Extremophiles (1998) 2:23–32 © Springer-Verlag 1998

ORIGINAL PAPER

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Extracellular α-amylase from *Thermus filiformis* Ork A2: purification and biochemical characterization

Received: June 2, 1997 / Accepted: September 16, 1997

Abstract An extracellular α-amylase produced by the thermophilic bacterium *Thermus filiformis* Ork A2 was purified from cell-free culture supernatant by ion exchange chromatography. The molecular mass was estimated to be 60000 Da by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The enzyme was rich in both basic and hydrophobic amino acids, presenting the following NH_2-terminal amino acid sequence: Thr-Ala-Asp-Leu-Ile-Val-Lys-Ile-Asn-Phe. Amylolytic activity on soluble starch was optimal at pH 5.5–6.0 and 95°C, and the enzyme was stable in the pH range of 4.0–8.0. Calcium enhanced thermostability at temperatures above 80°C, increasing the half-life of activity to more than 8 h at 85°C, 80 min at 90°C, and 19 min at 95°C. Ethylenediaminetetraacetic acid (EDTA) inhibited amylase activity, the inhibition being reversed by the addition of calcium or strontium ions. The α-amylase was also inhibited by copper and mercuric ions, and *p*-chloromercuribenzoic acid, the latter being reversed in the presence of dithiothreitol. Dithiothreitol and β-mercaptoethanol activated the enzyme. The α-amylase exhibited Michaelis-Menten kinetics for starch, with a K_m of 5.0 mg·ml^{-1} and k_{cat}/K_m of 5.2×10^5 ml·mg^{-1}s^{-1}. Similar values were obtained for amylose, amylopectin, and glycogen. The hydrolysis pattern was similar for maltooligosaccharides and polysaccharides, with maltose being the major hydrolysis product. Glucose and maltotriose were generated as secondary products, although glucose was produced in high levels after a 6-h digestion. To our knowledge this is the first report of the characterization of an α-amylase from a strain of the genus *Thermus*.

Key words *Thermus filiformis* · α-Amylase · Extracellular · Purification · Properties · Thermostability

Communicated by G. Antranikian

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Introduction

Bacteria of the genus *Thermus* have been isolated from natural hydrothermal environments associated with areas of volcanic or geothermal activity and from artificial hot water environments such as hot tap water or thermally polluted streams (Brock 1986). *Thermus aquaticus* YT-1 was the first *Thermus* strain to be isolated by Brock and Freeze (1969) from a hot spring in Yellowstone National Park, USA. Since then many strains have been isolated either in the United States (Munster et al. 1986), in Japan (Saiki et al. 1972, Taguchi et al. 1982), Iceland (Hudson et al. 1986), New Zealand (Hudson et al. 1986), Portugal, and in the Azores Islands (Santos et al. 1989). Taxonomic studies led thus far to the description of six species: *Thermus aquaticus*, *Thermus brockianus*, *Thermus filiformis*, *Thermus scotoductus*, *Thermus oshimai*, and *Thermus thermophilus* (Nobre et al. 1996).

Several enzymes produced by *Thermus* strains have been purified and characterized, some of which have biotechnological applications. While there have been numerous reports on extracellular proteases (Cowan et al. 1987a; Matsuzawa et al. 1988; Peek et al. 1992; Saravani et al. 1989; Taguchi et al. 1983), little information is available on the amylolytic activities of *Thermus* species. The purification and characterization of a cell-associated pullulanase produced by *Thermus aquaticus* YT-1 has been described (Plant et al. 1986). Another pullulanase possessing α-1,4 activity is produced extracellularly by a presumed *Thermus* strain AMD 33, a strain isolated in Japan (Nakamura et al. 1989). Recently, another pullulanase produced by *Thermus caldophilus* GK-24 has been described (Kim et al. 1996).

Amylolytic activity appears to be an unusual property of *Thermus* (Manaia and da Costa 1991), although Munster et al. (1986) reported that a number of strains isolated in the USA were able to grow on starch-containing medium.

On the other hand, amylase production is rather common in *Bacillus* strains, where this class of enzyme has been extensively studied. Some of the enzymes possessing higher thermostability, such as the α-amylases from *Bacillus licheniformis* or *Bacillus stearothermophilus,* are already used in the industrial degradation of starch to glucose (Zamost et al. 1991). Highly thermostable amylases are also produced by several hyperthermophiles. These enzymes present maximum activity levels between 80° and 100 °C, e.g., *Thermococcus profundus* amylase (Chung et al. 1995) and *Pyrococcus furiosus* amylase (Laderman et al. 1993), with measurable activity at 130°–140°C (Brown and Kelly 1993).

In this paper we describe the isolation, purification, and characterization of an α-amylase produced by *Thermus filiformis* strain Ork A2, a strain isolated in New Zealand.

Material and methods

Bacterial strain, medium, and culture conditions

Strain Ork A2 used in this study was isolated by D.A. Cowan (unpublished results) from hydrothermal sediments taken from the Orakei Korako thermal field of New Zealand. The isolate Ork A2 produced yellow colonies, was gram negative, and formed nonmotile, nonsporulating rods typical of the genus *Thermus*. This strain was shown to belong to *Thermus filiformis* on the basis of a complete 16S rDNA sequence analysis (F.A. Rainey, personal communication). For α-amylase production the strain was grown aerobically at 70°C in *Thermus* medium (Brock 1978) supplemented with 1% (w/v) soluble starch (Sigma St. Louis, MO, USA).

