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Characterisation of a highly stable α-amylase from the halophilic archaeon *Haloarcula hispanica*

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Abstract Intracellular and extracellular proteins from halophilic archaea face very saline conditions and must be able to maintain stability and functionality at nearly saturated salt concentrations. Haloarchaeal proteins contain specific adaptations to prevent aggregation and loss of activity in such conditions, but these adaptations usually result in a lack of stability in the absence of salt. Here, we present the characterisation of a secreted α-amylase (AmyH) from the halophilic archaeon *Haloarcula hispanica*. AmyH was shown to be very halophilic but, unusually for a halophilic protein, it retained activity in the absence of salt. Intrinsic fluorescence measurements and activity assays showed that AmyH was very stable in high-salt buffer and even maintained stability upon the addition of urea. Urea-induced denaturation was only achieved in the absence of NaCl, demonstrating clearly that the stability of the protein was salt-dependent. Sequencing of the *amyH* gene showed an amino acid composition typical of halophilic proteins and, moreover, the presence of a signal peptide containing diagnostic features characteristic of export via the Twin-arginine translocase (Tat). Analysis of the export of AmyH showed that it was translocated post-translationally, most likely in a folded and active conformation, confirming that AmyH is a substrate of the Tat pathway.

Keywords α-Amylase · Halophilic adaptation · *Haloarcula hispanica* · Signal peptide · Twin-arginine translocase

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Introduction

Extremely halophilic archaea (haloarchaea) are obligate halophiles that require more than 15% salt for optimal growth. Haloarchaea can be found in environments such as the Dead Sea, the Great Salt Lake (UT, USA), or man-made solar evaporation ponds that are used for the production of sea salt. The salt concentration in these environments sometimes reaches saturation, and haloarchaea maintain their osmotic balance through the accumulation of large amounts of KCl in the cytoplasm (Lanyi 1974). Thus, proteins from haloarchaea face extremely saline conditions both inside and outside the cell. Halophilic proteins are adapted to these conditions and this is reflected in their unique amino acid composition (for reviews, see Lanyi 1974; Eisenberg 1995; Madern et al. 2000; Mevarech et al. 2000). First, the surface of halophilic proteins is highly acidic, and, second, halophilic proteins have reduced overall hydrophobicity when compared to non-halophilic proteins. These features are important to prevent aggregation and, at the same time, retain structural flexibility at high salt concentrations (Mevarech et al. 2000). Strikingly, haloarchaeal proteins require high salt for activity and stability; at less than 1–2 M NaCl or KCl most haloarchaeal proteins unfold and lose their activity (Madern et al. 2000).

Many starch-degrading proteins have been identified in various organisms, but relatively few are from haloarchaea. An α-amylase from the haloalkaliphilic strain *Natronococcus amylolyticus* (sp. Ah-36) has been characterised biochemically and its corresponding gene has been cloned and sequenced (Kobayashi et al. 1992, 1994). Furthermore, haloarchaeal amylases have been purified and characterised from *Halobacterium salinarum* (*halobium*) (Good and Hartman 1970), *Haloferax mediterranei* (Perez-Pomares et al. 2003), and *Haloarcula* sp. S-1 (Fukushima et al. 2005), but none of their corresponding genes have been cloned or sequenced. *Haloarcula hispanica*, an archaeon that was isolated from a solar saltern in

Spain (Juez et al. 1986), was known to have starch-degrading activity in the culture medium, but the enzyme responsible for this was never isolated or characterised. In the present study we describe the purification of the enzyme responsible for this activity and show its biochemical characteristics. This enzyme is of interest from the perspective of its ability to maintain structure and function under extremely saline conditions. However, this amylase is unusual in that it is able to sustain both structural integrity and activity even after exposure to environments that would usually be considered unfavourable, such as low-salt buffers or high concentrations of urea. Furthermore, we identified the gene encoding the amylase and analysed the secretion of the protein in vivo, showing that the protein is most likely exported via the Twin-arginine translocase (Tat).

Materials and methods

Strains and growth conditions

H. hispanica (Juez et al. 1986; DSM No. 4426) was kindly provided by Dr. Mike Dyall-Smith (University of Melbourne, Australia). A strain overproducing amylase, denoted *H. hispanica* B3, was constructed by transposon mutagenesis using the method described by Woods et al. (1999). Rich growth medium contained 5% peptone, 1% yeast extract, and 23% salt water (18.4% NaCl, 2.7% $MgSO_4$, 2.3% $MgCl_2{\cdot}6H_2O$, 0.54% KCl, and 0.056% $CaCl_2$). *H. hispanica* B3 was grown in the presence of 2 μg/ml lovastatin (Calbiochem). Minimal medium was prepared by supplementing 23% minimal salts (16% NaCl, 6.4% $MgCl_2{\cdot}6H_2O$, 0.64% K_2SO_4) with 10 mM NH_4Cl, 2 mM $CaCl_2$, 0.5 mM K_2HPO_4, 5 mM $NaHCO_3$, 0.2% glycerol, trace elements (0.36 mg/l $MnCl_2{\cdot}4H_2O$, 0.44 mg/l $ZnSO_4{\cdot}7H_2O$, 2.3 mg/l $FeSO_4{\cdot}7H_2O$, and 0.05 mg/l $CuSO_4{\cdot}5H_2O$) and vitamins (1 mg/l thiamine and 0.1 mg/l biotin).

