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α-Amylase activity from the halophilic archaeon *Haloferax mediterranei*

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Abstract The halophilic archaeon *Haloferax mediterranei* is able to grow in a minimal medium containing ammonium acetate as a carbon and nitrogen source. When this medium is enriched with starch, α-amylase activity is excreted to the medium in low concentration. Here we report methods to concentrate and purify the enzyme. The relative molecular mass of the enzyme, determined by gel filtration, is 50 ± 4 kDa, and on SDS-PAGE analysis a single band appeared at 58 kDa. These results indicated that the halophilic α-amylase is a monomeric enzyme. The enzyme showed a salt requirement for both stability and activity, being stable from 2 to 4 M NaCl, with maximal activity at 3 M NaCl. The enzyme displayed maximal activity at pHs from 7 to 8, and its optimal temperature was in a range from 50 °C to 60 °C. The results also implicated several prototropic groups in the catalytic reaction.

Keywords α-Amylase · Archaea · Extracellular · *Haloferax mediterranei* · Purification

Introduction

Starch-degrading enzymes, such as amylases, are found widely among the three domains Eucarya, Bacteria, and Archaea, playing a central role in carbohydrate metabolism. However, relatively fewer studies are focused on archaeal α-amylases (Jones et al. 1999). There are a great variety of enzymes implicated in the hydrolysis of starch, and these are widely used in industrial processes (Kadziola et al. 1998; Machius et al. 1995). Moreover, α-amylases have become one of the most valuable enzymes in biotechnology, especially in the food- and starch-processing industries (Vihinen and Mäntsälä 1989).

α-Amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) are a family of endo-amylases that randomly catalyze the hydrolysis of the 1–4 glycosidic bonds in starch components, glycogen, and other related carbohydrates, yielding oligosaccharides and glucose as end products (Sunna et al. 1997). The primary physiological role of this enzyme is the metabolism of the starch present in the media, where it is excreted; therefore, many organisms depend on their amylases for survival (Jones et al. 1999; Strobl et al. 1998).

In the present report, we describe the α-amylase from the halophilic archaeon *Haloferax mediterranei*. This organism requires high salt concentrations (mainly sodium chloride) for optimal growth, and its metabolic machinery is adapted to that extreme condition. Enzymes excreted into the media, such as the α-amylase studied here, also must work at salt concentrations at which other enzymes would lose their properties. Usually, halophilic enzymes not only are able to deal with high ionic strength in their environment but also need it to maintain function and structure (Dym et al. 1995).

Furthermore, an understanding of protein–water interactions is of increasing importance in the biotechnological industry, where the use of enzymes, for example, in biotransformations and biosensors, often requires stable and active proteins in low-water or non-aqueous systems. Archaea provide us with a ready source of proteins that may serve as model proteins that exist in high-salt concentrations, which is in effect a low-water environment (Danson and Hough 1997; Marhuenda-Egea and Bonete 2002).

The salt requirements for the halophilic α-amylase described here focus both on salt-dependent stability and activity. The behavior of the amylase at different pHs and temperatures has also been determined in order to compare it with other halophilic enzymes and other

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amylases in the search for amylases that possess unique new properties.

Materials and methods

Growth conditions and crude enzyme preparation

Cells were grown in 25% (w/v) salts, at 37 °C, pH 7.2. The minimal media included 1% (w/v) ammonium acetate as a carbon and nitrogen source and was supplemented with 0.2% (w/v) soluble starch, adding a previously filter-sterilized starch stock solution. Cells were harvested by centrifugation at 10,000 rpm for 30 min and supernatant was used as crude enzyme preparation.

Determination of α-amylase activity

α-Amylase activity was routinely assayed by the iodine-binding assay, in 50 mM Tris-HCl buffer pH 7.5, at 50 °C. The reaction mixture contained 0.1% (w/v) soluble starch and was terminated by cooling in ice. Color was developed by the addition of iodine solution (4% potassium iodide [w/v], 1.25% iodine [w/v]), and the loss of starch was determined spectrophotometrically at 600 nm. One unit of activity was defined as the amount of protein that hydrolyzed 1 mg of starch in 1 min (Haseltine et al. 1996). When the iodine-binding assay was not possible, amylase activity was determined with an assay for the production of sugar-reducing ends (Haseltine et al. 1996). The sample (1% starch in 0.05 M Tris-HCl buffer, pH 8.0, 3 M NaCl plus enzyme) was adjusted to 0.4 M sodium carbonate, 20 mM potassium cyanide, and 4 mM potassium ferricyanide and heated at 90 °C for 10 min. The absorbance was determined at 420 nm and corrected by subtracting the absorbance of a sample lacking added substrate. One unit of activity was defined as the amount of protein that produced 1 μmol of reducing ends in 1 min. Maltose was used as reducing ends standard. Other possible substrates were also tested, pullulan α-cyclodextrine (cyclomaltohexaose) and β-cyclodextrine (cyclomaltoheptaose), incubating the enzyme with each of them at concentration 0.5% (w/v) for 15–60 min at 50 °C. All assays were performed, at least, in duplicate and average values were obtained.