Protein assay

The protein concentration was estimated by the Bio-Rad (Hercules, CA, USA) protein assay method as described by Bradford (1976) using the microassay procedure. Bovine serum albumin (Sigma) was used as the standard protein.

Amylolytic activity

Amylase activity was determined by the rate of absorbance decrease of the colored starch-I_2 complex, using the modified procedure of Blanchin-Roland and Masson (1989). An appropriate enzyme dilution was made up to a final volume of 100 μl with 15 mM sodium acetate buffer, pH 5.8, and added to 1 ml of 0.25% (w/v) soluble starch in the same buffer, which had been boiled and filtered through Whatman No.1 filter paper while hot. The reaction mixture was incubated at 70°C, and 50-μl aliquots were removed at various times and diluted into 1-ml volumes of iodine solution (freshly prepared by adding 200 μl of a 2.2% I_2/4.4% KI (w/v) solution into 100 ml of 2% (w/v) KI). The rate of decrease in absorbance was measured at 620 nm, where one unit of enzyme activity corresponded to the hydrolysis of 1 mg of starch·min^{-1} at 70°C. The pH of the substrate solution was adjusted at the assay temperature. Enzyme samples were diluted as necessary to maintain linearity over the assay interval used.

When chemical additions interfered with the assay, amylase activity was determined by measuring the increase in reducing sugar formed by hydrolysis of soluble starch. The amount of reducing sugar was quantified by a modification of the dinitrosalicylic acid method (Bernfeld 1955). An appropriate enzyme dilution was made up to a final volume of 100 μl with 15 mM sodium acetate buffer, pH 5.8 and added to 1 ml of 0.25% (w/v) starch in 15 mM sodium acetate buffer, pH 5.8, prepared as already described, and incubated at 70°C. At various times, 1 ml of the solution of 1% (w/v) dinitrosalicylic acid; 0.4 M NaOH; 30% (w/v) sodium, potassium tartrate was added. This mixture was boiled for 5 min and diluted with 5 ml of water. The absorbances were measured at 540 nm, where one unit of enzyme activity corresponded to the release of 1 μmol of reducing sugar (as maltose) per min at 70°C.

Amylase purification

Cells were removed from the culture medium by centrifugation at 13000 × *g*, for 10 min at 4°C. The supernatant was diluted 1:1 with Milli Q water and added, in 500-ml batches, to 40 ml of S-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden), previously equilibrated in 10 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (Pipes)-NaOH, 2 mM $CaCl_2$, pH 6.5. The amylase was eluted with 200 ml of a linear 0–0.7 M NaCl gradient in 10 mM Tris-HCl, 2 mM $CaCl_2$, pH 8.0. Active fractions were bulked between salt concentrations of 0.12–0.2 M; the NaCl was removed by dialysis against 10 mM Tris-HCl, 2 mM $CaCl_2$, pH 8.0; and the sample applied to a Mono Q anion-exchange column (1 × 10 cm, Pharmacia) equilibrated in 10 mM Tris-HCl, 2 mM $CaCl_2$, pH 8.0, connected to a fast protein liquid chromatography (FPLC) system (Pharmacia). The amylase fraction was recovered in the nonadsorbed protein fraction. All purification steps were conducted at room temperature.

Gel electrophoresis and molecular mass determination

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed on a Phast System (Pharmacia) using Phast Gels Homogeneous 20. Amylase samples were boiled for 5 min in the presence of 2.5% SDS and 10 mM phenylmethylsulfonyl fluoride (PMSF). Proteins were stained with silver nitrate using the Phastgel Silver Kit (Pharmacia). The molecular mass of the enzyme was estimated from the positions of standard proteins (low molecular weight calibration kit, Pharmacia). The native molecular mass was determined by size-

exclusion chromatography in a Bio-Gel P-100 column (1.5 × 86 cm, Bio-Rad) equilibrated in 50 mM Tris-HCl, 2 mM $CaCl_2$, pH 8.0. Due to low protein concentration, the amylase sample was applied simultaneously with 1 mg of the following standard proteins: thyroglobulin, 669 000 Da; bovine albumin, 66 000 Da; egg albumin, 45 000 Da; deoxyribonuclease I, 31 000 Da; and cytochrome *c*, 12 400 Da, all from Sigma, and its chromatographic profile was followed by determination of amylolytic activity in eluted fractions.

Isoelectric point determination and activity staining

Isoelectric focusing (IEF) was performed in a Phast System (Pharmacia) using Dry IEF Gels (Pharmacia), which were hydrated with 75 µl of ampholine solution (Pharmalyte 8–10.5) in 1.025 ml of water during 30 min at room temperature. Protein bands were stained with silver nitrate using the Phastgel Silver Kit (Pharmacia). The isoelectric point of α-amylase was determined from the position of standard protein markers from 5–10.5 (High pI kit, Pharmacia). Activity staining was performed by overlaying a gel made of 0.25% (w/v) soluble starch, 15 mM acetate buffer, 1.5% (w/v) agar, pH 5.8 on an IEF gel, for 30 min at room temperature, followed by a 60-min incubation at 70°C. Hydrolyzed starch fractions were visualized as clear bands on a blue gel, after staining with I_2/KI solution.