Amylase purification

H. hispanica B3 cultures (2 l) were grown for 6–7 days. Cells were removed by centrifugation, and the supernatant was concentrated 20-fold by tangential ultrafiltration using Vivaflow 200 filtration units with a 30 kDa cut-off (Vivascience). The concentrate was loaded onto a β-cyclodextrin-sepharose column, which was made by coupling β-cyclodextrin to epoxy-activated sepharose 6B (Sigma, MO, USA). The column was washed with 10-column volumes of buffer A (50 mM BisTris–HCl pH 6.5, 4 M NaCl, and 5 mM $CaCl_2$), and protein was eluted using buffer A supplemented with 10 mg/ml β-cyclodextrin. β-Cyclodextrin was subsequently removed by dialysis. If required, the enzyme was further concentrated using Centriprep (Millipore) or Vivaspin (Vivascience) filter units.

Protein determination

Protein concentration was determined with the BCA protein assay (Pierce), using bovine serum albumin as the standard.

Dynamic light scattering

A 100-μl amylase sample in buffer A was centrifuged at 14,000 *g* for 10 min and then analysed in a DynaPro-801 molecular sizing instrument equipped with a microsampler. Samples from three different batches of purified amylase were measured 20 times at 20°C, and data were analysed using Dynamics 5.0 software.

Amylase activity assays

Amylase activity was determined by measuring released reducing sugars using the DNS (dinitrosalicylic acid) method. An amount of 10 μl enzyme was added to 0.5 ml 1% starch in buffer A that had been pre-incubated at 50°C for 5 min. The enzyme–starch mixture was incubated for 20 min at 50°C, and then 0.5 ml DNS reagent (0.4 M NaOH containing 1% 3,5-DNS and 30% sodium potassium tartrate tetrahydrate) was added. Samples were boiled for 10 min, cooled to room temperature, and diluted five times with H_2O. The absorbance of the samples at 540 nm was determined. One unit of activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per minute. The optimal conditions above were determined by varying several parameters. For determining activity at different pH values, the following buffers were used. At pH 3–5.5: buffer A in which BisTris–HCl was replaced by 50 mM acetate-NaOH; pH 6–7: buffer A; pH 7.5–9: buffer A in which BisTris–HCl was replaced by 50 mM Tris–HCl.

For some activity assays the starch-iodine method was used. In those cases, 20 μl enzyme was added to 580 μl buffer A containing 0.05% starch. This was incubated for 10–30 min, after which the reaction was stopped by adding 200 μl iodine solution (50 mM HCl containing 2.5 mM KI and 5 mM I_2) and 1 ml water. Absorbance was measured at 620 nm.

Fluorescence spectroscopy

Fluorescence emission spectra were obtained on a Perkin-Elmer LS50 luminescence spectrometer. Intrinsic fluorescence was measured using an excitation wavelength of 280 nm. Excitation and emission bandwidths were both 4 nm. Usually, ten scans were averaged for each spectrum, and all spectra had the background obtained with buffer alone subtracted. All samples were pre-incubated for 16 h in the various salt and urea concentrations.

Sequencing of the amylase gene

A number of peptide sequences derived from the amylase were determined using in-gel tryptic digestion and tandem mass spectrometry. Based upon these peptide sequences degenerate oligonucleotides were designed. Two oligonucleotides that were successfully used had the sequences GCNCCNGGNHTNACNWTBGCNG and CKYTCRTANACRTANAKRTCYTKRTC. In these oligonucleotides, the following single letter codes were used: B is C, G, or T; H is A, C, or T; K is G or T; N is A, G, C, or T; R is A or G; W is A or T; and Y is C or T. Using the two degenerate primers and *H. hispanica* genomic DNA as a template, a fragment of about 650 bp was amplified by PCR. This PCR product was sequenced, proving that the fragment did indeed specify an internal part of a gene homologous to α-amylase. The remainder of the sequence was determined using partial inverse PCR (Pang and Knecht 1997). In short, partial digests of *H. hispanica* genomic DNA were made with *Alu*I, and the DNA fragments generated by the digests were ligated under dilute conditions in order to obtain only self-ligating fragments. These fragments were used as a template in a PCR reaction containing two primers located in the internal fragment of the amylase gene, but facing opposite directions. The amplified fragments were sequenced in order to obtain the sequences of the flanking regions. The sequence of the amylase gene was verified by amplifying the entire gene in a normal PCR reaction and direct sequencing of both strands. All PCR reactions were performed with Dynazyme EXT DNA polymerase (Finnzymes).

Western-blot analysis

Proteins were separated by SDS-PAGE and immunoblotted using a semi-dry system as described by Kyhse-Andersen (1984). Amylase was visualised with specific antibodies raised in rabbits and horseradish peroxidase anti-rabbit IgG conjugates, using the ECL detection system (Amersham Biosciences).

Pulse-chase labelling

Cells of *H. hispanica* B3 were grown overnight in rich medium at 45°C, after which cells were washed in 23% minimal salts (see above) and subcultured for several hours in minimal medium. Cells at an optical density (660 nm) of approximately 0.7 were pulsed with 25 μCi/ml Promix (Amersham Biosciences; a mixture of [^{35}S]-methionine and [^{35}S]-cysteine). After 10 min an excess of non-radioactive methionine and cysteine (1 mg/ml) was added to stop further incorporation of radioactivity. Samples were then taken after 0 and 30 min. These were immediately mixed with cold TCA (final concentration 10%), and kept on ice for 30 min. Cells and proteins in the medium were pelleted by centrifugation, washed with acetone, dissolved in 50 mM Tris–HCl (pH 8), 2 mM EDTA, 2% sodium dodecyl sulphate, and boiled for 5 min. Amylase was immunoprecipitated as described by Edens et al. (1982) and visualised using SDS-PAGE and a Fuji FLA-5000 phosphorimager.