Analysis of reaction products and NMR spectroscopy

The hydrolysis products of the amylase from *Haloferax mediterranei* were analyzed by high-performance liquid chromatography (HPLC) in a carbohydrate column (4.6×250 mm Waters, Milford, Mass.) in 50 mM phosphate buffer pH 7, 2 M NaCl. The standards were maltose, maltotrioside, and maltohexaoxide prepared in concentrations of 1 mM in the same conditions as the reaction products.

Spectra were recorded on an Avance DRX-300 nuclear magnetic resonance (NMR) spectrometer (Bruker, Germany) operated at 40 °C and at 300 MHz for protons in deuterated 10 mM sodium phosphate buffer ($p^2H = 7.3$). The spectral width, data point, and number of accumulation were 4,500 MHz, 16 K, and 16, respectively. Chemical shifts were measured relative to the calibrated resonance of internal sodium-3-(trimethylsilyl)-1-propane sulfonate (Merck). The substrate used was p-nitrophenyl-α-D-maltohexaoside (Sigma).

Optical rotation (specific rotatorial activity, $[\alpha]_D^{20}$) was registrated in a digital polarimeter (Jasco DIP-1000), with starch 1% as substrate and pullulan 1% as substrate in 50 mM Tris-HCl, 3 M NaCl.

Purification of α-amylase

The supernatant from 2 l of culture was treated with hydroxylapatite previously equilibrated with 50 mM Tris-HCl buffer pH 7.0, 3 M NaCl. The proteins bound to hydroxylapatite were recovered by centrifugation at 3,000 rpm for 5 min. The α-amylase was eluted from hydroxylapatite in 10 ml of 0.2 M phosphate buffer, pH 7.5, and 2.5 M ammonium sulfate and was used as starting material for next purification steps.

The sample was applied to a Sepharose-4B column and eluted using a linear gradient from 2.5 M ammonium sulfate to 0.5 M ammonium sulfate containing 20% (w/v) glycerol. The more active fractions were pooled and concentrated using a DEAE-cellulose column. The concentrated sample was further purified by gel filtration in a Sephadex G-50 column. The protein concentration was determined by the Bradford method (Bradford 1976).

Samples were analyzed by SDS-PAGE and the relative molecular mass was determined by gel-filtration chromatography in Sepharose CL-6B.

Effect of salt concentration, pH, and temperature

The effect of salt concentration on enzyme activity was tested by measuring the activity at 50 °C in 50 mM Tris-HCl, pH7.3, buffers containing different NaCl concentrations. For each salt concentration, starch concentration was varied from 0.02 to 0.2% (w/v).

The stability of the amylase at different salt concentrations was determined by incubating the enzyme in buffers containing the studied salt concentration and measuring the activity of aliquots at different times. To get samples of enzyme at different salt concentrations, highly active samples were applied to small amounts (2 ml) of hydroxylapatite and the bound amylase protein was eluted in 0.05 M phosphate buffers, each of them containing the desired salt concentration.

For pH studies different buffers were used: 0.2 M citric acid/phosphate for pHs from 4.5 to 7, 0.2 M Tris-HCl buffers for pHs from 7 to 9, and 0.2 M CHES buffers for pHs 9 to 10. All of them contained 3 M NaCl. The pH was checked after each reaction and showed no changes with respect to the initial values. For each pH, the starch concentration varied from 0.02 to 0.2% (w/v).

To measure the stability of the enzyme at different pHs, the same buffers were used. To adjust the pH of the enzyme sample, a small amount of a highly concentrated enzyme preparation was placed in the buffer at the pH tested (50 μl to 1 ml) and pH was checked after the addition. Once mixed, an aliquot was taken and its activity measured. Other aliquots were collected at different times, and their activity was compared with the initial activity value.

The assays to study the dependence of temperature on the amylase activity were carried out in 0.2 M phosphate buffer, pH 7.5, 3 M NaCl, at different temperatures. For each temperature, starch concentration was varied from 0.02 to 0.2% (w/v).

The stability of the amylase at different temperatures was determined by incubating an enzyme sample in a thermostatic bath at the desired temperature. At different times aliquots were collected and ice cooled, and their activities were measured and referred to the initial activity of the sample.

Effect of EDTA and metal ions on the amylase activity

The enzyme sample was adjusted to different EDTA concentrations by adding a highly concentrated stock solution of EDTA. The concentrations tested were 25 mM, 12 mM, 6 mM, and 3 mM. The activity of aliquots of each sample containing EDTA was measured at different times and its activity compared with the initial activity.

When loss of activity was achieved, the sample was adjusted to 50 mM magnesium or calcium chloride, and its activity was measured at different times. When calcium chloride was added, a small precipitate appeared after addition, and it was removed by centrifuging the sample.