The molecular weight determination of the active band obtained in IEF was performed by dissolution of the acrylamide of the IEF gel with 10 µl hydrogen peroxide 30% (v/v) for 12 h at 85°C. The resulting mixture was boiled for 5 min in 50 mM Tris-*N*,*N*'-(bis-2-hydroxyethyl)-glycine (Bicine), 1% (w/v) SDS, 1 mM β-mercaptoethanol, 4 M urea, and 10 mM PMSF. The samples were separated by SDS-PAGE on a 15% gel, according to the method of Weber and Osborn (1969) in 100 mM Tris-Bicine, 0.1% (w/v) SDS buffer. Proteins were detected by silver staining (Coligan et al. 1996).

Amino acid analysis

The amino acid composition of the purified α-amylase was determined according to the method of Heinrickson and Meredith (1984). After acid hydrolysis with 6 N HCl for 18 h at 115°C under N_2, the resulting amino acids were derivatized using the phenylisothiocyanate method, and separated and identified by reverse phase (RP)-HPLC using a Superspher column (244 × 4 mm, Merck Darmstadt, Germany).

NH_2-terminal sequence

The NH_2-terminal amino acid sequence of the purified α-amylase was determined by stepwise Edman degradation (Edman and Begg 1967) in an automatic protein sequencer (Applied Biosystems 473 A, Foster City, CA, USA), using an electroblotted sample of the enzyme. After electrophoretic separation by SDS-PAGE, the acrylamide gel was incubated for 5 min in 10 mM 3-cyclohexylamine-1-propanesulfonic acid (Caps), 10% (v/v) methanol and overlaid on an Immobilon (Millipore Bedford, MA, USA) membrane, treated in the same way as the gel. The transfer was performed at 500 V for 60 min at 7°C in 10 mM Caps, 10% methanol. The membrane was stained with 0.1% (w/v) Coomassie brilliant blue R-250 in 40% (v/v) methanol and destained in 50% (v/v) methanol.

Effects of pH and temperature

The amylolytic activity was determined by varying the buffer of the substrate solution in the standard assay: 15 mM acetate buffer for the pH range 4.0–6.0, phosphate buffer for pH 6.0–8.0, and borate buffer for pH 8.0–9.0. The same buffers were used for determination of the pH stability of the enzyme over 60 min at 70°C. Due to the low amount of protein present in each assay (66 ng), bovine serum albumin (BSA) was added at a concentration of 50 $\mu g \cdot ml^{-1}$ in the thermostability assays.

The optimal temperature for activity was determined by assaying activity between 40° and 95°C, in the presence and absence of 2 mM $CaCl_2$. For thermostability determinations, amylase samples were incubated for 8 h in 15 mM acetate buffer, pH 5.8 in the presence of BSA, 50 $\mu g \cdot ml^{-1}$, at several temperatures. At specific intervals, samples were withdrawn and assayed for residual amylolytic activity.

Analysis of hydrolysis products

The purified α-amylase was incubated at 70°C for 6 h in the presence of 0.25% (w/v) soluble starch (Sigma), amylose (Merck), amylopectin (Merck), glycogen (Merck), pullulan (Sigma), maltose (Merck), maltotriose (Sigma), maltotetraose (Sigma), maltopentaose (Merck), or maltohexaose (Merck). At specific time intervals samples were withdrawn and hydrolysis was terminated by immersion in ice. Samples were analysed for DP1-DP6 maltooligosaccharides by HPLC, on a Tracer Carbohydrate column (Tecknokroma, Barcelona, Spain). Samples were deionized for 5 min with AG 501-X8 (Bio-Rad, 0.05 g resin$\cdot ml^{-1}$ of sample). After sample dilution with an equal volume of acetonitrile, centrifugation for 3 min, and filtration through 0.2-µm filters (Schleicher and Schuell GmbH, Dassel, Germany), the samples were applied to the carbohydrate column and eluted isocratically at 1 $ml \cdot min^{-1}$ in 55% (v/v) aqueous acetonitrile, at room temperature. The separated maltooligosaccharides were detected by refractometry (ERC-7512, Erma, Tokyo, Japan) and quantified by comparison with standard maltooligosaccharides using a microprocessor equipped with data analysis software (JCL 6000, Jones Chromatography, Colorado, USA).

Kinetic determinations

The initial rates of hydrolysis of starch, amylopectin, amylose, and glycogen were measured by the dinitrosalicylic acid method. The kinetic parameters K_m and V_{max} were

calculated from Lineweaver-Burk and Eaddie-Hofstee plots (Ferscht 1985).

Effect of metal ions, inhibitors, and denaturing agents

The effect of metal ions on amylolytic activity was determined by addition of 1 mM of each ion to the standard assay. All metals were used in the chloride form except for Mn^{2+} and Zn^{2+}, which were assayed in the sulfate form. The activity of the enzyme alone in acetate buffer was taken to be 100%.

The effect of inhibitors on amylolytic activity was determined by preincubating the enzyme in the presence of inhibitor for 20 min at room temperature and then performing the assay in the presence of the same inhibitor concentration.

The effect of denaturing agents was determined by assaying residual activity after incubation for 20 min at 25°C or 70°C in the presence of the agent. The activity of the enzyme alone in acetate buffer was taken to be 100%.

Results

Amylase production

In the presence of starch, α-amylase production by *Thermus filiformis* Ork A2 began during the exponential growth phase, reaching highest values at the beginning of the stationary phase of growth (Fig. 1). After a few hours the α-amylase activity found in the culture supernatant decreased abruptly to low values. Simultaneously, the culture pH dropped from 7 to 4.5 and cell viability also decreased. In cultures where starch was omitted from the medium, enzyme titer accompanied growth throughout, reaching highest levels in the stationary phase of growth. In this medium, α-amylase production was maintained at high levels during stationary phase of growth (Fig. 1). Highest enzyme levels were similar in both media, with 2.1 U of amylolytic activity per ml of culture supernatant. Starch-containing medium was selected for further enzyme production, since the onset of the pH decrease was a useful indicator of maximum enzyme production. The culture supernatant, which was shown to also contain proteolytic activity, was used for enzyme purification.

