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Purification, crystallization and preliminary crystallographic analysis of the marine α -amylase AmyP

AmyP is a raw-starch-degrading α -amylase newly identified from a marine metagenome library. It shares low sequence similarity with characterized glycoside hydrolases and was classified into a new subfamily of GH13. In particular, it showed preferential degradation to raw rice starch. Full-length AmyP was cloned and overexpressed in *Escherichia coli*, then purified and crystallized in the presence of its substrate analogue β -cyclodextrin. X-ray diffraction data were collected to a resolution of 2.1 Å. The crystal belonged to space group $P2_12_12$, with unit-cell parameters $a = 129.824$, $b = 215.534$, $c = 79.699$ Å, $\alpha = \beta = \gamma = 90^\circ$, and was estimated to contain two molecules in one asymmetric unit.

1. Introduction

Starch is extensively used in the food industry as well as other industries such as pharmaceuticals, paper, adhesives, packaging and biofuels (Jobling, 2004; Qin *et al.*, 2011). Most of these applications require disruption of starch granules through high temperature or the presence of an acid, alkali or enzyme (Robertson *et al.*, 2006; Tawil *et al.*, 2010). Enzyme hydrolysis of raw starch is energy efficient and environmentally friendly. Therefore, raw-starch-degrading enzymes have attracted more and more attention. α -Amylases (EC 3.2.1.1) are widely occurring enzymes, which hydrolyse α -1,4-glycosidic linkages in starch and related carbohydrates with a random *endo*-mechanism. Thus, they are among the most important raw-starch-degrading enzymes. However, only a limited number of α -amylases are able to degrade raw starch (Robertson *et al.*, 2006; Sun *et al.*, 2010) and most of them are produced by terrestrial organisms. Thus, it is attractive to mine new α -amylases with unique properties from non-terrestrial environments such as the oceans.

Marine environments vary in many aspects such as pressure, temperature, salinity, nutrient availability and so on. Marine organisms, especially microbes, thus provide a huge potential source of novel enzymes with unique properties. However, more than 99% of the microbes in the oceans cannot be cultured, meaning that the full potential of this vast enzyme pool is still under-explored. Hence, metagenomics, a culture-independent method, has become a powerful tool to mine new microbial enzymes from marine environments (Kennedy *et al.*, 2008; Langridge, 2009). Using a metagenomic strategy, a lot of enzymes including α -amylases have been obtained from marine sources, some of which exhibit properties distinct from terrestrial enzymes. So far, only three α -amylases from marine sources (Vidilaseris *et al.*, 2009; Puspasari *et al.*, 2011; Liu *et al.*, 2012) have been reported to be able to digest raw starch. AmyP, the most recently found one of the three α -amylases, was attributed to a new subfamily of the glycoside hydrolysis enzyme family 13 (Liu *et al.*, 2012) and showed a remarkable raw-starch-degrading activity especially towards raw rice starch (Lei *et al.*, 2012). It exhibited the highest sequence identity (about 70%) to the putative glycosidases from deep-sea bacteria *e.g.* *Photobacterium profundum* and *Vibrio splendidus*. However, most of these glycosidases were revealed by whole-genome sequencing and none of them has been biochemically characterized. Of the structurally characterized α -amylases, the most similar to AmyP is *Bacillus stearothermophilus* neopullulanase (PDB code 1j0h_A, Hondoh *et al.*, 2003), which shares only 28% sequence

identity with AmyP (covering 74% of full length). Notably, compared with *B. stearothermophilus* neopullulanase, AmyP is truncated at the N-terminus by about 100 residues and elongated at the C-terminus by about 150 residues which are identified to be a novel starch-binding domain (unpublished data). To test whether the exclusive features of AmyP that are not present in other known α -amylases are structurally relevant, we recombinantly overexpressed and purified AmyP, and crystallized it in the presence of its substrate analogue β -cyclodextrin. Preliminary crystallographic analysis of an AmyP crystal was carried out. Structural and biochemical analysis may lead to an understanding of how AmyP functions.

2. Materials and methods

2.1. Cloning

The gene of AmyP (GenBank ID HM572234) was amplified by PCR from a marine metagenome library of the South China Sea as described previously (Liu *et al.*, 2012) using the primers 5'-CCCGGATCCATGTGCGATAGCGCTTTGA-3' with a *Bam*HI site and 5'-CCCGTCGACTTACGGACACTTAGAGACC-3' with a *Sal*I site. The PCR was carried out with Pyrobest DNA polymerase (Takara). The product was digested with *Bam*HI and *Sal*I and ligated into a pET32a vector (Novagen) with a VDKLAAALE HHHHHH His tag at the C-terminal to facilitate purification. The insertion of AmyP into the vector was confirmed by DNA-sequence analysis (Sangon).

