Extremophiles (2009) 13:633–641
DOI 10.1007/s00792-009-0246-2

ORIGINAL PAPER

Identification of several intracellular carbohydrate-degrading activities from the halophilic archaeon *Haloferax mediterranei*

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Received: 4 November 2008 / Accepted: 1 April 2009 / Published online: 26 April 2009

Abstract Three different amylolytic activities, designated AMY1, AMY2, and AMY3 were detected in the cytoplasm of the extreme halophilic archaeon *Haloferax mediterranei* grown in a starch containing medium. This organism had also been reported to excrete an α-amylase into the external medium in such conditions. The presence of these different enzymes which are also able to degrade starch may be related to the use of the available carbohydrates and maltodextrins, including the products obtained by the action of the extracellular amylase on starch that may be transported to the cytoplasm of the organism. The behavior of these intracellular hydrolytic enzymes on starch is reported here and compared with their extracellular counterpart. Two of these glycosidic activities (AMY1, AMY3) have also been purified and further characterized. As with other halophilic enzymes, they were salt dependent and displayed maximal activity at 3 M NaCl, and 50°C. The purification steps and molecular masses have also been reported. The other activity (AMY2) was also detected in extracts from cells grown in media with glycerol instead of starch and in a yeast extract medium. This enzyme was able to degrade starch yielding small oligosaccharides and displayed similar halophilic behavior with salt requirement in the range 1.5–3 M NaCl.

Keywords *Halophilic archaea* · *Haloferax mediterranei* · Amylolytic enzymes · Isolation and biochemical characterization

Communicated by F. Robb.

Electronic supplementary material The online version of this article (doi:10.1007/s00792-009-0246-2) contains supplementary material, which is available to authorized users.

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Introduction

Polysaccharides are widely used as a carbon and energy source by organisms from the three domains of life, including *Archaea*, to support heterotrophic growth. The diversity of oligo and polysaccharides provides an abundance of biological roles for these carbohydrates such as structure, food storage and utilization, viral invasion or highly selective cellular signaling events (Henrissat and Davies 1997). The utilization by the cell of the polysaccharides available in the medium generally involves its extracellular hydrolysis by glycosyl hydrolases, thus generating a great variety of oligosaccharides. The next steps are the uptake of these sugars, carried out by specific transporters and finally their intracellular hydrolysis by specific intracellular glycoside hydrolases to generate mainly hexoses and pentoses.

Different enzymes are able to hydrolyze glycosidic bonds in carbohydrates. Among them there are, for example, amylases, that may be classified according to the manner in which the glycosidic bond is attacked. Alpha-amylases hydrolyze alpha-1,4-glycosidic linkages, randomly yielding alpha-limit dextrins, oligosaccharides, and monosaccharides and are exoamylases, which hydrolyze the glycosidic linkage only from the non-reducing outer polysaccharide chain ends. Exoamylases include beta-amylases and glucoamylases (gamma-amylases, amyloglucosidases).

Beta-amylases yield beta-limit dextrins and maltose and gamma-amylases yield glucose. Due to the complex structure of starch, an appropriate combination of intracellular and extracellular enzymes is required for its conversion to oligosaccharides and smaller sugars (van der Maarel et al. 2002).

Our report is focused on the starch and other carbohydrate-degrading enzymes from the halophilic archaeon *Haloferax mediterranei*. This extremophile grows efficiently on starch, lactose, sucrose, fructose, and glucose (Danson 1989; Rodríguez-Valera et al. 1983; Altekar and Rangaswamy 1990). Although a lot of effort has been invested in the study of these degrading enzymes from hyperthermophilic archaeal organisms, fewer studies are focused on their counterparts from other physiological groups of extremophiles such as halophiles and alkaliphiles (Mijts and Patel 2002; Verhees et al. 2003; Sakuraba et al. 2004; Labes and Schönheit 2007). However, general descriptions and information about the glycolytic pathways are available for archaeal organisms (Siebers and Schönheit 2005). Halophilic archaea glycolytic pathways are considerably different from each other, and their variety depends on the acquisition or non-acquisition of the genes implied in the uptake and degradation of various sugars (Falb et al. 2008). Archaeal halophiles include carbohydrate-utilizing organisms, such as *Haloarcula marismortui*, *Halococcus saccharolyticus* or *Hfx. mediterranei* (Rodríguez-Valera et al. 1983), which are able to catabolize hexoses, pentoses, sucrose, and lactose (Rawal et al. 1988; Altekar and Rangaswamy 1990; Johnsen et al. 2001; Johnsen and Schönheit 2004) and non-carbohydrate-utilizing ones such as *Halobacterium salinarum* (Rawal et al. 1988), indicating the great diversity in their sugar-degrading capabilities.