Enzyme purification

The culture supernatant was subjected to cation-exchange chromatography on S-Sepharose. The amylase was recovered from the resin in the first absorbance peak obtained with the salt gradient elution. The active fraction was dialyzed and applied to a Mono Q column, where the

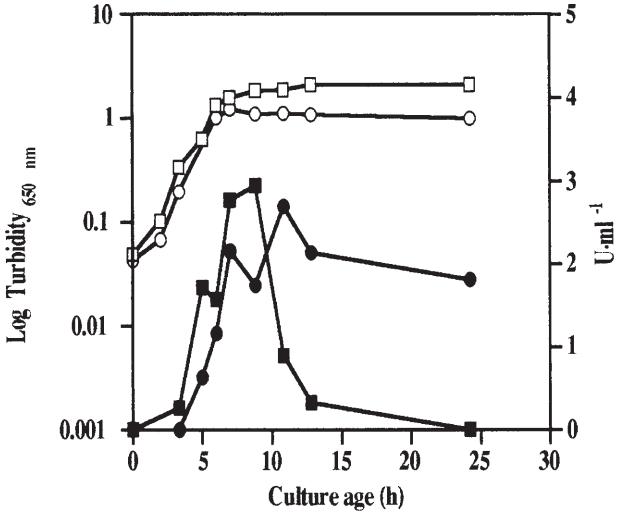

Fig. 1. Bacterial growth (*open symbols*; log turbidity at 650 nm) and amylase production (*closed symbols*; $u \cdot ml^{-1}$). The bacteria were grown in *Thermus* media in the absence (*circles*) and presence (*rectangles*) of 1% starch at 70°C

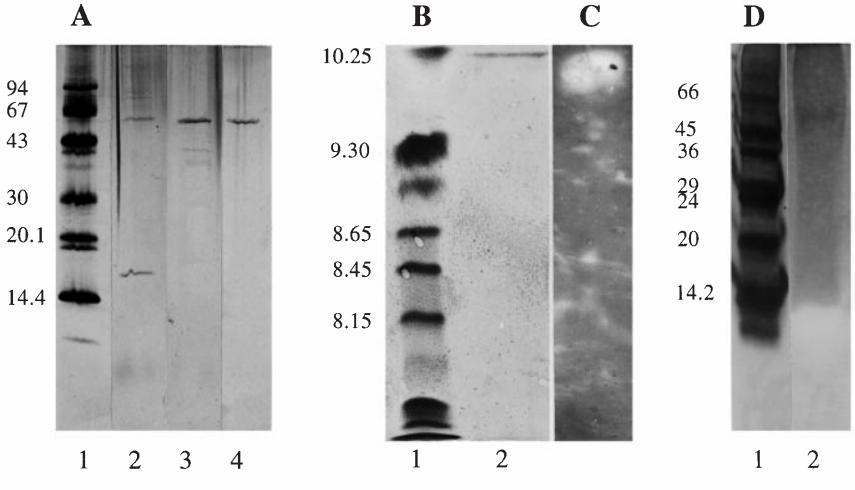

Fig. 2. Electrophoresis of enzyme samples. **a,b,d** Proteins were stained with silver nitrate. **a**, Polyacrylamide gel electrophoresis in sodium dodecybulfate (SDS-PAGE) of α-amylase samples throughout the purification steps. *1*, molecular weight markers (kDa), *2*, culture supernatant; *3*, S-Sepharose; *4*, Mono Q. **b** Isoelectric focusing (IEF) of purified α-amylase. *1*, pI markers, *2*, α-amylase. **c** Activity staining of an α-amylase sample separated by IEF. **d** SDS-PAGE of amylase sample after IEF. *1*, molecular weight markers (kDa); *2*, α-amylase

Table 1. Summary of the purification steps of the α-amylase from the culture supernatant of *Thermus filiformis* Ork A2[a]

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture supernatant	1208	4.6	263	100	1.0
S-Sepharose	971	0.165	5934	81	22.6
Mono Q	661	0.104	6352	55	24.2

[a] Purification was initiated with 500 ml of culture supernatant.

α-amylase was recovered in the nonadsorbed protein fraction. Sample purity was evaluated by SDS-PAGE, showing a single band in the fraction obtained after the Mono Q chromatography in the presence of PMSF (Fig. 2a). The same sample was separated on IEF, also yielding a single band (Fig. 2b), which was shown to possess amylolytic activity by activity staining (Fig. 2c). A summary of the purification is shown in Table 1. These procedures yielded a purified amylase fraction with a specific activity of 6352 $U \cdot mg^{-1}$ of protein, a purification factor of 24, and a yield of 55%. The extracellular contaminating proteolytic activity detected in the culture supernatant was reduced to 0.5% in the final amylase fraction. The protease was inhibited by 10 mM PMSF, suggesting a serine enzyme. Several chromatographic steps were performed in an attempt to remove the residual contaminating protease, without success. The effect of the protease on amylolytic activity and thermostability was investigated; these experiments showed that there was no interference between the two activities. The only detectable effect of proteolytic activity occurred on the preparation of samples for SDS-PAGE, with partial digestion of the amylase band, prevented by preparation of the sample in 10 mM PMSF. The amylase fraction obtained by chromatography on Mono Q was used for amylase characterization.