Results

Purification of *H. hispanica* amylase

Using transposon mutagenesis, we isolated a mutant strain of *H. hispanica* that had approximately fivefold increased levels of amylolytic activity. The increase in activity was shown by an increase in size of the halo that is visible around a colony after growth on starch plates followed by staining with iodine solution (Fig. 1a) and activity measurements (Fig. 1b). The enzyme responsible for this activity, which we termed AmyH (see section on the sequencing of the amylase gene below), was purified from the culture medium. For this purpose, culture supernatant was concentrated by ultrafiltration, and AmyH was then purified using affinity chromatography. After purification, only one band was visible on an

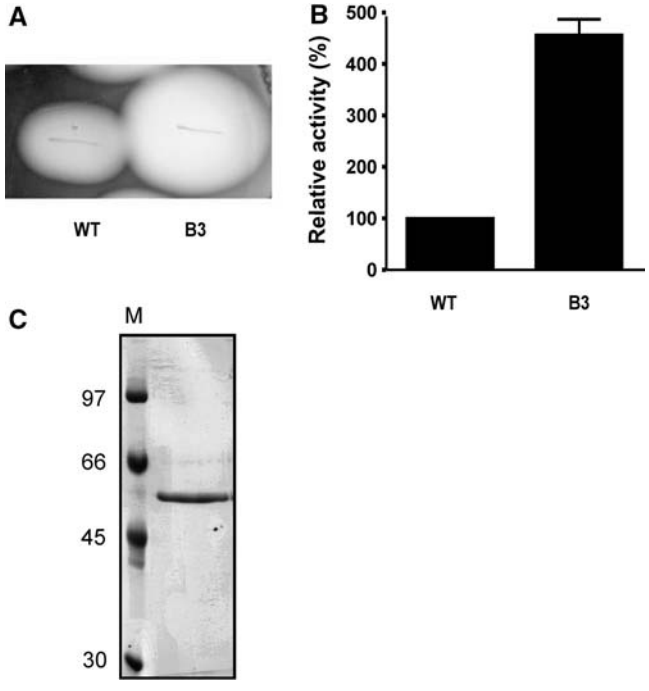

Fig. 1 Overproduction and purification of *H. hispanica* α-amylase. **a** Wild-type (*WT*) and overproducing strain B3 were grown on plates containing rich medium supplemented with 0.2% starch. Amylase activity was visualised by staining with an iodine solution (2% KI, 0.2% I_2). The size of the clear halo around the colony is indicative of the amount of amylase secreted. **b** Amylase activity in the medium of the wild-type and mutant strain B3 was measured using the starch-iodine method. Activity of the wild-type strain was set at 100%. **c** Purified AmyH was visualised by SDS-PAGE and Coomassie Brilliant Blue R250 staining. *M* molecular-weight markers, in kDa

SDS-PAGE gel, with an apparent molecular mass of approximately 50 kDa (Fig. 1c).

Gel filtration chromatography was performed to determine the oligomeric state of this protein. Unfortunately, in a high-salt buffer (4 M NaCl) in which AmyH has its highest activity (see below), the protein eluted at a volume larger than the void volume of the column, indicating that the protein interacted with the column. In a low-salt buffer (200 mM NaCl) the protein eluted with an apparent molecular mass of 41 kDa, which demonstrated that under these conditions the protein is monomeric. Although the protein was still partially active under these conditions (see below), it was not clear whether the low salt concentration would affect oligomerisation. Therefore, the molecular mass of the protein in high salt was estimated using dynamic light scattering (Table 1). This indicated a molecular mass of 43.3 kDa, showing that the protein is monomeric in solution. Notably, the molecular mass estimated by dynamic light scattering is lower than the mass estimated from SDS-PAGE, but it is not unusual for halophilic proteins to migrate with a reduced mobility on SDS-PAGE gels (see Lichi et al. 2004).

Enzymatic properties of *H. hispanica* amylase

AmyH was active on starch, amylose, amylopectin, and glycogen, whereas no detectable activity was found on β-cyclodextrin or pullulan. Further experiments to analyse biochemical characteristics of the enzyme (see below) were performed with starch as the substrate.

The pH optimum of the enzyme was determined with buffers with pH values ranging from 3 to 9 (Fig. 2a). The optimal activity was at a pH of 6.5. Activity dropped off quickly in more acidic conditions, as more than 65% of activity was lost at a pH of 5 or lower. At pH 6.5, the temperature optimum was 50°C (Fig. 2b), with more than 50% of activity remaining between 37 and 60°C. At higher temperatures the activity dropped quickly, with only 10% activity remaining at 70°C.

The most characteristic feature of halophilic proteins is the ability to function under very saline conditions. As shown in Fig. 2c, AmyH is indeed extremely halophilic as it functions optimally at 4–5 M NaCl. In contrast to most other halophilic proteins, the enzyme did not lose its activity at low salt concentrations; in the absence of NaCl the enzyme still had more than 30% of its activity.