The effect of calcium and magnesium ions on the enzyme activity was determined by adjusting the reaction medium to different ion concentrations from 0 to 200 mM.

Data processing

Reciprocal initial velocities were plotted versus reciprocal substrate concentrations. All plots were linear. Initial velocities (v) obtained at each salt concentration, pH, or temperature, respectively, by varying the substrate concentration (S), were fitted to Eq. 1 to obtain values for maximum velocity (V), the Michaelis constant (K) for the substrate, and the apparent first-order constant for the interaction of enzyme and substrate (V/K).

$$v = V \cdot S/(K + S) \quad (1)$$

Data for the pH dependence of V and V/K were fitted to Eq. 2 using the algorithm of Marquardt-Levenberg with the SigmaPlot program (Jandel Scientific, v. 1.02):

$$\log Y = \log[C/([H^+]/K_1 + K_2/[H^+])] \quad (2)$$

where Y represents the value of V or V/K for each pH and C is the pH-independent value of the parameter at the optimum state of protonation. K_1 and K_2 are dissociation constants associated with ionizing groups that, to show activity, must be protonated or deprotonated, respectively.

The values of pK obtained at different temperatures were fitted to Eq. 3:

$$\mathrm{pK} = \Delta H^{\circ}/(2.303 \cdot R \cdot T) - \Delta S^{\circ}/(2.303 \cdot R) \quad (3)$$

in order to determine values for the ionization enthalpy (ΔH^{o}_{ion}) and the ionization entropy ΔS^{o}.

Data from the stability studies, salt concentration, pH, and temperature, were fitted as a logarithm of the residual activity versus time for each salt concentration, pH, or temperature studied. The half-life and the pseudo-first-order constant for the denaturating process were determined from the slope of the straight lines obtained.

In the study of the inactivation of the enzyme with EDTA, the pseudo-first-order inactivation constant was determined for each concentration and fitted to the equation (Bhattacharyya et al. 1992; Pérez-Pomares et al. 1999):

$$\log\ k_{obs} = n \log[\mathrm{EDTA}] + \log\ k \quad (4)$$

where n represents the number of moles of EDTA required to react with essential metal ions in the enzyme.

Results

Different nitrogen sources were tested to obtain the best results for amylase activity. The optimal yield was obtained when the cells were grown in a minimal medium containing ammonium acetate at concentrations from 0.5 to 1.0% (w/v) plus soluble starch. No amylase activity was found in cell extract obtained by sonicating the cell pellet.

The activity was optimal with starch as substrate. No activity at all was found when pullulan was used as substrate. The α-amylase displayed endo-acting activity since it was also able to hydrolyze α-cyclodextrine and β-cyclodextrine. The activity with α-cyclodextrine was 46% and with β-cyclodextrine was 48% of the activity with starch in the same conditions at 0.5% (w/v) substrate concentrations.

The HPLC analysis of the products of the reaction (during 1 h) showed that the main products were maltose (65%), in lower proportion maltohexaose (9%), and other minority components.

The NMR spectra were recorded for both the reaction mixture without enzyme and the reaction mixture after the reaction with α-amylase in deuterated 10 mM phosphate buffer, pH 7.3. In the anomeric proton region of *p*-nitrophenyl α-D-maltohexaoside (Fig. 1) appeared a proton signal of the hydrolyzed product at 5.2 ppm (doublet, $J = 3.57$ Hz) that did not appear in the control spectrum.

Optical rotation of the reaction solution containing initially 1% soluble starch decreased sharply after the complexion of the reaction from a specific rotatorial activity $[\alpha]_D^{20} + 141.5$ to $[\alpha]_D^{20} + 56.5$.

The enzyme was excreted into the media in low concentrations. Therefore, in order to concentrate it, the culture medium was treated with hydroxylapatite since this process was very fast and easy. Hydroxylapatite binds proteins that may be eluted using phosphate concentrations that compete with proteins for binding. The maximum yield was obtained for 0.2 M phosphate, but 0.05 M was enough to get a high yield. Data for this purification step are summarized in Table 1.