Hfx. mediterranei excretes at least an extracellular α-amylase that releases mainly maltose as a final cleavage product (Pérez-Pomares et al. 2003). However, the way the sugars are introduced into the cytoplasm of the cell, the oligosaccharides that can be transported and the transporters implied remain unknown. The approaches followed to unravel the routes or steps leading to the further degradation and usage of the polysaccharides include the identification, characterization and purification of enzymes involved in this task. These findings may give us useful information about the specific properties required for activity under these conditions. Furthermore, archaeal organisms 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 (Marhuenda-Egea and Bonete 2002; Danson and Hough 1997). Here we report the presence of several intracellular carbohydrate degrading enzymes that display amylolytic activity.

Materials and methods

Growth conditions and crude enzyme preparation

Hfx. mediterranei strain R4 (ATCC 33500) (Rodríguez-Valera et al. 1983) was grown in 25% (w/v) salts, at 37°C, pH 7.2 and supplemented with different carbon and nitrogen sources. The different media used, named as A, B, and C were: (A) medium including 1% (w/v) ammonium acetate as carbon and nitrogen source and supplemented with 0.2% (w/v) soluble starch, adding a previously filter-sterilized starch stock solution; (B) medium with 1% (w/v) ammonium acetate but supplemented with 2% (v/v) glycerol and (C) complex medium prepared containing 0.5% (w/v) yeast extract.

Cells were harvested by centrifugation at 10,000 rpm for 30 min at 4°C and washed three times with 25% (w/v) salt solution. The cell pellet was resuspended in 0.05 M phosphate buffer, 2.5 M ammonium sulfate, sonicated and centrifuged at 30,000 rpm for 60 min. The cell extract was used as crude enzyme preparation.

Determination of degrading activity

The activities were routinely assayed by the iodine binding assay, in 50 mM Tris–HCl buffer pH 7.5, at 40°C, 3 M NaCl (activity buffer). The reaction mixture was adjusted to the adequate starch concentration with potato-soluble starch (Sigma) 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 which hydrolyzed 1 mg of starch in 1 min (Haseltine et al. 1996).

The activity was also measured by the dinitrosalicylic acid method (Bernfeld 1955) in order to determine the release of reducing end sugars. The reaction mixture contained the different concentrations of sugars tested: soluble starch, maltotriose, maltohexaose, α-cyclodextrin and β-cyclodextrin. The reaction was kept at the temperatures tested in a thermostatic bath and stopped in ice. The dinitrosalicylic acid reagent (100 µl) was added to the samples and this mixture was heated at 100°C in a boiling bath for 10 min. The development of color was followed spectrophotometrically at 540 nm. One unit of activity was defined as the amount of protein which produced 1 µmol of reducing ends in 1 min. Maltose was used to produce a standard curve. All assays were performed, at least, in duplicate and average values obtained.

The degradation of starch by the different enzymes was determined by both methods. Each enzyme was added to a starch solution (10 mg/ml) in Tris–HCl buffer pH 7.5, 3 M

NaCl and aliquots were tested for the remaining starch or reducing ends formed at different times.

Enzyme purification

The crude enzyme preparation 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 further purified. Each pool was applied to a starch column prepared with insoluble starch packed in a 2.5 × 10 cm column. This column was intensively washed with 3 M NaCl 0.02 M Tris–HCl pH 8.0 buffer and the enzyme was recovered with 0.5 M maltose prepared in the same buffer. The active fractions pooled were further purified by gel filtration in a Sephacryl S-300 column. The protein concentration was determined by the Bradford method (Bradford 1976).

The degrading enzyme marked as AMY2 did not significantly bind to the starch column. In this case, the sample was concentrated with a DEAE–cellulose column and the enzyme further purified by gel filtration on Sephacryl S-300 column. The active fractions were concentrated to a final volume of 50 µl by ultrafiltration in an Amicon concentrating unit using a 10,000-MW cut-off membrane, and the enzyme applied to a SDS-PAGE for further studies.