Table 1 Dynamic light scattering

D_T (10^{-9} cm^2 s^{-1})	R_H (nm)	MW (kDa)	Polydispersity (%)
552 ± 80	2.97 ± 0.12	43.3 ± 4.0	12.3 ± 5.6

Dynamic light scattering measures the translational diffusion coefficient (D_T) of particles which, presuming the particles are spherical, is converted into the hydrodynamic radius (R_H) and an estimated molecular mass. Polydispersity is a measure of the size distribution

Activity in KCl was comparable to that of NaCl (data not shown), indicating that the type of monovalent cation is not critical.

Many α-amylases require Ca^{2+} ions for activity (van der Maarel et al. 2002), and therefore we measured the activity in the standard assay buffer with different Ca^{2+} concentrations (Fig. 3). Maximum activity was obtained with 2 mM $CaCl_2$ or more, whereas in the absence of $CaCl_2$ the enzyme retained only around 30% of its activity. Addition of 10 mM EDTA completely abol-

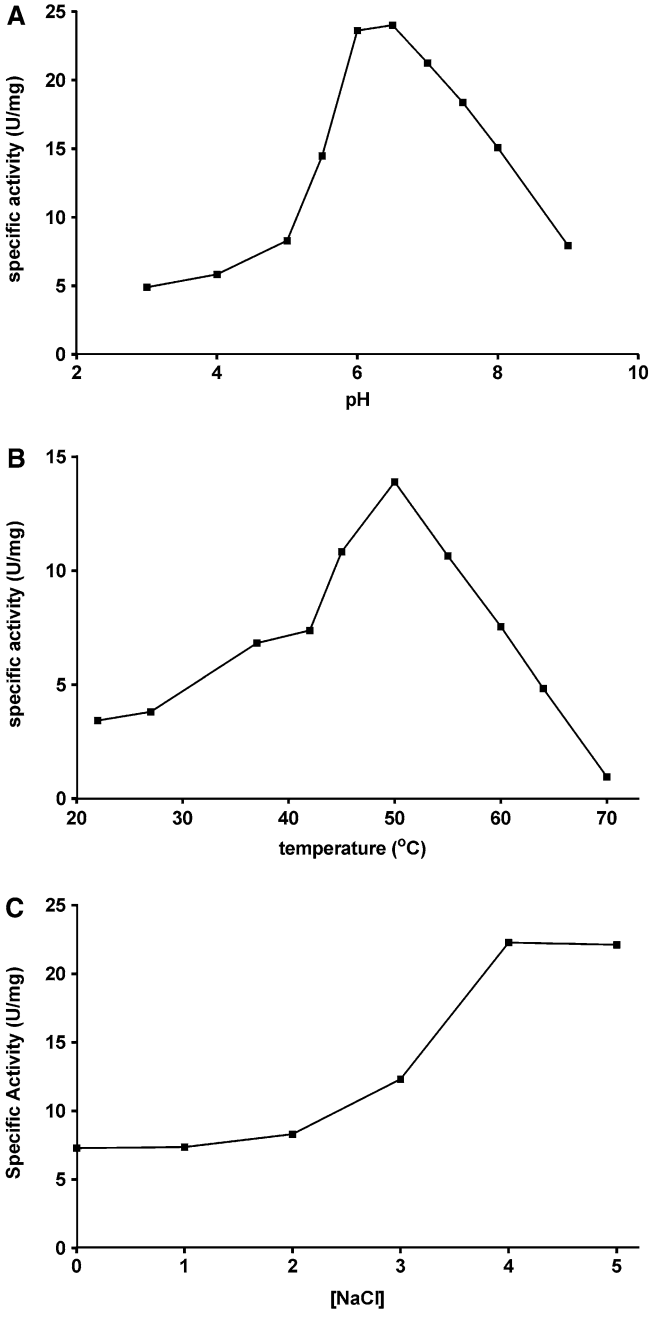

Fig. 2 Characterisation of the activity of AmyH. Enzyme assays were measured with the DNS assay using starch as the substrate. The effects of pH (**a**), temperature (**b**), and salt concentration (**c**) were tested

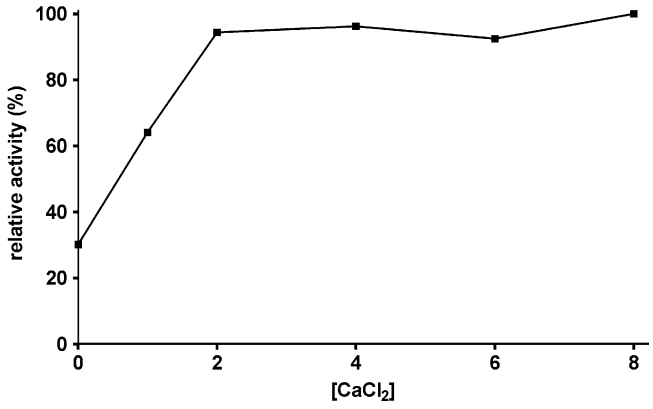

Fig. 3 Calcium dependence of AmyH. Activity of AmyH in the presence of different concentrations of $CaCl_2$ in buffer containing 4 M NaCl and 50 mM BisTris pH 6.5 was measured with the starch-iodine assay