Structural properties

The molecular weight of the amylase was determined by SDS-PAGE, indicating a 60000-Da protein. However, a molecular weight of 31000 Da was obtained by size-exclusion chromatography. The molecular weight of the denatured enzyme was further confirmed by SDS-PAGE of an enzyme fraction separated on IEF, yielding a single band of 60000 Da (Fig. 2d). The isoelectric point of the α-amylase was found to be 10.1.

The amino acid analysis showed a high content of basic and hydrophobic amino acids, with a particularly high level of leucine (Table 2). The NH_2-terminal amino acid sequence of the α-amylase was Thr-Ala-Asp-Leu-Ile-Val-Lys-Ile-Asn-Phe. A comparison of this sequence with the NH_2-terminal sequences of other bacterial α-amylases showed low levels of identity. This amino acid sequence was also compared with the amino acid sequences available on the Swiss-Prot (EMBL) database, revealing high level of similarity with several proteins unrelated to amylolytic activity.

Table 2. Amino acid composition of *Thermus filiformis* Ork A2 α-amylase

Amino acid	Number of residues/molecule
Asx	10
Glx	14
Ser	32
Gly	38
His	9
Arg	49
Thr	23
Ala	27
Pro	52
Tyr	15
Val	26
Ile	14
Leu	166
Phe	30
Lys	73

Physicochemical properties

The α-amylase displayed optimal activity at pH 5.5–6.0 (Fig. 3a), with high activity within a broad pH range. The enzyme also presented broad pH stability, maintaining over 80% activity after 60 min at 70°C in the pH range 4.5–8.0 (Fig. 3b).

The α-amylase showed optimal activity at 95°C, in the presence of calcium. In its absence, maximal values were obtained between 85° and 95°C (Fig. 4a). The presence of calcium did not contribute significantly to activity up to 90°C, but at 95°C its presence was essential for maintaining enzyme activity (Fig. 4b). Enzyme thermostability was enhanced by the presence of calcium, increasing half-life times of activity at temperatures above 80°C. The presence of 5 mM $CaCl_2$ increased $t_{1/2}$ to more than 8 h at 85°C, 80 min at 90°C, and 19 min at 95°C. The presence of starch also enhanced amylase thermostability at temperatures above 80°C, but not as effectively as calcium (Table 3).

Catalytic properties

The substrate specificity of the α-amylase was determined against maltooligosaccharides and polysaccharides. The smallest maltooligosaccharide hydrolyzed was maltotriose, although at very low rates. The pattern of hydrolysis was similar for all substrates tested, with higher production of maltose, followed by glucose and lower levels of maltotriose

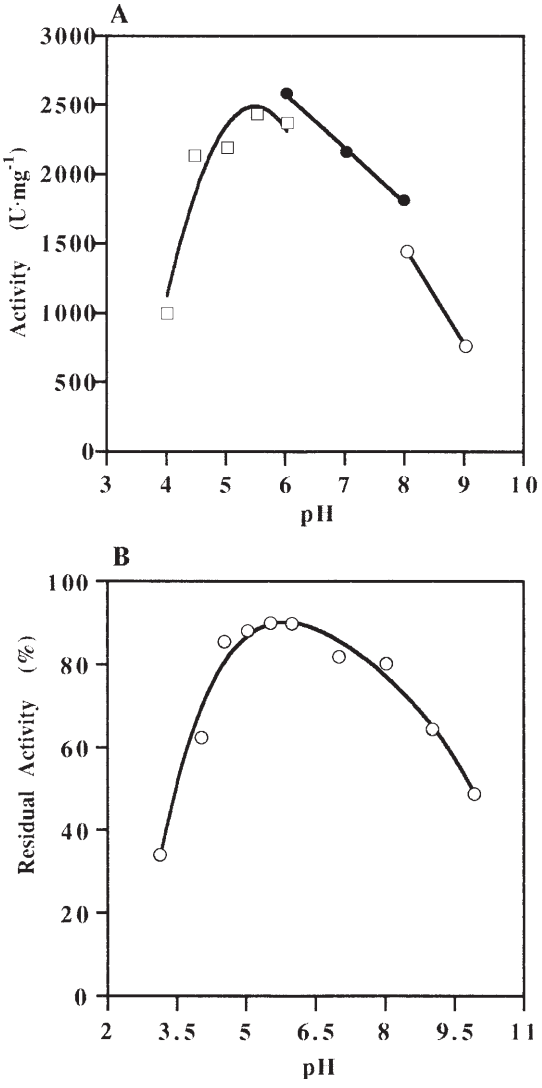

Fig. 3. Effect of pH on the α-amylase. **a** pH profile. Activity was determined according to the standard assay with different buffers: for pH 4.0–6.0 15 mM acetate (*squares*); for pH 6.0–8.0 15 mM phosphate (*closed circles*), and for pH 8.0–9.0 15 mM borate(*open circles*). **b** pH stability. Residual activity was determined after a 60-min incubation at 70°C in the same buffers