The three purified enzymes were analyzed by SDS-PAGE and the relative molecular mass was determined by gel filtration chromatography in a Sephacryl S300 column.

Identification of the products

Thin-layer chromatography (TLC) was used to analyze products resulting from the action of the carbohydrate degrading enzymes on different substrates: 0.5% (w/v) starch, 50 mM maltotriose, maltohexaose, α, and β-cyclodextrin. Each purified enzyme was incubated overnight with the substrate, as described previously, and spotted in silica gel plates. The plate was developed with solvent mixtures containing different proportions of isopropanol–acetone–lactic acid. Standard 50 mM or 1% solutions of the carbohydrates used as substrates were also included in the plates.

The oligosaccharides were detected by spraying 1% diphenylamine, solved in acetone containing 10% phosphoric acid to the TLC plate, and heated at 160°C for 10 min as described by Kobayshi et al. (2000).

Effect of salt concentration, pH and temperature

The effect of salt concentration on enzyme activity was tested at 40°C in 50 mM Tris–HCl, pH 7.5 buffers containing different NaCl concentrations. For each salt concentration, starch was varied from 0.02 to 0.2% (w/v). The maltooligosaccharides concentrations tested ranged from 0.02 to 50 mM.

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 containing 3 M NaCl. The pH was checked after each reaction and showed no changes with respect to the initial values. For each pH, carbohydrate concentrations were varied as described earlier.

The assays to study the dependence of temperature on the activity were carried out in 0.2 M Tris–HCl buffer pH 7.5, 3 M NaCl at different temperatures. For each temperature, carbohydrate concentrations were varied as described previously.

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 Michaelis Menten equation 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).

Results

The halophilic archaeon *Hfx. mediterranei* was grown in three different media (A, B and C) in order to compare the intracellular carbohydrate-degrading enzymes produced in response to the conditions assayed. The cells were also able to grow when ammonium acetate was substituted for 0.5 M potassium nitrate; however, the growth rate and yield of degrading enzymes were lower. The amylolytic activity was measured routinely using the iodine method. When cells were grown in medium A, the activity displayed by the extracellular amylolytic activity was far higher than that found in the correspondent cell extract obtained by sonicating the cell pellet. Due to this low activity, compared with the extracellular activity, longer reaction times were required. The cell extract from the cells grown in medium A, was applied to a Sepharose-4B column and eluted with an ammonium sulfate gradient from 2.5 to 0.5 M ammonium sulfate. The amylolytic activities were tested on the fractions obtained and the results are shown in Fig. 1a. Under these conditions three main activities, reported as AMY1, AMY2, and AMY3, were observed and pooled separately in order to be analyzed and further purified.