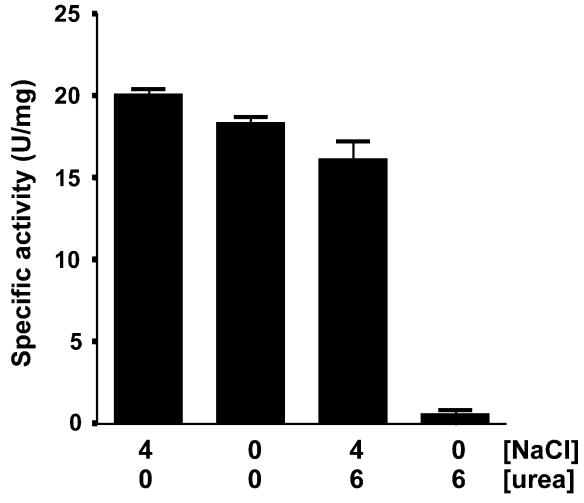

Fig. 4 Stability of AmyH. AmyH was dialysed for 24 h against buffer containing 50 mM BisTris pH 6.5, 5 mM $CaCl_2$, and different amounts of NaCl and/or urea as indicated (in molar). After dialysis, activity was measured with the DNS assay in standard conditions (i.e. 50 mM BisTris pH 6.5, 5 mM $CaCl_2$, and 4 M NaCl)

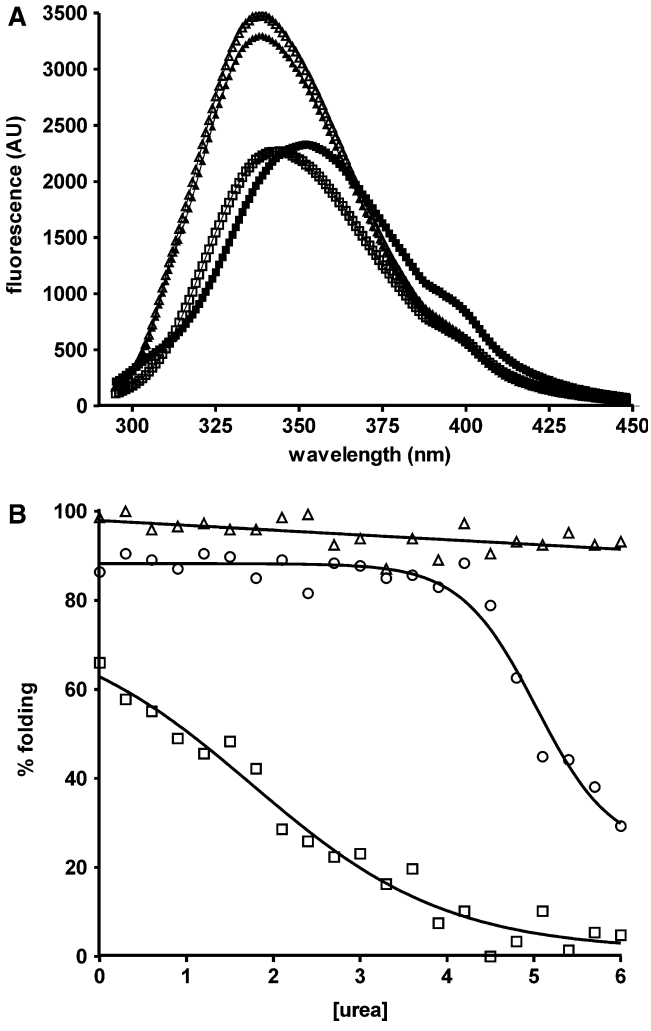

Fig. 5 Intrinsic fluorescence measurements. Fluorescence emission spectra of AmyH were recorded with an excitation wavelength of 280 nm. **a** *Open squares* AmyH in buffer B (50 mM BisTris pH 6.5, 5 mM $CaCl_2$); *open triangles* AmyH in buffer B plus 4 M NaCl; *closed triangles*: AmyH in buffer B plus 4 M NaCl and 6 M urea; *closed squares*: AmyH in buffer B plus 6 M urea. **b** Urea-induced unfolding of AmyH in the presence of buffer containing 4 M NaCl (*open triangles*), 2 M NaCl (*open circles*), or 0 M NaCl (*open squares*). Denaturation curves were obtained by plotting the wavelength of maximal intensity versus urea concentration

ished activity, indicating that the activity in the absence of calcium is due to residual Ca^{2+} ions that remain bound to AmyH. Subsequent addition of an excess of $CaCl_2$ restored activity to about 50%, whereas addition of $MgCl_2$ or $MnCl_2$ did not (data not shown). This demonstrated a specific requirement of AmyH for Ca^{2+} ions.

Stability of α-amylase

As shown above, AmyH was partially active at low salt concentrations. To test whether this partial loss of activity in low salt was recoverable, samples were dialysed for 24 h against buffers containing 0 or 4 M NaCl. Next, enzyme activity was determined under standard (high-salt) conditions (Fig. 4). As expected, after dialysis against a buffer containing 4 M NaCl all activity was retained. Interestingly however, nearly all activity was also recovered after dialysis in the absence of salt, showing that the partial loss of activity in low-salt buffers is completely reversible.

