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Crystallization of an α-amylase, AmyA, from the thermophilic halophile *Halothermothrix orenii*

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This report is the first crystallographic study of an amylase from an organism that is both thermophilic and halophilic. α-Amylase from the thermophilic halophile *Halothermothrix orenii* (AmyA) is a 515-residue protein. It is stable and significantly active at 338 K in starch solution containing NaCl [up to 25%(*w*/*v*)]. Purified recombinant AmyA protein crystallizes in the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 55.126$, $b = 61.658$, $c = 147.625$ Å, using the hanging-drop vapour-diffusion method. The crystal diffracts X-rays to a resolution limit of 1.89 Å.

1. Introduction

Enzymes with activity and stability characteristics suitable for high-temperature industrial processes have great commercial potential. Amylase is an extremely important enzyme in the starch-hydrolysis process. Most starch-degrading enzymes are from thermophilic and hyperthermophilic prokaryotes and much less research has been devoted to enzymes from other extremophiles such as halophiles. Amylases from extremely halophilic and moderately halophilic bacteria (Good & Hartman, 1970; Kobayashi *et al.*, 1992; Coronado *et al.*, 2000) are either relatively stable and active at low salt levels (halotolerant) or are inactivated at low salt levels (extremely halophilic). However, none of the halotolerant or extremely halophilic amylases are active and stable at high temperature (333 K and above). AmyA was cloned from the anaerobic, thermophilic (growth at temperature above 333 K) and moderately halophilic (optimum NaCl requirement of 10%) bacterium *Halothermothrix orenii* (Mijts & Patel, 2002). The biochemical characteristics of purified recombinant AmyA protein showed that it was active at temperatures up to 352 K (optimally active at 338 K) and was halophilic, maintaining 90% activity at 25% NaCl and 45% activity without NaCl, while optimally active at 5% NaCl (Mijts & Patel, 2002). *H. orenii* is therefore a potential source of enzymes uniquely adapted to activity at high temperatures and high salt concentrations. AmyA is the first extracellular enzyme from an anaerobic and moderately halophilic bacterium to belong to the amylase family. By crystallizing the protein and solving its structure, we can understand the specific structural properties required for its activity under such extreme conditions.

2. Material and methods

2.1. Expression and purification of recombinant AmyA

The *AmyA* gene was cloned in pTrcHisB vector (Invitrogen) and the protein was over-expressed with an N-terminal hexahistidine tag in *Escherichia coli* strain TOP10 cells (Invitrogen) as detailed in an earlier report (Mijts & Patel, 2002). One colony of TOP10 cells containing the pTrcHis-*AmyA* construct was used to inoculate 10 ml of LB medium with 100 µg ml^{-1} of ampicillin (LB-Amp medium) and the cells were grown at 310 K for 16 h. This 10 ml culture was added to 1 l of fresh LB-Amp medium and the cells were grown to an OD_{600} of 0.6. The protein was induced for 4 h by adding IPTG to a final concentration of 1 m*M*. The cells were harvested by centrifugation at 5000*g* and resuspended in 20 ml phosphate buffer (20 m*M* sodium phosphate pH 7.8, 500 m*M* NaCl). The cells were lysed at 277 K using a French press at 6.9 MPa. DNaseI was added to the lysate at a concentration of 5 µg ml^{-1} and the sample was incubated on ice for 30 min. Any insoluble material was removed by centrifugation at 10 000*g* for

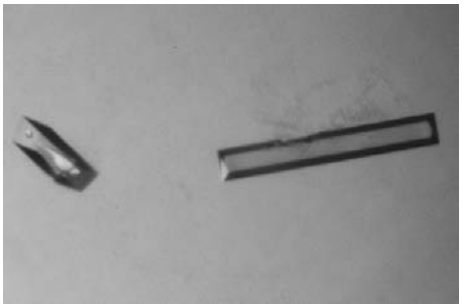

Figure 1
Crystals of AmyA from *H. orenii* with maximum dimensions of 0.1 × 0.1 × 0.6 mm.