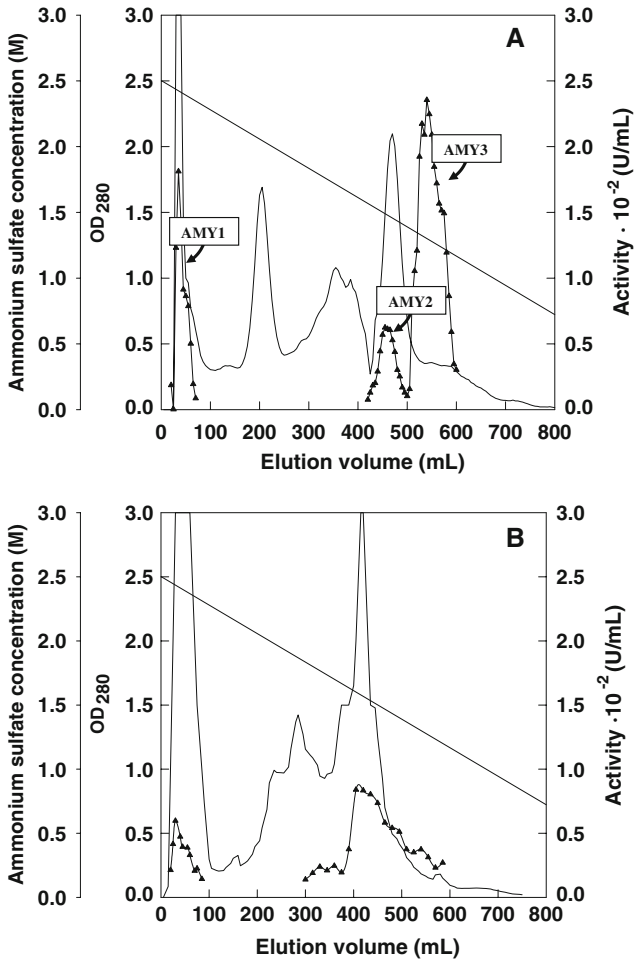

Fig. 1 Isolation of the cytoplasmic amylolytic activities AMY1, AMY2, and AMY3 from *Hfx mediterranei*, growth in **a** medium with 1% ammonium acetate and 1% starch and **b** medium with 1% ammonium acetate and 2% glycerol; eluted from a Sepharose 4B column with an ammonium sulfate 2.5 to 0.5 M gradient. The activities *filled triangle* were determined as stated in "Materials and methods" with starch as substrate

When the cells were grown in media lacking starch (B and C) no external amylase activity was found, although the cell extract displayed starch degrading activity. The cell extract from medium B was also applied to a Sepharose-4B column and the fractions tested as described for medium A. The results, shown in Fig. 1b, displayed two main starch-degrading activities in the same position as those found for AMY1 and AMY2 from medium A (Fig. 1a), albeit with lower activities. These activities in the same position as AMY1 and AMY2 were pooled to be further purified and characterized. The activity in the same position as AMY2 displayed the same features as AMY2. However, the activity found in the same position as AMY1 displayed different features, including the fact that it was not retained in the starch column and so could not be purified to homogeneity.

On the other hand, when the pooled activities AMY1 and AMY3 were applied separately to a column with insoluble starch, they were retained in the column, washed and eluted using Tris–HCl buffer containing 0.5 M maltose. As summarized in Table 1, the purification factor achieved by this single step was 3,400-fold for AMY3, and its molecular mass 64 ± 7 kDa (Table 2). This purified sample was used to determine kinetic parameters Km and *V*/*K* for both starch and maltohexaose as shown in Table 2. The same procedure applied to the activity named AMY1 also yielded a very high purification factor (Table 1) and its molecular mass was 38 ± 3 kDa. The subunit molecular masses for AMY3 and AMY1, determined by SDS-PAGE, were 71 ± 7 and 45 ± 3 kDa, respectively.

The activity AMY2 was not retained in the starch column; however, the elution volume was larger than the total volume of the starch bed, thus indicating some affinity for the starch column. The pooled samples of AMY2 were applied to a Sephadex S300 column for further purification

Table 1 Purification of the amylolytic activity AMY1 and AMY3 from *Hfx. mediterranei* grown in a medium containing 1% ammonium acetate and 0.2% starch

	Volume (ml)	Activity (U/ml)	Yield (%)	Protein concentration (mg/ml)	Specific activity (U/mg)	Purification factor
Cell extract						
	15	0.20	100	18	2.6×10^{-3}	1
Sepharose-4B A.S. gradient						
AMY1	20	0.025	17	4.5	5.6×10^{-3}	2.2
AMY3	45	0.016	24	0.081	0.20	76
Starch column						
AMY1	6	0.071	14	0.015	4.7	1,810
AMY3	12	0.053	21	6.0×10^{-3}	8.8	3,400
Sephacryl-S300						
AMY1	6	0.041	8.0	5.0×10^{-3}	8.0	3,080
AMY3	19	7.2×10^{-3}	4.6	0.7×10^{-3}	10.2	3,900

Table 2 Kinetic parameters and molecular masses for the amylolytic activities in *Hfx. mediterranei*