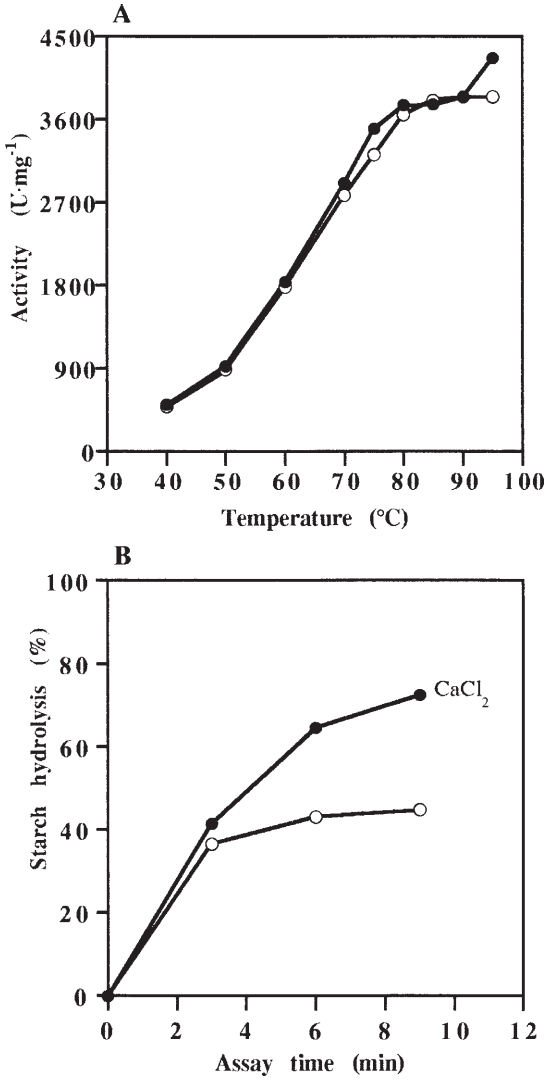

Fig. 4. Effect of temperature on the α-amylase. **a** Temperature profile. Activity was determined according to the standard assay at temperatures from 40°–95°C in the absence (*open circles*) and presence of 2 mM $CaCl_2$ (*closed circles*). **b** Effect of calcium on amylase activity at 95°C. Starch hydrolysis was determined in the absence (*open circles*) and presence of 2 mM $CaCl_2$ (*closed circles*) at 95°C

Table 3. Thermostability of the α-amylase of *Thermus filiformis* Ork A2[a]

	$t_{1/2}$ (min)				
	70°C	80°C	85°C	90°C	95°C
No addition	>480	476	116	5.9	2.5
2 mM $CaCl_2$	>480	>480	>480	59.7	10.2
5 mM $CaCl_2$	>480	>480	>480	80.8	19.2
1.5% Starch		455	212	27.9	6.6

[a] Half-life values of activity were determined between 70° and 95°C over an 8-h incubation in 15 mM sodium acetate buffer, pH 5.8.

(Fig. 5). Maltotetraose was a major hydrolysis product of all substrates in the first hour of digestion, but was completely degraded after 3 h. The hydrolysis of maltotetraose produced high levels of maltose but approximately one-fifth was hydrolyzed into maltotriose and glucose. Maltopentaose was cleaved more slowly than maltohexaose, but both were completely degraded after 3 h of digestion. Amylose was hydrolyzed at a higher rate than the other polysaccharides tested, glycogen being the more slowly degraded. The hydrolysis patterns of starch and amylopectin were identical. For amylose, amylopectin, and

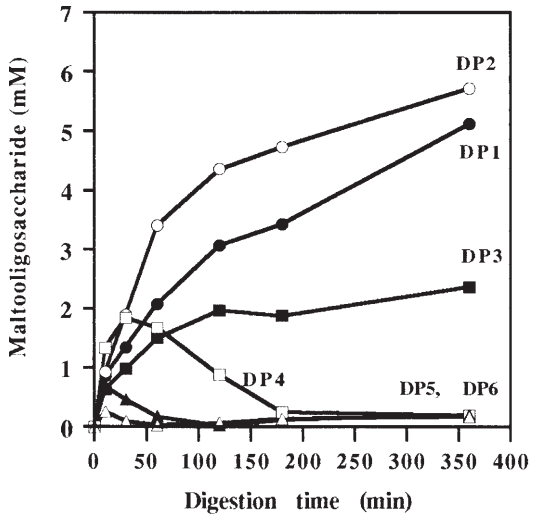

Fig. 5. Hydrolysis pattern of starch. The α-amylase was incubated in the presence of 0.25% starch for 6 h at 70°C. At various times, samples were removed and analyzed by HPLC for the various maltooligosaccharides. *DP*, degree of polymerization. Glucose (*DP1*, *closed circles*); maltose, (DP2, *open circles*); maltotriose, (DP3, *closed squares*), maltotetraose, (DP4, *open squares*); maltopentaose, (DP5, *closed triangles*); maltohexaose, (DP6, *open triangles*)

starch digestions, the level of glucose almost equaled that of maltose, but these only after 6 h of digestion. Small amounts of maltopentaose and maltohexaose were produced, but these were constantly degraded into smaller products. Maltooligosaccharides with a polymerization degree of more than six were produced during the 6-h period of sampling, although in small amounts. These products were not quantified. No activity was detected against α-1,6-glycosidic bonds in pullulan. No reversion products of amylolytic activity were found. These results, in combination with the rapid reduction in blue value in the assay, suggest that this enzyme is an α-amylase, cleaving α-1,4-glycosidic bonds randomly in the chain.

The amylase showed Michaelis-Menten kinetics for starch, amylose, amylopectin, and glycogen hydrolysis. The apparent K_m and V_{max} calculated by Lineweaver-Burk and Eaddie-Hofstee plots were in close agreement. The kinetic parameters obtained are presented in Table 4.