Halophilic enzymes are usually unstable in chaotropic salts such as urea or guanidine hydrochloride (Fukuchi et al. 2003). AmyH is barely active in 6 M urea (data not shown), but to test the effect of urea on the stability of AmyH, the enzyme was dialysed against buffers containing 6 M urea in the presence or absence of 4 M NaCl (Fig. 4). Surprisingly, after dialysis against a buffer containing 4 M NaCl and 6 M urea, activity was fully restored following transfer to normal assay

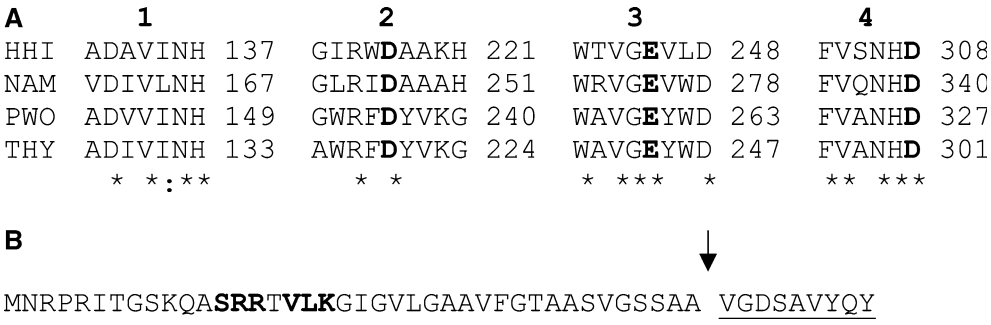

Fig. 6 Sequence of the *H. hispanica* amylase AmyH. **a** Partial alignments of the amino acid sequences of α-amylases from *H. hispanica* (*HHI*), *N. amylolyticus* (*NAM*), *P. woesei* (*PWO*), and *T. hydrothermalis* (*THY*). Identical (*) and conserved (:) residues are indicated, and the positions of the boxes in the proteins are indicated on the right of each box. The three active site residues are indicated in *bold*. **b** Signal peptide of AmyH. Residues corresponding to the twin-arginine consensus motif are indicated in *bold*, and the processing site is indicated by an *arrow*. The amino-terminal residues of the mature protein that were determined by N-terminal sequencing are *underlined*

conditions, indicating that either AmyH refolds very efficiently after unfolding in urea, or that AmyH remains folded in the presence of urea. After dialysis against a buffer containing 6 M urea, but lacking NaCl, AmyH activity was hardly detectable. Activity could also not be recovered if the first dialysis was followed by a second dialysis in order to slowly decrease the urea concentration and slowly increase the NaCl concentration. Thus, urea-induced unfolding in the absence of salt was irreversible under the conditions tested.

To address the effects of the concentration of salt on the structure of AmyH, intrinsic fluorescence spectra were determined to assess global structural changes in different NaCl concentrations. The fluorescence emission maximum in 4 M NaCl is around 338 nm (Fig. 5a). In a low-salt buffer (100 mM NaCl), the emission maximum wavelength red-shifted to 344 nm with an intensity of about 65% of that of protein in high salt, indicating that tryptophan residues which are particularly sensitive to changes in the solvent had become more exposed to water. Emission spectra were also determined in the presence of 6 M urea (Fig. 5a). Surprisingly, both fluorescence intensity and emission maximum wavelength in buffer containing 4 M NaCl and 6 M urea were virtually identical to those in high-salt buffer without urea, showing that the enzyme remains fully folded in the presence of urea. In a buffer containing low salt and 6 M urea the emission maximum shifted to 352 nm, which is typical for a fully unfolded protein with exposed tryptophan residues.

To investigate the effect of urea on the unfolding *H. hispanica* amylase in more detail, intrinsic fluorescence was measured at 0, 2, and 4 M NaCl in the presence of various concentrations of urea (Fig. 5b). Normally, urea-induced unfolding of a protein results in a characteristic sigmoid-shaped curve. With three different salt concentrations, three different parts of a sigmoidal curve were observed. At 4 M NaCl, the protein remained fully folded. At 2 M NaCl, the protein was also fully folded in the absence of urea, but started to unfold at urea concentrations of 4 M or higher. Without salt, the protein was less structured in the absence of urea and started to lose its structure with increasing urea concentrations.

Sequence of the amylase-encoding gene

A number of sequences of amylase-derived peptides were determined using in-gel tryptic-digestion and tandem mass spectrometry. Degenerate oligonucleotides based upon the sequences of two of the peptides (APG[L/I]T[F/M]AD[L/I]PR and HVD[K/Q]D[L/I]YVYER) were successfully used to amplify a 650-bp product from the genome of *H. hispanica*. The sequence of this product showed clear homology to α-amylase genes. Using partial inverse PCR (Pang and Knecht 1997), we determined the sequences of the flanking regions and obtained the full-length sequence of the amylase gene, which we termed *amyH* (amylase from *H. hispanica*). The sequence was deposited in the EMBL/Genbank/DDBJ databases with accession number AJ890146. The *amyH* gene specifies a protein of 433 residues that has its highest level of similarity to α-amylase from the haloalkaliphilic organism *N. amylolyticus* (63% of identical and conserved residues). Furthermore, the *H. hispanica* amylase is highly similar to α-amylases from thermophilic archaea, such as those from *Thermococcus hydrothermalis* and *Pyrococcus woesei*. All proteins belonging to the α-amylase family have four conserved regions (see MacGregor et al. 2001), three of which contain the active site residues: two aspartate residues in boxes II and IV respectively, and a glutamate in box III. Furthermore, boxes I and IV contain two additional conserved histidine residues. As shown in Fig. 6a, all of these residues are conserved in AmyH.