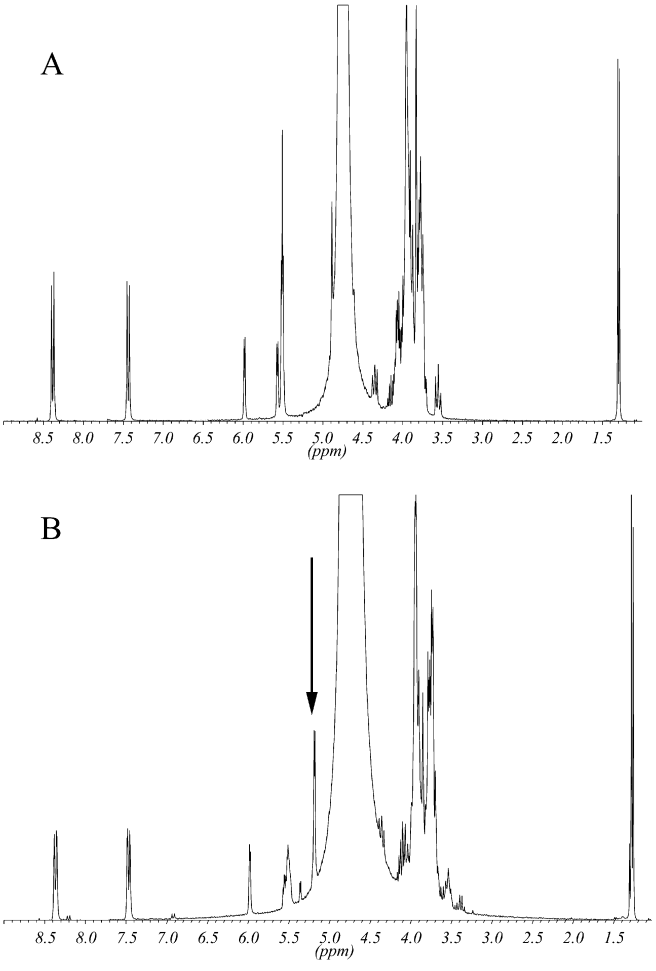

Fig. 1 NMR spectra recorded for the reaction mixture containing p-nitrophenyl α-D-maltohexaoside as substrate, without enzyme **(A)** and after reaction with the α-amylase **(B)**. Both reaction mixtures were performed in deuterated 10 mM phosphate buffer, pH 7. The *arrow* in **B** corresponds to the proton signal of the hydrolyzed product that did not appear in **A**

Table 1 Description of the purification steps for α-amylase from *Haloferax mediterranei*

Purification step	Volume (ml)	Activity (U/ml)	Yield (%)	Protein conc. (mg/ml)	Specific activity (U/mg)	Purification factor
Medium supernatant	1000	0.50	100	0.020	25.0	1
Hydroxylapatite	15	14.3	43	0.620	23.1	0.9
Sepharose-4B	25	0.65	3.3	0.006	108.3	4.3
DEAE-cellulose	6	1.9	2.3	0.02	95.0	3.8
Sephadex-G50	15	0.60	1.8	0.0005	1200	48

The purification of the enzyme was analyzed by SDS-PAGE, and one main band consistent with the value obtained by gel filtration was observed. The relative molecular mass for this band was 58 ± 2 kDa. This method usually yields abnormally high molecular weight values for halophilic enzymes (Bonete et al. 1996), so this was an overestimated value for this parameter.

Determination of the relative molecular mass

The relative molecular mass for the α-amylase was estimated to be 50 ± 4 kDa by gel filtration on a Sepharose CL-6B column, previously equilibrated in 50 mM Tris-Cl buffer, pH 7.3, 3 M NaCl, and calibrated with different proteins as molecular weight markers.

Effect of salt concentration on α-amylase

To test α-amylase dependence on salt concentration for activity, its kinetic parameters were determined at different NaCl concentrations. The α-amylase activity was tested at 50 °C in 50 mM Tris-HCl buffers, pH 7.3, and different NaCl concentrations in a range from 0.25 M to 4 M. For each salt concentration, the starch concentration was varied from 0.02% to 0.2% (w/v). Data for all the assays fitted Michaelis-Menten kinetics (Eq. 1). The kinetic parameters V, K, and V/K were determined as described in Materials and Methods. The dependence of V versus salt concentration is displayed in Fig. 2. The optimal salt concentration for maximal activity was 3 M NaCl, and very close activities were also reached at higher salt concentrations. The V value determined at 0.25 M NaCl was 0.47 U/ml, and it increased very slowly until the NaCl concentration was 1 M. At higher salt concentration, the maximal velocity increased sharply until the concentration reached 3 M NaCl. A similar dependence was observed for the dependence of K versus NaCl concentration (Fig. 2).

The effect of salt concentration on the amylase stability was also tested. When highly active amylase samples were dialyzed against a buffer with no salts added (NaCl or KCl), no remaining activity at all was observed. However, the presence of a NaCl concentration of 1 M was enough to prevent loss of activity for months, with a half-life time of 34 days. The stability increases at higher salt concentrations, 2 M NaCl and 4 M NaCl reaching half-life times of 43 days and 83 days, respectively.