	MW (kDa)	Km starch (mg/ml)	*V*/*K* starch	Km maltohexose (mM)	*V*/*K* maltohexose
AMY1	38 ± 3	16 ± 4	$3.7 \times 10^{-4} \pm 0.2 \times 10^{-4}$	2.2 ± 0.6	$1.3 \times 10^{-3} \pm 0.2 \times 10^{-3}$
AMY2	116 ± 34	11 ± 2	$2.8 \times 10^{-4} \pm 0.2 \times 10^{-4}$	0.35 ± 0.08	$7.1 \times 10^{-3} \pm 1.3 \times 10^{-3}$
AMY3	64 ± 7	1.1 ± 0.3	$2.7 \times 10^{-4} \pm 0.4 \times 10^{-4}$	13 ± 5	$2.5 \times 10^{-3} \pm 0.7 \times 10^{-3}$
AMYEXT	70 ± 10	2.7 ± 0.7	$4.6 \times 10^{-3} \pm 0.7 \times 10^{-3}$	5.6 ± 1.1	$9.6 \times 10^{-4} \pm 1.1 \times 10^{-4}$

and to estimate its molecular mass, 116 ± 34 kDa (Table 2). The sample was analyzed by SDS-PAGE but no single band of protein was achieved. However, this enzyme preparation was used to determine the kinetic parameters and molecular masses shown in Table 2. In order to isolate AMY2, a non-denaturing electrophoresis on polyacrylamide containing 1% (w/v) starch was carried out and the activity was monitored by staining the gel with iodine. The degradation of the starch and the related lack of blue color indicated the presence of the enzyme. The sample was also applied to other lanes of the electrophoresis gel to be stained with Coomassie Blue. The band stained with Coomassie Blue corresponding to the zone that displayed lack of color in the other part of the gel stained with iodine was cut out and analyzed by LC–MS/MS technique, but no consistent results were obtained from this analysis.

Finally, the method developed for the study of these enzymes was also applied, as a control, to the culture supernatant. The activity thus obtained, named AMYEXT, was also retained by starch and released by 0.5 M maltose.

Effect of salt concentration, pH, and temperature on the carbohydrate-degrading activities

In order to study the dependence on salt concentration for activity, the kinetic parameters (*V*, *K*, *V*/*K*) were determined at different NaCl concentrations. Data for all the assays fitted Michaelis–Menten kinetics. The optimal salt concentration for AMY1 and AMY3 was 3 M NaCl, although they are still active at concentrations as high as 4 M NaCl (Fig. 2a). On the other hand, AMY2 displayed high activity in the range from 1 to 3 M NaCl (Fig. 2a). AMY1, AMY2, and AMY3 remained stable when stored at concentrations from 1 to 4 M NaCl, but drastically lost their activities when dialyzed against activity buffer without NaCl.

Similar assays were performed in order to determine the effect of pH on the carbohydrate-degrading enzymes, in a range from 4 to 10. The initial velocity patterns were determined for each pH, by varying the starch concentration. As displayed in Fig. 2b, the optimal pH was 7 for all the three enzymes, although they remained very active at pH 8 and the enzyme AMY3 was highly active from pH 5 to pH 7.

The effect of temperature on the kinetic parameters for these glycosidic enzymes was also determined at temperatures from 10°C to 70°C. As shown in Fig. 2c, the activity was very low at temperatures below 30°C and increased very sharply reaching a maximum at 50°C. The three enzymes remained active at 60°C although activity declined sharply at temperatures as high as 70°C.

Analysis of the products formed in the carbohydrate degradation

The reaction of degradation of starch by the three amylolytic internal enzymes and the excreted enzyme was monitored measuring the concentration of remaining starch (Fig. 3a) and the simultaneous production of reducing ends (Fig. 3b). When there was no remaining starch, as determined by iodine staining, in the reaction mixture of AMY1 and AMY3, the concentration of reducing ends in these reaction mixtures was much lower than that measured in the same conditions for AMY2. So, AMY1 and AMY3 would be producing less but larger dextrins, whilst AMY2 would produce more yet smaller dextrins. Moreover, AMY2 displayed lower Km value (0.35 ± 0.08 mM) for maltohexaose than AMY1 and AMY3, indicating higher affinity for this smaller carbohydrate as a substrate. As a matter of fact, all these internal degrading enzymes displayed Km values for maltohexaose (oligosaccharide) far lower than those found for the external amylase. Meanwhile, the Km for starch was far greater than that found for the external amylase (large polysaccharide) (Table 2) indicating less affinity for this polymer. To confirm these findings and visualize the products formed, thin layer chromatography was performed.

