 11.3 83.7</td>
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<td>46.0 3.64 32.6 12.4 14.0 23.0 13.5 0.9</td>
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* The incubation period was 46 h.
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 gran 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* amylppullulanase (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 Ca2+ 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 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* amyllopullulanases 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 amylolpullulanase (16)—have now been characterized. We propose that *P. furiosus* amylolpullulanase and PFA are involved in starch degradation. A putative integral membrane protein encoded by an ORF located upstream of the amylolpullulanase gene (16) might participate in transporting the starch hydrolysis products inside the cells, where α-glucosidase hydrolyses them to G₆ (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).

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