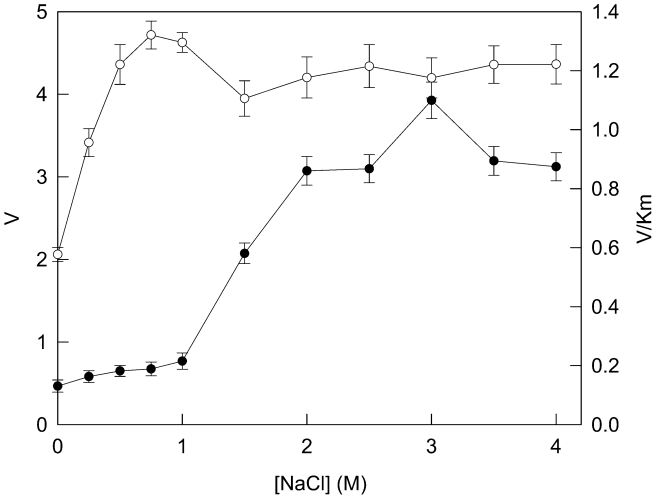

Fig. 2 Effect of the salt concentration (NaCl) on the kinetic parameters of the starch hydrolysis reaction. Each data point was determined at 50 °C in 50 mM Tris-HCl buffers, pH 7.3, by varying starch concentrations. The values for V (●) and for V/K (○) were determined at different salt concentrations from 0 to 4 M NaCl

Effect of pH on the halophilic amylase

Similar assays were performed in order to determine the effect of pH, in a range from 4.5 to 10.0, on the amylase activity. The initial velocity patterns were determined for each pH by varying the starch concentration, and experiments were carried out at 30 °C, 40 °C, and 50 °C.

As displayed in Fig. 3, log V decreased at both high and low pH values. Similar patterns were found for log V/K (Fig. 3), also indicating that the protonation states of two residues were important for catalysis.

Data fitted the equation for bell-shaped pH profiles (Eq. 2), and values for the acid side pK_1 as well as for the basic side pK_2 were obtained (Table 2). The pK_1 values for the log V profiles decreased as temperature increased, from 5.58 to 5.35, and according to their dependence on temperature (Eq. 3), a value of 5.2 ± 1.3 kcal/mol was obtained for the ionization enthalpy of the groups involved in the α-amylase reaction. Similar values were found for the log V/K profiles, where they ranged from 6.05 to 5.72, and the ionization enthalpy was 7.4 ± 1.8 kcal/mol. This pK_1 may be related to a group that must be deprotonated for binding or catalysis.

The pK_2 values for the log V profiles also decreased from 10.02 to 9.41 as the temperature increased. From

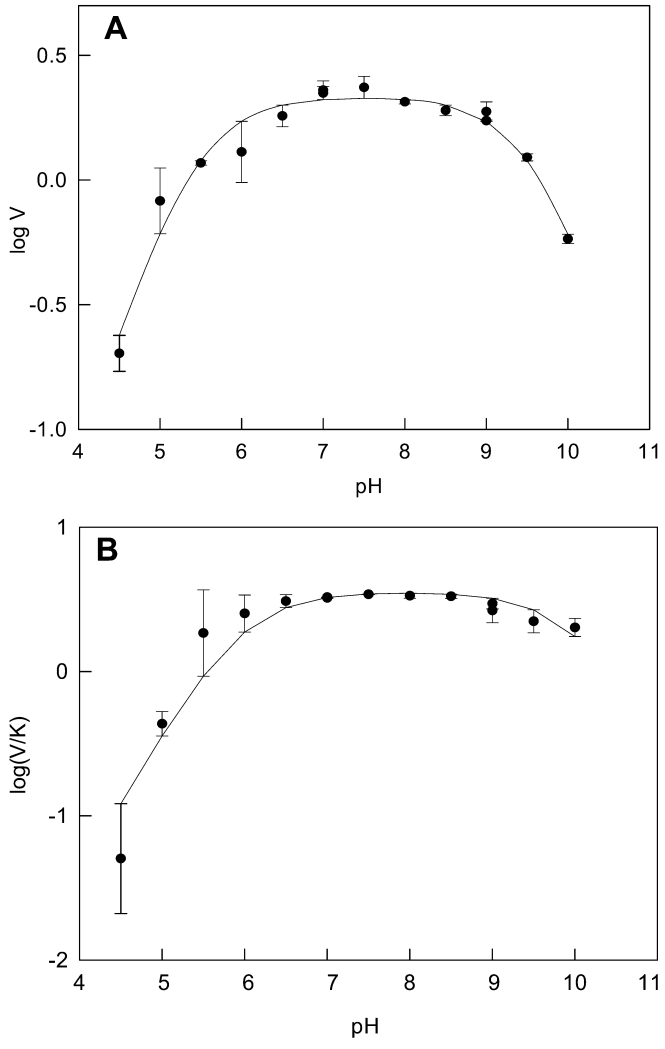

Fig. 3 Effect of pH on the kinetic parameters determined at 40 °C by varying starch concentration for each pH. The values for log V, at a pH from 4.5 to 10, are those plotted in Fig. 2 as (•). The curve displayed was obtained by fitting data to Eq. 2. The values for log V/K, in the same pH range, are displayed in Fig. 2 as (○), and the curve was also obtained by fitting data to Eq. 2. These profiles are similar to those obtained at 30 °C and 50 °C