TLC plates were developed using different solvent systems containing isopropanol, acetone, and lactic acid. When mixed in a volume of proportion 4:2:1, respectively, the separation of oligosaccharides of lower molecular mass, such as glucose (G1) and maltose (G2) was optimal. To obtain an optimal separation of oligosaccharides from G1 to G6 (maltohexaose) the volume proportion used was 2:2:1 isopropanol, acetone, and lactic acid, respectively. The degradation of starch by AMY1 yielded maltose in a very low concentration, and dextrins larger than maltohexaose since they could not be separated by TLC under

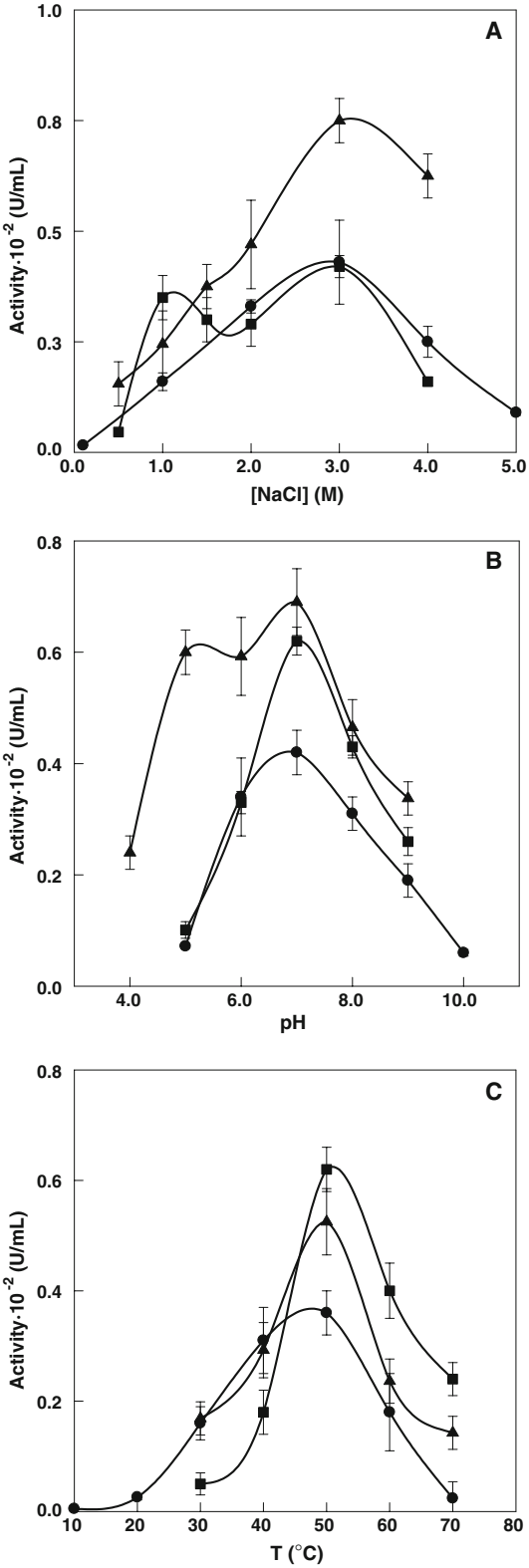

Fig. 2 Effect of the salt concentration (NaCl) on the activity for the starch hydrolysis reaction catalyzed by AMY1 (*filled circle*) AMY2 (*filled square*), and AMY3 (*filled triangle*) **a**. Each data point was determined by varying starch concentrations at different salt concentrations. **b** Effect of pH. **c** Effect of temperature