Virtually all secretory proteins are synthesised as pre-proteins with a transient amino-terminal extension (signal peptide) that is important for targeting the translocation channel and initiation of translocation. The signal peptide is usually removed during or shortly after translocation. To determine the site of processing,

we sequenced the N-terminus of the mature secreted protein. The sequence was determined as NH_2–VGDSAVYQY, which corresponds to amino acids 41–49 of the full-length protein encoded by the *amyH* gene. Thus, the signal peptide comprises the first 40 residues of the precursor protein (Fig. 6b), and the mature protein that is secreted into the culture medium contains 393 residues with a predicted molecular weight of 44,159 Da. This is close to the estimated molecular weight of 43.3 kDa that was determined by dynamic light scattering (see above). Interestingly, the signal peptide contains the sequence SRRTVLK. This is nearly identical to the consensus sequence (S/TRRxFLK) of bacterial proteins that are secreted via the Tat pathway (Robinson and Bolhuis 2004), and it is therefore likely that AmyH is a Tat-dependent substrate.

Halophilic enzymes are more acidic than their non-halophilic counterparts, which is reflected both in an increase of aspartic and glutamic acid residues and a decrease of lysine residues (the frequency of arginine residues is usually not significantly different in halophilic proteins). Furthermore, halophilic proteins have a relatively low hydrophobicity as revealed by a low level of large hydrophobic and aromatic residues (Phe, Trp, Tyr, Leu, Ile, and Met), and an increase in small hydrophobic residues (Ala and Val). To verify the halophilic character of AmyH, we compared its amino acid composition to that of other α-amylases of archaeal, bacterial, and eucaryal origin. As shown in Table 2, AmyH is more acidic in nature than α-amylases from the moderate halophile *Halomonas meridiana* (Coronado et al. 2000) and non-halophilic organisms. AmyH is not as acidic as the α-amylase from *N. amylolyticus*, which is an enzyme that functions not only in high salt, but also at a high pH (optimum pH 8.7; Kobayashi et al. 1992). Like *N. amylolyticus* α-amylase, AmyH appears also to be less hydrophobic than non-halophilic amylases, with fewer large hydrophobic and aromatic residues, and an increase in small hydrophobic residues. Similar differences can, however, be seen for the amylase of *Streptomyces griseus*, which is not a halophile but has, like haloarchaea, a very GC-rich genome (Lezhava et al. 1995). Thus, the difference in composition of hydrophobic residues may be a result of codon bias. Nevertheless, the high GC content, and thus the difference in levels of hydrophobic residues, is still likely to be an important part of the halophilic adaptation in haloarchaea.

Translocation AmyH

As mentioned above, the signal peptide of AmyH contains a twin-arginine motif, suggesting that the protein is exported via the Tat pathway. A characteristic feature of Tat substrates is that they fold before translocation (Robinson and Bolhuis 2004), and therefore we analysed the presence of the precursor of AmyH (pre-AmyH) by Western-blotting and activity measurements. As shown in Fig. 7a, cell-associated AmyH is predominately in a form with slower mobility than the mature protein in the medium, indicating that this is the precursor that still contains the signal peptide. A small portion of pre-AmyH is also visible in the medium, but this is probably the result of some cell lysis. The ratio of pre-AmyH in cells versus mature AmyH in the medium, as judged from band intensity on the Western-blot, is approximately 1:4. This is, interestingly, the same as the ratio of amylase activity in the same fractions (Fig. 7a), showing that pre-AmyH has the same specific activity as mature AmyH. This indicates that pre-AmyH in the cytoplasm is fully active and folded.

To test whether cell-associated pre-AmyH was export-competent, we performed a pulse-chase analysis. As shown in Fig. 7b, after 0 min of chase most of the protein (91%) was in the precursor form. After a further 30 min of chase, a significant proportion (46%) of this precursor was processed into mature AmyH, showing that the full-length precursor can be chased to a processed and thus exported form.

Discussion

In this study, we have described a novel α-amylase from *H. hispanica*. The enzyme is secreted by a transposon mutant that produces approximately fivefold more than

Table 2 Amino acid compositions of α-amylases

Organism	PI	DE (%)	*K* (%)	FWYLIM (%)	AV (%)
Haloarcula hispanica	4.20	16.6	2.0	22.4	16.0
Natronococcus amylolyticus	4.02	24.3	0.7	20.9	17.4
Pyrococcus woesei	4.82	12.9	4.4	31.9	13.8
Thermococcus hydrothermalis	4.89	12.0	4.6	30.9	14.1
Bacillus licheniformis	6.05	12.9	5.8	25.1	13.8
Halomonas meridiana	4.51	13.0	1.3	23.5	17.6
Streptomyces griseus	5.45	10.1	3.9	20.0	19.2
Salmonella typhymurium	5.66	11.4	3.8	26.1	12.6
Aspergillus oryzae	4.48	11.3	4.2	26.8	13.8

Theoretical isoelectric points and amino acid compositions (mol%) of various amylases (without the signal peptide). SwissProt accession numbers are: *N. amylolyticus*: Q60224 (archaeon); *P. woesei*: Q7LYT7 (archaeon); *T. hydrothermalis*: O93647 (archaeon); *B. licheniformis*: P06278 (bacterium); *H. meridiana* (bacterium): Q9L4I9; *S. griseus* (bacterium): S14063; S. *typhymurium*: Q8ZL87 (bacterium); *A. oryzae* P10529 (eucaryon)