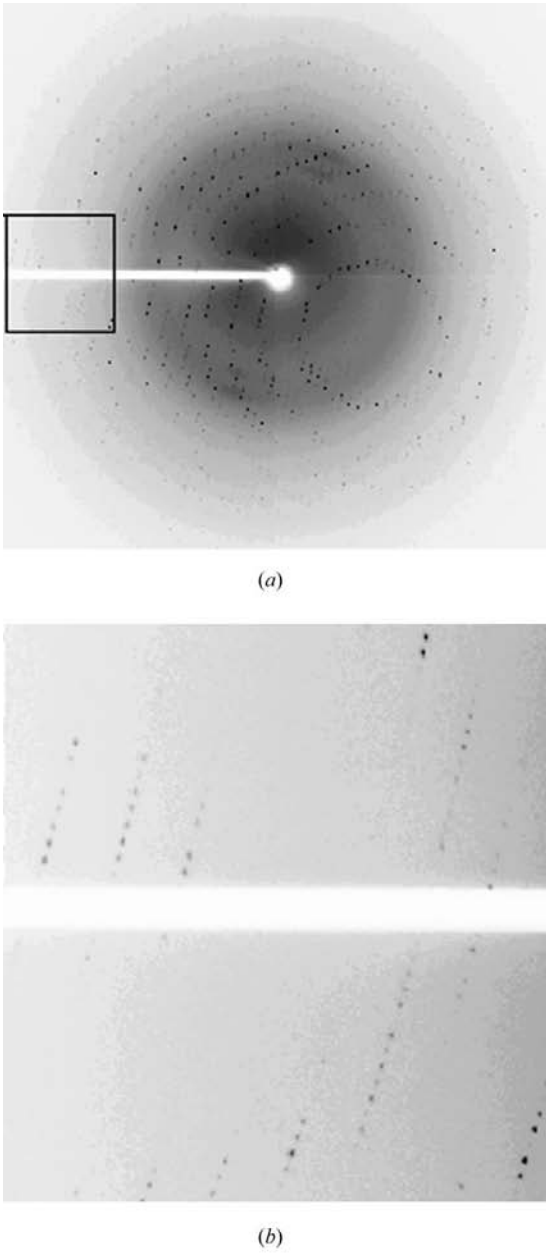

Figure 2
(*a*) X-ray diffraction pattern from a crystal of AmyA; (*b*) close-up view of high-resolution region with limit at 1.89 Å.

15 min. The supernatant was incubated at 341 K for 30 min to denature *E. coli* proteins and transferred to ice for 15 min to maximize protein precipitation. AmyA remains active at temperatures of up to 343 K and therefore most of the *E. coli* host proteins were precipitated by the heat-precipitation technique and removed by subsequent centrifugation at 10 000*g* for 20 min. The clear supernatant was loaded onto a column containing 2 ml Ni–NTA agarose affinity resin (Qiagen). After washing with phosphate buffer containing 10 m*M* imidazole, the recombinant AmyA protein was eluted from the column with 200 m*M* imidazole in the phosphate buffer. It was further purified by gel-filtration chromtography using a Hiload 16/60 Superdex75 column (Amersham Pharmacia Biotech) with buffer consisting of 10 m*M* HEPES pH 7.5, 200 m*M* NaCl. The pure protein fractions were pooled together and concentrated using Centriprep and Centricon YM-10 devices (Millipore).

2.2. Crystallization and diffraction data collection

AmyA protein sample (5.5 mg ml^{-1}, Bradford method) was set up for crystallization using a Crystal Screen Cryo kit (Hampton Research) with the vapour-diffusion hanging-drop method. 1 µl of protein sample was mixed with 1 µl of reservoir solution and equilibrated against 700 µl of reservoir solution at 296 K. Single crystals formed under the condition 70 m*M* sodium acetate pH 4.6, 5.6% PEG 4000 and 30% glycerol. The condition was further optimized by mixing 4 µl of protein sample with 1 µl of reservoir solution. Crystals were obtained with maximum dimensions of 0.1 × 0.1 × 0.6 mm after 1 d (Fig. 1). The mother liquor with glycerol increased by 5% was used as cryoprotectant. The crystals were flash-cooled in liquid nitrogen. X-ray diffraction data were collected at SPring8, Japan (beamline BL40B2, ADSC Quantum4 CCD detector, 100 K). All data were indexed, integrated and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Result and discussion

The expression level of recombinant AmyA protein was quite low. However, the protein could be harvested efficiently after it was bound to an Ni–NTA agarose affinity column and most of the contaminants could be removed on the column by washing with 10 m*M* imidazole in the washing buffer.

Table 1
Diffraction data statistics of AmyA crystal.

Values in parentheses refer to the highest resolution shell (1.96–1.89 Å).

Synchrotron-radiation source	SPring8, Japan (BL40B2)
Wavelength (Å)	1.0
No. of imaging plates	180
Unit-cell parameters (Å)	a = 55.126, b = 61.658, c = 147.625
Space group	$P2_12_12_1$
Mosaicity of crystal (°)	0.613
Resolution range (Å)	20–1.89
Total No. of reflections	85503
No. of unique reflections	38334
Redundancy	2.2
Completeness (%)	95.1 (89.4)
R_{sym}† (%)	2.7 (6.5)

† $R_{sym} = \sum_j \sum_i |\langle I_i \rangle - I_i| / \sum_i I_i$.

Heat precipitation was the key step in the purification procedure, as it removed most of the unwanted host proteins and reduced the non-specific binding on the Ni–NTA affinity column. Finally, high purity of the protein was achieved through the gel-filtration chromatographic step.

The crystals diffract to 1.89 Å (Fig. 2). Analysis of the data shows that the crystal belongs to orthorhombic space group $P2_12_12_1$. The molecular weight of AmyA is 52 kDa (according to its sequence). The Matthews coefficient (Matthews, 1968) is calculated to be 2.28 Å^3 Da^{-1}, with one monomer molecule in the asymmetric unit and a solvent content of 45.67%. Data-collection and crystallographic statistics are summarized in Table 1.

Attempts are being undertaken to solve the structure by the molecular-replacement method. By comparing it to known amylase-family protein structures, we hope to elucidate the specific structural details that afford AmyA its thermophilic and halophilic biochemical characteristics.

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