Table 2 pK values obtained from the kinetic parameters, determined for the α-amylase reaction at different pHs and different temperatures. These values have been obtained by fitting data to Eqs. 2 and 3, performed by the computer program Sigma Plot

Temperature (°C)	Profile	pK1 ± SE	pK2 ± SE	s
30	log V	5.58 ± 0.06	10.02 ± 0.19	0.298
30	log V/K	6.05 ± 0.11	10.1 ± 0.3	0.455
40	log V	5.41 ± 0.06	9.59 ± 0.09	0.216
40	log V/K	5.95 ± 0.13	10.0 ± 0.3	0.529
50	log V	5.35 ± 0.06	9.41 ± 0.07	0.205
50	log V/K	5.72 ± 0.06	9.81 ± 0.19	0.238

the dependence of this parameter on temperature, the ionization enthalpy calculated was 13.7 ± 0.3 kcal/mol.

The values for pK_2 found in the V/K profiles at different temperatures ranged from 10.1 to 9.81, and a value of 6.5 ± 0.2 kcal/mol for the enthalpy of ionization was calculated from the dependence of pK_2 on temperature.

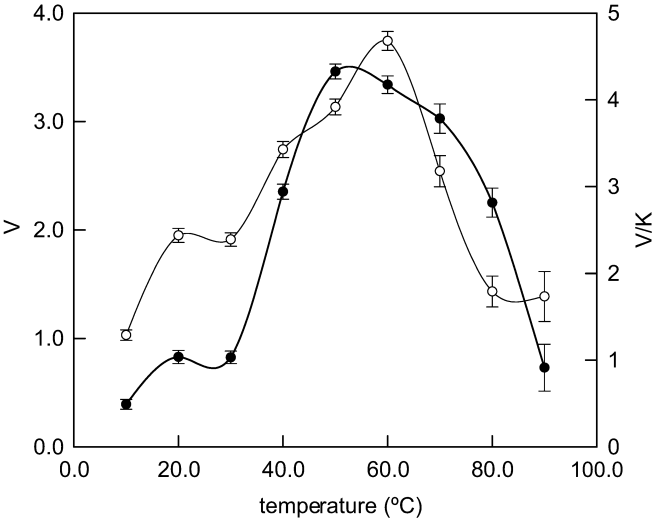

Fig. 4 Effect of temperature on the kinetic parameters for the reaction catalyzed by the α-amylase. Data points were determined in 0.2 M phosphate buffer, pH 7.5, 3 M NaCl, by varying starch concentrations at each temperature. The values calculated for V (•) and for V/K (○) were determined at temperatures from 10 °C to 90 °C

The dependence of stability of the enzyme on pH, at 3 M NaCl, was also investigated, and the half-life calculated for low pHs were as follows: pH 4.2, 5.7 ± 0.2 h; pH 4.6, 138 ± 6 h; and pH 5, 1500 ± 60 h. For pHs from 6 to 8, the half-life was the same as at pH 7.5 (storage pH), about 100 ± 10 days. For higher pHs, the stability increases, reaching a maximum at pH 9 with a half-life of 280 ± 50 days and decreasing to 240 ± 50 days at pH 10.

Effect of temperature on α-amylase

In order to determine the effect of temperature on the kinetic parameters of α-amylase, the activity was determined at different starch concentrations for temperatures from 10 °C to 90 °C and data were fitted to Eq. 1. As shown in Fig. 4, the activity was very low at temperatures up to 30 °C and increased very sharply, reaching a maximum at 50–60 °C. The activity remained very high (65% of the maximal value reached), even at temperatures as high as 80 °C. For V/K (Fig. 4), the maximum obtained was at 60 °C.

The dependence of stability on temperature was also determined by incubating the enzyme in 50 mM Tris-ClH buffer, pH 7.5, at temperatures from 40 °C to 80 °C. Activity of aliquots at different times was plotted as a logarithm of residual activity versus time, and the half-life was determined from the slopes. The values calculated were 2,100 ± 300 h at 40 °C, 10.1 ± 0.8 h at 50 °C, 0.88 ± 0.07 h at 60 °C, 7.2 ± 0.6 min at 70 °C, and 4.1 ± 0.5 min at 80 °C. From the slopes (the

pseudo-first-order constants), the enthalpy for the inactivation by temperature, calculated using Eq. 3, was 390 ± 7 kJ/mol and the entropy was 0.44 ± 0.08 kJ/mol·K.