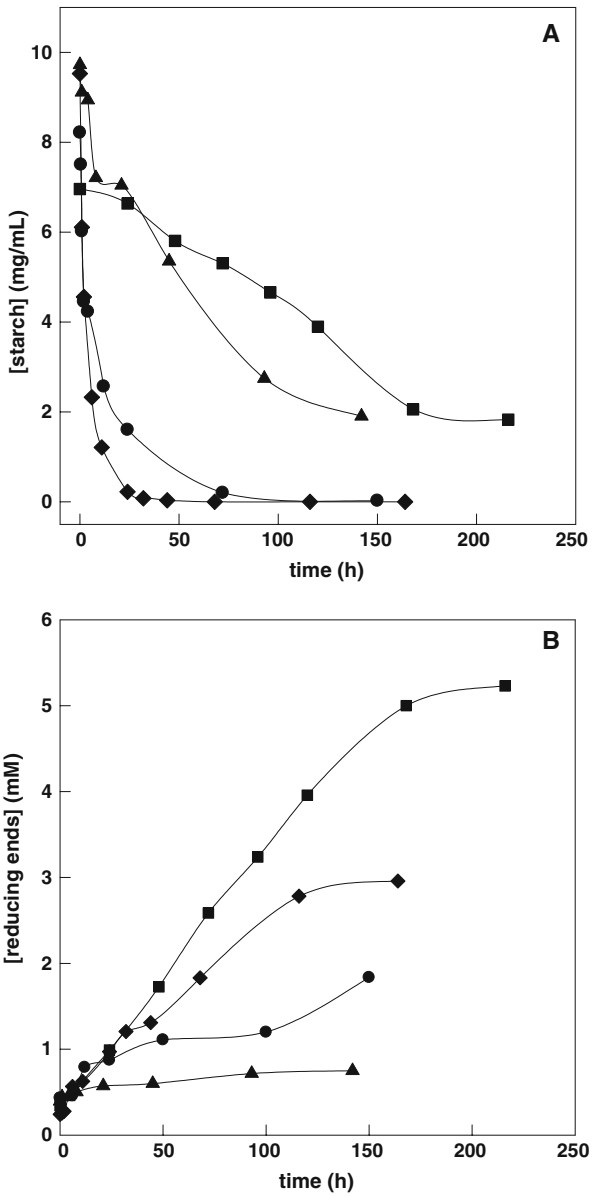

Fig. 3 Degradation of starch by AMY1 (*filled circle*), AMY2 (*filled square*), AMY3 (*filled triangle*), and AMYEXT (*filled diamond*) versus reaction time. **a** Remaining starch concentrations measured by the iodine method. **b** Reducing ends formed in the hydrolysis reaction. Concentrations were determined by the dinitrosalicylic acid method

the test conditions. The degradation of maltohexaose by AMY1 yielded such a low concentration of reducing ends that they were not detectable by TLC. The degradation of starch by AMY2 produced mainly glucose and dextrins larger than maltohexaose and the degradation of maltohexaose also produced glucose. The enzyme AMY3 degraded starch producing mainly maltotetraose or maltopentaose and dextrins larger than maltohexaose, and produced no detectable amounts of oligosaccharides with

maltohexaose as a substrate, under the conditions used for TLC analysis (Fig. 1, supplementary material).

Discussion

The use of starch may be essential for the cell in order to obtain carbon and energy when other sources are not available or are not abundant in the medium. The halophilic archaeon *Hfx mediterranei* enables comparative studies with different carbon and energy sources, because it grows readily in controlled media with fewer nutritional requirements than other halophilic organisms, and since it can use a wide range of carbohydrates, such as starch, lactose, fructose, and glucose as carbohydrate substrates (Verhees et al. 2003). One of these controlled media only contained glycerol and ammonium acetate as energy and carbon sources. Glycerol was chosen due to its abundance and because it is widely used by many organisms including *Hfx. mediterranei* as an energy and carbon source (Oren 1993). We have compared the adaptation of this halophilic archaeon to these media: starch, glycerol, and yeast extract medium by producing glycosidic activities both intra and extracellular.

The purification steps for many halophilic enzymes (Bonete et al. 1986, 1987, 1996; Camacho et al. 1993; Ferrer et al. 1996; Serrano et al. 1998; Martínez-Espinosa et al. 2001) include the elution of the sample loaded in a Sepharose-4B column with an ammonium sulfate gradient from 2.5 to 0.5 M. This step allowed us to separate the amylolytic enzymes present in the crude extract and also enables us to compare the production of each enzyme under the different test conditions. When starch was present in the medium, the activity AMY3 was much higher than that found when grown in a complex medium and was not significantly detected when starch was replaced by glycerol. Accordingly, this enzyme may be related to the optimal use of starch.