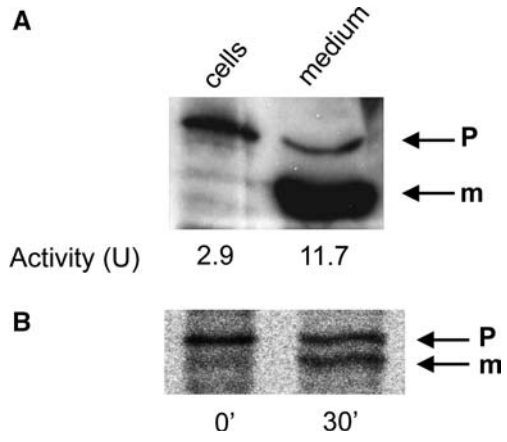

Fig. 7 Export of AmyH. **a** Cells of *H. hispanica* B3 were grown in rich medium, after which cells and culture medium were separated by centrifugation. Cell-associated and secreted amylase were visualised by SDS-PAGE and Western-blotting. The ratio of cell-associated pre-AmyH (*p*) versus mature AmyH (*m*) in the medium was estimated using ImageJ 1.33 software (http://rsb.info.nih.gov/ij/). **b** Processing of pre-AmyH was analysed by pulse-chase labelling and subsequent immunoprecipitation, SDS-PAGE, and visualisation using phosphorimaging. The ratios of precursor versus mature AmyH were determined using Aida software (Fuji)

the wild-type strain. The mutant strain has not, as yet, been fully characterised, but it is the synthesis and not the secretion of the protein that has increased. Therefore it seems likely that, in the mutant strain, a gene with a regulatory function has been disrupted.

Interestingly, AmyH is most likely secreted via the Twin-arginine translocase, since the signal peptide of AmyH has a clear Tat-consensus motif. The Tat pathway is specifically used for the export of a subset of proteins that are translocated in a folded conformation (Robinson and Bolhuis 2004). This may involve proteins that must fold before translocation, such as those that have enzymatically incorporated cofactors, or proteins that simply fold very rapidly in the cytoplasm. In most organisms the Tat pathway exports only a small percentage of secreted proteins; most secretory proteins make use of the Sec pathway, in which proteins are threaded through a channel in an unfolded conformation. Surprisingly, haloarchaeal proteins appear to be exported mostly through the Tat pathway, as most putative secretory proteins contain a double arginine and other diagnostic features in their signal peptide (Bolhuis 2002; Rose et al. 2002). The only other haloarchaeal amylase for which the sequence is known, that of the haloalkaliphile *N. amylolyticus* (Kobayashi et al. 1994), was not secreted when the RR-motif in the signal peptide was changed to KK (Rose et al. 2002). This showed that this protein is indeed a Tat-dependent substrate, since conserved changes that maintain the overall charge distribution would normally not affect a Sec substrate. The reason for the extensive use of the Tat pathway in haloarchaea is, most likely, an adaptation to the very saline conditions in which haloarchaea thrive. Haloarchaea accumulate 4–5 M KCl in the cytoplasm (Lanyi 1974), and newly synthesised proteins, which have exposed hydrophobic stretches that are buried once the protein is folded, may be prone to aggregation. Thus, newly synthesised proteins have to fold quickly, and many secretory proteins could therefore fold before reaching the cytoplasmic membrane. We showed that cell-associated pre-AmyH is active and export-competent, demonstrating that pre-AmyH is translocated post-translationally in, most likely, a folded and active conformation. The Sec pathway is incompatible with folded proteins, thus explaining the need for AmyH to be secreted by the Tat pathway. Notably, we have been unable to restore AmyH activity after urea-induced denaturation, indicating that its refolding is very inefficient. It is therefore conceivable that the folding of AmyH and other secretory proteins from haloarchaea depends on cytoplasmic chaperones because of the very saline conditions. If that is the case, folding of these proteins would have to take place before translocation, and translocation could, in turn, only occur via the Tat pathway.

Most halophilic proteins unfold at salt concentrations below 1–2 M (Madern et al. 2000). In contrast, AmyH remains active at low salt concentrations. Thus, unlike most other halophilic proteins, such as the amylases from *H. mediterranei* (Perez-Pomares et al. 2003) and *N. amylolyticus* (Kobayashi et al. 1992), the protein does not unfold at low levels of salt. Amylases from *H. meridiana* and *H. salinarum (halobium)* also do not unfold at low salt concentrations, but both of these enzymes are only moderately halophilic as they have an optimal activity in 10% salt or less (Coronado et al. 2000; Good and Hartman 1970). Both intrinsic fluorescence measurements and the increased sensitivity to urea showed that AmyH is more loosely folded at low salt concentrations. Compared to non-halophilic amylases, AmyH has an increased number of acidic residues (16.2%), although it is not as acidic as some other haloarchaeal proteins, such as the α-amylase from *N. amylolyticus* (24.3%) or the well-studied malate dehydrogenase from *Haloarcula marismortui* (20.4%). At low salt concentrations, an abundance of acidic residues destabilises proteins, presumably through electrostatic repulsion. The medium level of acidic residues in AmyH may provide part of the explanation for retaining partial activity and structure in the absence of salt. However, this does not explain why the protein functions optimally in nearly saturated salt or why it is remarkably resistant to urea in the presence of salt. One other haloarchaeal protein that has been shown to resist high concentrations of urea is nucleoside diphosphate kinase from *Natrialba magadii* (Polosina et al. 2002), a protein that has a salt optimum of 3.5 M and is, strikingly, also stable under low salt conditions. Thus, a subset of haloarchaeal proteins appears to retain structural integrity and activity under conditions that are generally adverse for halophilic proteins. Detailed structural studies will be required to understand the biochemical and biophysical properties of these proteins.

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