Effect of EDTA and metal ions on amylase activity

The enzyme was incubated at different EDTA concentrations in 50 mM Tris-HCl buffer, pH 7.5, 3 M NaCl. The addition of EDTA supposed a loss of amylase activity with time. The activities measured were expressed as residual activity, and its logarithm was plotted versus EDTA concentration. The half-life for each concentration was obtained from the slopes, and the values were as follows: 29 ± 5 h at 25 mM EDTA; 64 ± 10 h at 12 mM; 670 ± 40 h at 6 mM; and 3500 ± 500 at 3 mM. The pseudo-first-order constants were determined for each concentration and fitted to Eq. 4, yielding a value of $n = 2{,}4 \pm 0.3$ (i.e., $n \cong 2$), the number of moles of EDTA required to react with essential metal ions in the enzyme.

When loss of activity was achieved, the sample was adjusted to 50 mM magnesium or calcium chloride, and no recovery of activity was observed, therefore indicating an irreversible loss of activity with EDTA.

Magnesium chloride behaved as an inhibitor when added in concentrations from 50 mM to 200 mM. The maximum inhibitory effect was obtained at 100 mM, inhibiting only 10% of the initial activity. Calcium chloride was a slight activator at concentrations below 150 mM, activating only a maximum of 3% at 100 mM, and a slight inhibitor at higher concentrations (8% of the initial activity at 200 mM).

Discussion

The production of this halophilic α-amylase is related to the presence of starch in the media and subsequently to the use of this carbon and energy source by *Haloferax mediterranei*.

The enzyme displayed amylase activity, as it was able to hydrolyze starch but was not able to hydrolyze pullulan, showing its ability to hydrolyze 1–4 bounds but not 1–6 bounds. The anomeric form of the products, according to the NMR spectra obtained and the polarimetry results, clearly showed their α-configuration (Igarashi et al. 1998), and hence, our enzyme can be classified as an α-amylase.

The purification procedure was very similar to those used for other halophilic enzymes (Bonete et al. 1986, 1987, 1996; Camacho et al. 1995; Ferrer et al. 1996; Serrano et al. 1998; Martínez-Espinosa et al. 2001) indicating the same halophilic behavior. The apparent relative molecular mass for the α-amylase by gel filtration was very similar to that found by SDS-PAGE; hence, the enzyme seems to be a monomeric protein. Similar relative molecular masses have been determined by SDS-PAGE for other α-amylases such as those from *Thermus filiformis Ork A2* (Egas et al. 1998), with a relative molecular mass of 60 kDa; from *Thermococcus hydrothermalis*, 53.6 kDa (Lévêque et al. 2000); and *Bifidobacterium adolescentis*, 66 kDa (Lee et al. 1997). Halophilic amylases from *Halothermothrix orenii* had a relative molecular mass of 56 kDa (Mijts and Patel 2002).

A decrease in the salts required by halophilic enzymes may lead to the loss of their structure and function (Danson and Hough 1997), as they usually need high salt concentration for stability and optimal activity (Bonete et al. 1986, 1987, 1996; Ferrer et al. 1996; Martínez-Espinosa et al. 2001; Serrano et al. 1998). The α-amylase reported here has to be adapted to the high NaCl concentration in the medium, where the enzyme plays its function. The increase of activity with salt concentration is a common feature of archaeal halophilic enzymes (Dym et al. 1995), showing the highly halophilic character of the α-amylase from *Haloferax mediterranei*.

On the other hand, K values increased at higher NaCl concentrations. More concentrated solutions thus seem to affect the binding mechanism between the enzyme and the substrate (starch). However, as described before, V/K increases at higher salt concentrations, indicating that salt concentration has an overall enhancing effect on the reaction rate.

Most of the halophilic enzymes studied are inactivated when the NaCl or KCl concentration decreases to less than 2 M (Madern et al. 2000). However, the high stability of the α-amylase from *Haloferax mediterranei* at lower salt concentrations, as well as the broad range of salt concentrations it is able to endure (both for activity and stability), may be related to fact that it is an excreted enzyme. Since salt dilution is a common stress factor in the ever-changing salt environment (D'Souza et al. 1997), this behavior may enable its adaptation to its natural habitat, mainly salterns (Rodriguez-Valera et al. 1983).

This behavior is very close to that reported for the amylases from the mesophilic, extremely halophilic Archaea, namely, *Halobacterium halobium* (Good and Hartman 1970) and *Natronococcus amylolyticus* (Kobayashi et al. 1992) and the moderately halophilic, mesophilic aerobic Bacteria *Halomonas meridiana* (Coronado et al. 2000), which are also relatively stable and active at low salt concentrations.

Studies on pH dependence of the kinetic parameters V and V/K at several temperatures have provided both useful information for optimal conditions for amylase activity and a better understanding of the role of ionizing amino acid residues in the catalytic reaction (Karsten and Viola 1991; Pérez-Pomares et al. 1999).

The pK_1 values for the log V profiles, as well as those for log V/K, at different temperatures allowed us to determine the ionization enthalpy of the groups involved in the α-amylase reaction. The pK_1 values, related to a group that must be deprotonated for binding or catalysis, were in the same range as those found in other

proteins of an imidazolium group (5.5–7.0). The enthalpy of ionization calculated was also very close to the values that this prototropic group, in histidine residues, displays in other enzymes (6.9–7.5) (Segel 1993).