Calcium, strontium, magnesium, sodium, cobalt, and manganese ions had no effect on the activity of the α-amylase at 70°C. On the other hand, Ba^{2+} and Fe^{3+} were slightly inhibitory, while Zn^{2+} inhibited 50% of the amylolytic activity. The sulfhydryl oxidants, Hg^{2+} and Cu^{2+}, inhibited enzyme activity by 95%. α-Amylase activity was also inhibited by 80% in the presence of *p*-chloromercuribenzoic acid (pCMB) (0.5 mM). This inhibition was reversed in the presence of dithiothreitol (DTT, 5 mM). DTT and β-mercaptoethanol (5 mM) increased amylolytic activity by 32% and 70%, respectively. The chelating agent ethylenediaminetetraacetic acid (EDTA, 10 mM) also inhibited amylase activity by 88%. Inhibition was reversed in the presence of Ca^{2+} or Sr^{2+} (15 mM). The other inhibitors tested, iodoacetamide (0.1 mM) and *o*-phenanthroline (5 mM), showed no effect on amylase activity.

The effect of denaturing agents on amylolytic activity was also determined. The enzyme was denatured by urea (8 M) and guanidine chloride (5 M) after incubation at high temperatures. However, only 7% and 20%, respectively, of the amylolytic activity was lost after 20 min at 25°C. Under these conditions, SDS (1%) induced rapid and complete enzyme denaturation.

Discussion

Thermus filiformis strain Ork A2 produced an extracellular α-amylase in the liquid culture, where production paralleled culture growth during metabolically active growth phases. Enzyme production appeared to be independent of the presence of starch, as similar activity titres were obtained in its presence or absence. Addition of starch to the medium resulted in a decrease in amylolytic activity, pH of the culture, and cell viability, during the stationary phase. The basis for the decrease in culture pH was not investigated, although this behaviour is frequently observed in *Thermus* cultures grown on complex media containing monosaccharides (D. Cowan, personal communication).

The amylase was purified by cation-exchange followed by anion exchange chromatography. The S-Sepharose chromatography was effective both in the concentration of the culture supernatant and in initial purification of the amylase. A significant reduction (95%) in contaminating protease activity was also obtained in this step. Chromatography on Mono Q was effective in removing minor protein contaminants present in the enzyme fraction as well as

Table 4. Kinetic parameters of *Thermus filiformis* Ork A2 α-amylase. The kinetic parameters were calculated from the Lineweaver-Burk plot

Substrate	K_m ($mg \cdot ml^{-1}$)	V_{max} ($10^4 \mu mol \cdot min^{-1} mg^{-1}$)	k_{cat} ($10^6 s^{-1}$)	k_{cat}/K_m ($10^5 ml \cdot mg^{-1} s^{-1}$)
Starch	5.01	1.18	2.60	5.20
Amylose	2.94	1.01	2.23	7.59
Amylopectin	3.69	0.72	1.59	4.30
Glycogen	1.15	0.33	0.72	6.25

a further reduction of protease activity to 0.5%, yielding a single protein band on SDS-PAGE if samples were prepared in the presence of PMSF. In the absence of this protease inhibitor, several bands of lower molecular weight were observed. Proteolytic degradation was significant only in the presence of SDS, where partial or complete denaturation of the amylase probably exposes susceptible bonds as suggested by Laderman et al. (1993) for the α-amylase of *Pyrococcus furiosus*. Many microorganisms produce extracellular amylases and proteases and the difficulties in separating both enzymes have been reported by several researchers (Freer 1993; Kim et al. 1995; Koivula et al. 1993; Laderman et al. 1993; Schwermann et al. 1994).

The amylase showed a higher molecular weight (60 000 Da) under denaturing conditions than in native conditions (31 000 Da). Variable apparent molecular weights have been observed for the amylases produced by *Streptococcus bovis* (Freer 1993) and *Clostridium acetobutylicum* (Paquet et al. 1991), generally attributed to the interactions of the enzymes with gel filtration matrices, composed of glucose polymers. In this study, the use of an acrylamide-based matrix precludes the possibility of such interactions. Retardation on size-exclusion chromatography has also been reported for some microbial proteases (Burton et al. 1993; Cowan et al. 1987b; Peek et al. 1992), and was attributed to the hydrophobic nature of these proteins, in conjunction with highly basic pI values (Peek et al. 1992). We speculate that such a mechanism might induce the retardation of *Thermus* amylase on gel filtration since the enzyme has a very basic isoelectric point (10.1) and a high content of hydrophobic amino acids. The amylase isoelectric point was in agreement with the amino acid composition which revealed a high content of basic amino acids, as well as of hydrophobic amino acids. The low level of identity of the NH_2-terminal amino acid sequence of Ork A2 amylase with other bacterial α-amylases and the high levels of similarity with proteins not related to amylolytic activity are inconclusive.

The optimal pH for activity, 5.5–6.0, was within the range of pH values reported for most bacterial α-amylases and pullulanases (Brown and Kelly 1993; Canganella et al. 1994; Chung et al. 1995; Freer 1993; Kumar et al. 1990; Nakamura et al. 1989; Paquet et al. 1991; Vihinen and Mäntsälä 1990), and is in agreement with the acidic catalysis proposed for these enzymes (Kim et al. 1995; Schwermann et al. 1994). Stability was independent of pH between 4.0 and 8.0, suggesting an indirect relationship between the decrease in culture pH and the amylolytic activity observed in stationary phase in the presence of starch.

