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Crystallization of a novel α-amylase, AmyB, from the thermophilic halophile *Halothermothrix orenii*

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This is a report on the structure determination of AmyB, the second α-amylase from *Halothermothrix orenii*, by X-ray crystallography. This bacterium was isolated from saltpans where conditions consisted of both high temperatures and high NaCl content. AmyB is a 599-residue protein which is stable and significantly active at 358 K in starch solution containing up to 10%(*w*/*v*) NaCl. The purified recombinant AmyB protein crystallizes in the monoclinic space group *C*2, with unit-cell parameters $a = 225.85$, $b = 77.16$, $c = 50.13$ Å, $\beta = 99.32^\circ$, using the hanging-drop vapour-diffusion method. The crystal diffracts X-rays to a resolution limit of 1.97 Å.

1. Introduction

Thermostable enzymes have enormous commercial potential in high-temperature industrial processes. Amylases possessing such characteristics are extremely important enzymes in the starch-hydrolysis process and have been well characterized in terms of their biochemistry and structure. To date, most of the starch-degrading enzymes studied have been from thermophilic and hyperthermophilic prokaryotes, with much less research being devoted to enzymes from thermophilic halophiles. The amylases from moderate and extreme halophiles that have been studied thus far are either active at low salt levels (halotolerant) or are inactivated at low salt levels (extremely halophilic) (Good & Hartman, 1970; Kobayashi *et al.*, 1992; Coronado *et al.*, 2000). None are active and stable at high temperatures (358 K and above).

Biochemical characterizations of the two α-amylases, AmyA (Li *et al.*, 2002) and AmyB (this study), from the anaerobic, thermophilic (growth at temperature above 358 K) and moderately halophilic (optimum NaCl requirement of 10%) bacterium *Halothermothrix orenii* have been reported (Mijts & Patel, 2002). The signal peptides found at the N-terminus of both enzymes suggest that they are secreted enzymes which are stable under these extreme conditions without protection for the bacteria. Sequence alignment with other members of the α-amylase family confirms the presence of the four conserved regions of the α-amylase family in both enzymes. However, AmyA and AmyB only show 23% identity and phylogenetic analysis (Fig. 1) shows that the enzymes are distinct from each other even though they are isolated from the same bacterium. This has raised important questions about the origin and evolution of the genes and their adaptation to the dual extreme environmental conditions. By solving the structures of both these amylases (the crystal structure of AmyA is being refined) and comparing them with other amylases, we hope to identify the structural features that confer functional properties and stability under such extreme conditions upon them.

2. Materials and methods

2.1. Expression and purification of recombinant AmyB

The *AmyB* gene was cloned into the pTrcHisB vector (Invitrogen) and the protein was expressed with an N-terminal hexahistidine tag in *Escherichia coli* strain TOP10 cells (Invitrogen; Mijts & Patel, 2002). A single colony of TOP10 cells containing the pTrcHis-*AmyB* construct was inoculated into 30 ml of LB-Amp medium (100 mg ml^{-1} ampicillin) and the cells were grown at 310 K for 16 h. The 30 ml culture was subsequently used to inoculate 3 l of LB-Amp medium and the cells were grown to an OD_{600} of 0.6. The enzyme was induced for 4 h by adding IPTG to a final concentration of 1 m*M*. The cells were pelleted by centrifugation at 7000*g*, resuspended in 60 ml buffer (50 m*M* sodium phosphate, 500 m*M* NaCl, 10 m*M* imidazole pH 8.0) and lysed by sonication (Vibra-cell 100). Cell debris was removed from the lysate as a pellet by centrifugation at 10 000*g* for 30 min and the supernatant was used for enzyme purification.

The heat-treatment method that we had used previously for purifying AmyA (Mijts & Patel, 2002) was not successful in the case of AmyB. The clarified supernatant was purified by using Ni–NTA agarose affinity column chromatography. For this, 20 ml of the supernatant was added to 4 ml of Ni–NTA agarose resin (Qiagen), shaken gently for 60 min at