The pK_2 values for the log V and log V/K also decrease as the temperature increases. However, the ionization enthalpy for both parameters from the dependence of pK_2 on temperature was far different. These pKs for the basic side of the pH profiles, related to a group or groups preferentially protonated for catalysis, are in the same range as those found for the phenolic OH of a tyrosine (9.8–10.5) or the ϵ-amine of a lysine (9.5–10.6). The ionization enthalpy for the phenolic group is about 6 kcal/mol, very close to that obtained for the log V profile. The pK_2 from the log V/K profile was very similar to the ionization enthalpy for the ϵ-amine, which ranged from 10 to 13 kcal/mol (Segel 1993).

The optimal pH range for the catalytic reaction ranged from 7 to 8. These values are compared with those reported for an amylase from alkaliphilic *Bacillus* sp., at pH 8.0–8.5 and stable in a range from pH 6 to 10 (Igarashi et al. 1998), and are a little higher than those reported for the amylase from *Halobacterium salinarum*, with an optimal pH range from 6.4 to 6.6 (Good and Hartman 1970). Other prokaryotes such as the thermophilic and halotolerant bacteria *Halothermothrix orenii* are reported to have α-amylases that also display optimal activities in this range of pHs (Mijts and Patel 2002). The pH of the usual culture medium for *Haloferax mediterranei* is 7.2 (Rodriguez-Valera et al. 1980), which is in the range for optimal activity of the α-amylase reported here .

Regarding the stability of the enzymes at different pH, the enzyme was highly stable at pH 10. In fact, the stability sharply increases as pH rises. The alkaliphilic nature of the enzyme was much more remarkable in the stability of the enzyme than in its activity, being a highly interesting feature for possible industrial applications. The use of α-amylases in detergents for medium-temperature laundering demands enzymes with high stability and activity in washing environments, usually very alkaline and oxidizing and containing metal ion-chelating agents and proteases (Nielsen and Borchert 2000).

The α-amylase from *Haloferax mediterranei* was affected by the addition of EDTA at concentrations above 10 mM, but the loss of activity was not instantaneous, and even at 25 mM EDTA, the half-life (29 h) was very large. These results are similar to those reported for other liquefying α-amylases, such as those from alkaliphilic *Bacillus* species (Igarashi et al. 1998). This loss of activity has been related to the conserved calcium ion located in the interface between domains A and B in α-amylases. This calcium ion is bound very tightly and its role has been suggested to be mainly structural. One or more calcium ions have been found in several structures (Machius et al. 1998; Nielsen and Borchert 2000).

The optimal temperature for the α-amylase from *Haloferax mediterranei* was in the same range as the optimal temperature (55 °C) reported for the enzyme from *Halobacterium salinarum*. However, the activity dropped sharply in this case, and at 65 °C the activity was very low (Good and Hartman 1970). Also for the alkaliphilic *Bacillus* sp., the optimal temperature was 55 °C (Igarashi et al. 1998). Halophilic enzymes such as NAD and NADP glutamate dehydrogenases from *Halobacterium salinarum* display maximal activity at 70 °C, and their heat stability is also very high (Bonete et al. 1986, 1987). This value is even similar to that obtained for thermophilic enzymes such as the pullulanase and α-amylases from *Thermus* sp. AMD33, with an optima at 70 °C for both enzymes (Nakamura et al. 1989), or for the halophilic and thermophilic bacteria *Halothermothrix orenii*, with an optima at 65 °C (Mijts and Patel 2002), a value not too far from that reported here for the α-amylase from *Haloferax mediterranei*.

The enzyme also displayed this thermophilic nature in its stability at high temperatures, but its stability decays sharply at temperatures above 60 °C. This is a common feature among amylase enzymes from mesophilic halophilic organisms, limiting their use in industrial starch-degrading applications (Mijts and Patel 2002). The amylase enzyme from the thermophilic and halophilic bacteria *Halothermothrix orenii* displayed optimal activity at 65 °C, the temperature at which this organism grows, and therefore it should be more stable than our enzyme at this condition; however, both of them are very stable at high temperatures and are able to work at high salinity conditions.

This high optimal temperature may be considered an adaptive response to the high temperatures these enzymes have to endure in their natural salt environments, i.e., salterns exposed to intense sunlight. This thermophilic nature has been reported for several halophilic enzymes (Bonete et al. 1986, 1987; Camacho et al. 1995; Marhuenda-Egea et al. 2001).

In conclusion, the enzyme described here presented several features very close to those found in other halophilic enzymes, including salt-dependent activity and stability as well as alkaliphilic and thermophilic behavior. The residues implied in the chemical mechanism also seem to share some common features that may be related to the halophilic nature of the enzyme that allows it to function in such extreme and interesting conditions.

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