The optimum temperature for activity was obtained at 95°C, a value higher than the values reported for the amylases of several *Bacillus* strains (Kim et al. 1995; Koivula et al. 1993; Krishnan and Chandra 1983; Kumar et al. 1990; Schwermann et al. 1994; Srivastava 1987; Vihinen and Mäntsälä 1990) and superior to the value of the pullulanase of *Thermus* AMD 33, 75°C (Nakamura et al. 1989). The temperature of optimal activity of *Thermus* Ork A2 amylase was also close to those determined for amylases from hyperthermophiles, ranging from 80°C for the *Thermococcus profundus* amylase (Chung et al. 1995) to 100°C for the amylase produced by *Pyrococcus furiosus* (Laderman et al. 1993). Amylase thermostability was high at 85°C, with a half-life of activity over 8 h in the presence of calcium. However, at 95°C the half-life of activity was only 19 min where as the pullulanase from *Thermus aquaticus* YT-1 (Plant et al. 1986) had a $t_{1/2}$ of 4.5 h at 95°C. Higher thermostability values were also reported for the amylases of *Thermococcus celer* and *Thermococcus* strain TYS, with $t_{1/2}$ of 6 h at 95°C, and of 2 h and 3 h, respectively, at 105°C (Canganella et al. 1994).

The hydrolysis pattern presented by the amylase showed that it is an α-amylase, with a relatively nonspecific endohydrolytic action, producing short-chain dextrins, which are further degraded to smaller maltooligosaccharides. Maltose was the major hydrolysis product, followed by glucose and maltotriose, a pattern also determined for α-amylases from *Bacillus licheniformis* (Ivanova and Dobreva 1994) and *Clostridium acetobutylicum* (Paquet et al. 1991). The unusual high level of glucose produced at the end of the digestion period was only comparable to the pattern obtained by the α-amylase from *Clostridium perfringens* (Shih and Labbé 1995). The amylase did not possess detectable α-1,6-glycosyl activity nor were any reversion products found.

The amylase of *Thermus filiformis* Ork A2 showed similar values for the K_m, the V_{max} and for the specificity constant k_{cat}/K_m for starch, amylose, amylopectin, and glycogen, as might be expected since the same type of bond is cleaved in all substrates. The differences obtained probably reflect the accessibility of the enzyme cleavage site, and are in general agreement with the structures proposed for these polysaccharides. The enzyme possesses higher specificity for the α-1,4-glycosidic bond of amylose, a nonbranched polymer, than for glycogen, the most branched of the polysaccharides tested. Glycogen structure is thought to be composed of a dense core, inaccessible to the enzyme, and some surface chains which could be more easily hydrolysed (Parks and Rollings 1995), a model supported by the low V_{max} obtained for this substrate. The results obtained for starch reflect its heterogeneous composition: 11%–37% amylose and 64%–89% amylopectin. The values obtained for the K_m are within the values reported by several authors, from $0.5\,g\cdot l^{-1}$ for the *Thermococcus profundus* amylase (Chung et al. 1995) to $23.9\,g\cdot l^{-1}$ for the *Clostridium perfringens* α-amylase (Shih and Labbé 1995).

No significant effect was obtained by the presence of most salts at 70°C. The sulfydryl oxidant metals inhibited enzyme activity, suggesting the presence of a catalytic cysteine residue (Freer 1993; Krishnan and Chandra 1983; Paquet et al. 1991; Srivastava 1987), an observation supported by the activation of the enzyme in the presence of both dithiothreitol and β-mercaptoethanol and by pCMB inhibition.

EDTA inhibited enzyme activity, suggesting a requirement for divalent ions in amylase activity. The effect of calcium on the thermostabilization of several enzymes, including α-amylase (Brown and Kelly 1993; Coolbear et al. 1992; Kim et al. 1995; Kumar et al. 1990; Nakamura et al. 1989; Shih and Labbé 1995; Srivastava 1987) is well known.

A critical structural role is attributed to this ion in α-amylases, where by maintaining the correct geometry of the active site via chelation with four amino acid residues (Schwermann et al. 1994), the metal ion imparts the active site with the conformational rigidity required for catalysis (Hsiu et al. 1964).

Urea and guanidine chloride denatured the amylase but only at high temperatures. SDS was a much more effective denaturant, as also reported for the amylases of *Thermococcus profundus* (Chung et al. 1995), *Pyrococcus furiosus* (Laderman et al. 1993), and *Clostridium perfringens* (Shih and Labbé 1995). This result is consistent with the high proportion of hydrophobic amino acids in *Thermus* Ork A2 amylase and with the proposed role of hydrophobic core packing as a mechanism of molecular stabilization in thermophilic enzymes (Britton et al. 1995; Mozhaev et al. 1988).

To our knowledge this is the first report of the characterization of an α-amylase from a strain of the genus *Thermus*.

Acknowledgments This work was supported by Praxis XXI and FEDER Programs (Praxis 2/2.1/BIO/20/94, Portugal) and by the European Community Biotech Programme (Biotechnology of Extremophiles) Contract no. BIO2-CT93-0274 to M.S.C. M.C. Egas was supported by scholarships from Junta Nacional de Investigação Científica e Tecnológica e Praxis XXI, Portugal. We thank Dr. Paula Veríssimo for the NH_2-amino acid sequencing. We also thank Dr. Fred Rainey (Baton Rouge, USA) for the 16S DNA sequence analysis of strain Ork A2.

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