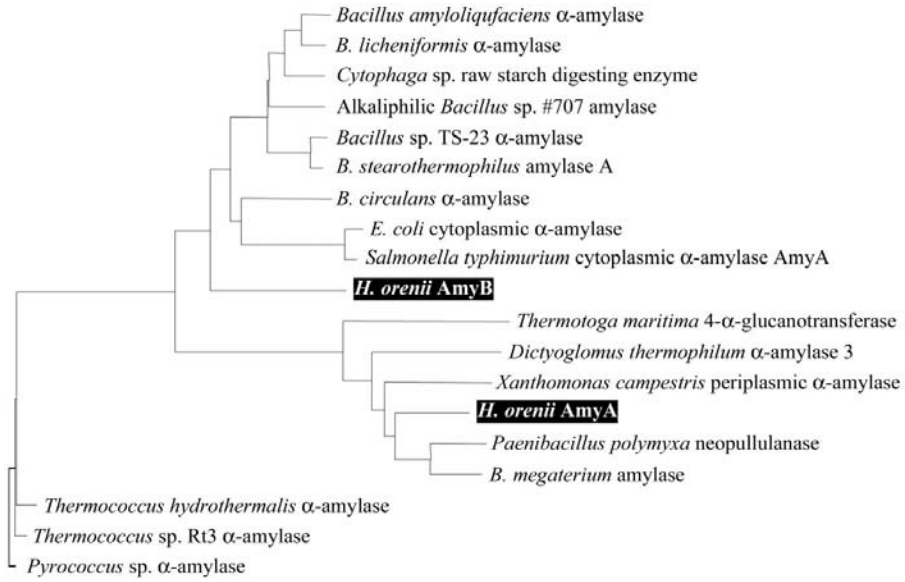

Figure 1
Phylogenetic link between AmyA and AmyB.

Figure 2
Crystals of AmyB from *H. orenii* with maximum dimensions of 0.25 × 0.18 × 0.04 mm.

277 K, loaded onto a column and washed twice with 10 ml wash buffer (50 m*M* sodium phosphate, 500 m*M* NaCl, 20 m*M* imidazole pH 8.0). AmyB was then eluted using elution buffer (50 m*M* sodium phosphate, 500 m*M* NaCl, 250 m*M* imidazole pH 8.0). Protein purity was confirmed by SDS–PAGE analysis before pooling and concentration using Centriprep and Centricon YM-10 devices (Millipore).

2.2. Crystallization and diffraction data collection

The protein was crystallized using Crystal Screen II (Hampton Research) by the sitting-drop vapour-diffusion method, in which 2 µl of AmyB (50 mg ml^{-1}) was mixed with 2 µl of the reservoir solution and equilibrated against 1 ml of the reservoir at 296 K. A crystal with dimensions of 0.25 × 0.18 × 0.04 mm (Fig. 2) was transferred into a mixture of paratone-N and mineral oil in a 1:1 ratio and was flash-cooled in liquid nitrogen. X-ray diffraction data were collected at the Advanced Photon Source (Argonne, USA) beamline 19BM with an SBC1 CCD detector. Data were collected at 100 K and were indexed, integrated and scaled using *HKL*2000 (Otwinowski & Minor, 1997).

3. Results and discussion

Purification of the AmyB protein was relatively easy because of its high expression level and its halophilic characteristics. This allowed the use of a high salt concentration for the affinity-purification step which helped to reduce the non-specific binding of other proteins. Additional purification of the protein was not necessary.

From the crystal screen, three crystals were observed after four months in a condition containing 30% PEG MME 5000, 0.1 *M* MES pH 6.5, 0.2 *M* ammonium sulfate. The crystal belongs to space group *C*2 and diffracted X-rays to 1.97 Å. The unit-cell parameters are a = 225.85, b = 77.16, c = 50.13 Å, β = 99.32°. The Matthews coefficient (Matthews, 1968) is calculated to be 2.98 Å^3 Da^{-1}, leading to one monomer molecule (MW = 72.3 kDa) in the asymmetric unit and a solvent content of 58.4%. Even though the crystal diffracted to 1.6 Å, the data statistics for high-resolution shells beyond 1.97 Å were not acceptable. A summary of crystallographic and data-collection statistics is reported in Table 1.

Table 1
Diffraction data statistics of an AmyB crystal.

Values in parentheses refer to the highest resolution shell (2.04–1.97 Å).

Synchrotron-radiation source	APS, USA (beamline 19BM)
Wavelength (Å)	0.978
No. of imaging plates	480
Unit-cell parameters (Å, °)	a = 225.85, b = 77.16, c = 50.13, β = 99.32
Space group	*C*2
Mosaicity of crystal (°)	0.86
Resolution range (Å)	50–1.97
Total No. of reflections	272674
No. of unique reflections	60572
Redundancy	4.5
Completeness (%)	97.7 (96.5)
$I/\sigma(I)$	10.6 (1.6)
R_{sym}† (%)	0.101 (0.320)

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

We are currently in the process of solving the structure using the molecular-replacement method (using PDB entry 1vjs as the search model). We hope to compare the structures of AmyB, AmyA and other members of the amylase family that are known to be either thermophilic or halophilic in order to understand the basis of their stability, structural characteristics and functional properties under thermo-halophilic conditions.

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References

Coronado, M., Vargas, C., Hofemeister, J., Ventosaa, A. & Nieto, J. J. (2000). *FEMS Microbiol. Lett.* **183**, 67–71.
Good, W. A. & Hartman, P. A. (1970). *J. Bacteriol.* **104**, 601–603.
Kobayashi, T., Kanai, H., Hayashi, T., Akiba, T., Akaboshi, R. & Horikoshi, K. (1992). *J. Bacteriol.* **174**, 3439–3444.
Li, N., Patel, B. K., Mijts, B. N. & Swaminathan, K. (2002). *Acta Cryst.* D**58**, 2125–2126.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mijts, B. N. & Patel, B. K. C. (2002). *Microbiology*, **148**, 2343–2349.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.