When the degradation of starch by these three intracellular enzymes AMY1, AMY2, and AMY3 seemed to be total, the concentration of reducing ends was still increasing, showing that these enzymes continued degrading starch into smaller saccharide molecules that could not be detected by the Lugol reagent (the minimum length of the amylose chain to produce its characteristic blue color is about 30–40 D-glucose units, providing a cavity corresponding closely in length to an 11-atom polyiodine chain). These smaller molecules were also detected by TLC. The amylolytic activities AMY1, AMY3, and the extracellular amylase degraded the starch into larger saccharide molecules, which agrees with their high Km values for maltohexose, being even higher than those for starch displayed by the extracellular amylase. However, AMY1 and AMY2 preferred shorter saccharide chains instead of starch. The maximal rate of production of reducing ends, as well as the maximal affinity for maltohexose, was found for AMY2, thus indicating that it released smaller oligosaccharides than the other glycolytic activities. The enzyme produced mainly glucose and maltotriose, and the rate of production of reducing ends was far higher than that found for the other internal activities. Meanwhile, iodine still developed color, indicating that starch molecules had not been totally degraded. This factor may indicate that this enzyme mainly cuts starch into small pieces and possibly in a less random way. Although we are still not able to classify it, this particular glucosidase activity would appear to be its main role.

The apparent relative molecular masses for the amylolytic activities AMY1 and AMY3 were very similar to those determined by SDS-PAGE, suggesting that these enzymes are monomeric proteins. Similar relative molecular masses have been reported for other starch degrading enzymes such as the α-amylases 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) or *Bifidobacterium adolescentis*, 66 kDa (Lee et al. 1997). The other amylolytic activity, AMY2, displayed a larger molecular mass 116 ± 34 kDa, but it requires further purification in order to elucidate its quaternary structure.

The common feature AMY1, AMY2, and AMY3 displayed was their halophilic behavior and certain thermophilic character. The activity for these intracellular enzymes was optimal at 3 M NaCl, a value similar to that found for the extracellular amylase (Pérez-Pomares et al. 2003), and the activity increased as salt concentration reached this particular salt concentration, becoming highly active at 4 M NaCl. These enzymes drastically lost their activity when dialyzed against a buffer without NaCl, indicating that they needed this in order to maintain their structures. The decrease in the salt concentration required may lead to the loss of the structure and function of the halophilic enzymes (Danson and Hough 1997; Bonete et al. 1986, 1987, 1996; Ferrer et al. 1996; Serrano et al. 1998; Martínez-Espinosa et al. 2001). Most of the halophilic enzymes studied are inactivated when the NaCl or KCl concentration decreases to less than 2 M (Madern et al. 2000).

The optimal pH for these enzymes was very close to pH 7, a value lower than that found for the extracellular amylase, which ranged from 7 to 8 (Pérez-Pomares et al. 2003). Moreover, AMY3 displayed an optimal pH in a range from 5 to 7, which is much lower than that for the extracellular amylase but very similar to the optimal pH which ranged from 6.4 to 6.6 as reported for the amylase from the halophilic archaeon *Hbt. salinarum* (Good and Hartman 1970).

The optimal temperature for these three degrading intracellular enzymes was 50°C, a little lower than that exhibited by α-amylase from *Hfx. mediterranei*, that was also reported to maintain its activity at temperatures as high as 80°C (Pérez-Pomares et al. 2003). This optimal temperature is also lower than that reported for several halophilic enzymes that usually exhibit certain thermophilic behavior (Bonete et al. 1986, 1987; Camacho et al. 1993; Marhuenda-Egea and Bonete 2002). However, their optimal temperature was very close to that found for the amylase from *Hbt. salinarum* (55°C), and the activity also decays at temperatures above 65°C (Good and Hartman 1970).

In conclusion, *Hfx. mediterranei* synthesizes intracellular enzymes which are able to degrade large dextrins, named AMY1 and AMY3, that are detected within the cell extract when cells are grown in starch containing media. This factor may allow the cell to use the dextrins that are larger than maltose produced by the action of the extracellular amylase. This extreme halophilic archaeon also produces another degrading enzyme, that was able to cut starch into smaller oligosaccharides, mainly glucose, named AMY2, and that is also present in cells cultured without starch. All these enzymes exhibit features common to extreme halophilic enzymes, such as salt dependence for activity and stability or certain thermophilic character. These three enzymes would allow the cell to totally degrade the dextrins and oligosaccharides that may be imported into the cell in order to obtain the maximal energy from these compounds.

Acknowledgments This work was supported by funds from BIO2005-08991-C02-01.

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