2.2. Protein expression and purification

Escherichia coli BL21(DE3) cells harboring pET32a-AmyP (Liu *et al.*, 2012) were used for enzyme production. The cells were grown with agitation at 310 K to an OD₆₀₀ of 0.7, then induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 289 K. After 20 h of induction, the cells were harvested by centrifugation at 5000 rev min⁻¹ for 8 min at 277 K. The cell pellets were resuspended in lysis buffer (20 mM Tris-HCl, 500 mM NaCl pH 7.8) and subsequently lysed by ultrasonication on ice. The lysate was centrifuged at 15 000 rev min⁻¹ for 20 min at 277 K. The supernatant was loaded onto a Ni²⁺-NTA nickel-chelating column (GE Healthcare, USA) pre-equilibrated with lysis buffer. The column was washed with about 20 column volumes of washing buffer (20 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole pH 7.8) to remove contaminants. The target

Table 1

Crystal parameters and data-collection statistics for the crystal of AmyP.

Values in parentheses are for the highest-resolution shell.

No. of crystals	1
Beamline	BL17U, SSRF
Wavelength (Å)	0.97919
Detector	ADSC Quantum 315r
Crystal-to-detector distance (mm)	250
Rotation range per image (°)	1.0
Total rotation range (°)	207
Exposure time per image (s)	1
Resolution range (Å)	50.00–2.10 (2.14–2.10)
Space group	<i>P</i> 2 ₁ 2 ₁ 2
Unit-cell parameters (Å, °)	<i>a</i> = 129.824, <i>b</i> = 215.534, <i>c</i> = 79.699, $\alpha = \beta = \gamma = 90$
Mosaicity (°)	0.99
Total no. of measured intensities	431484
Unique reflections	122204
Multiplicity	3.5 (3.4)
Average <i>I</i> / σ (<i>I</i>)	9.5 (3.5)
Completeness (%)	93.0 (94.6)
<i>R</i> _{merge} [†] (%)	12.6 (42.2)
Overall <i>B</i> factor from Wilson plot (Å ²)	25.0

[†] $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the *i*th observation of reflection *hkl* and $\langle I(hkl) \rangle$ is the weighted average intensity of all observations *i* of reflection *hkl*.

protein was eluted with about ten column volumes of elution buffer (20 mM Tris-HCl, 500 mM NaCl, 300 mM imidazole pH 7.8). Protein was concentrated and applied onto a Superdex 200 16/600GL size-exclusion column (GE Healthcare) equilibrated with the buffer of 20 mM Tris-HCl, 150 mM NaCl pH 7.5 for further purification. All purification steps were performed at 277 K and the resulting protein was concentrated to 10 mg ml⁻¹ by centrifugal ultrafiltration (Millipore; 5 kDa cutoff) for crystallization. The concentration of the protein was determined using the theoretical absorbance coefficient of AmyP at 280 nm (107 065 M⁻¹ cm⁻¹ or 1.522 mg ml⁻¹ cm⁻¹) calculated by *ProtParam* (<http://www.expasy.org/tools/#primary>).

2.3. Crystallization

Initial screening was performed by the sitting-drop vapour-diffusion method using Crystal Screen and Crystal Screen 2 kits from Hampton Research at 289 K. 1 μ l protein solution (10 mg ml⁻¹ in the buffer consisting of 20 mM Tris-HCl and 150 mM NaCl pH 7.5) with 4 mM β -cyclodextrin was mixed with 1 μ l reservoir solution. After several days, microcrystals appeared in condition No. 22 of Crystal

Figure 1

SDS-PAGE (a) and size-exclusion chromatography (b) for purified recombinant AmyP. The apparent molecular weight of AmyP calculated from the elution volume in size-exclusion chromatography is about 65 kDa, close to its theoretical value 70 kDa.

Figure 2
A crystal of AmyP.

Screen [0.2 M NaAc (sodium acetate), 0.1 M Tris-HCl pH 8.5, 30%(w/v) PEG 4000]. The crystallization condition was optimized using the hanging-drop vapour-diffusion method at 289 K. 1 μ l protein solution was mixed with 1 μ l reservoir solution and the mixture was equilibrated against 500 μ l reservoir solution. After several rounds of refinement, diffraction-quality single crystals were obtained.

2.4. Diffraction data collection and processing

The crystals of AmyP were harvested and soaked in a cryoprotectant solution consisting of 0.2 M NaAc, 0.1 M Tris-HCl pH 8.8, 27%(w/v) PEG 4000 and 20%(v/v) glycerol for several seconds. The crystal was flash-cooled in liquid nitrogen and used for X-ray diffraction data collection using synchrotron radiation at 100 K on beamline BL17U at the SSRF (Shanghai). A complete diffraction data set consisting of 207 diffraction images was collected from one crystal with an oscillation angle of 1° per image. Diffraction data were indexed, integrated and scaled using the program *MOSFLM* (Leslie, 1992). Data-collection and processing statistics are listed in Table 1. A self-rotation function was calculated to check the local non-crystallographic symmetry (NCS) of the molecules in the asymmetric unit by the program *MOLREP* (Vagin & Teplyakov, 1997).

3. Results and discussion

The purified AmyP was shown by SDS-PAGE to have an apparent molecular weight between 60 and 112 kDa, consistent with its theoretical molecular weight of about 70 kDa, and a purity higher than 95% (Fig. 1a). The size-exclusion experiment indicated that it was predominantly a monomer (Fig. 1b). The purified AmyP was concentrated to 10 mg ml⁻¹ for initial crystal screening experiments. Several days after the crystallization experiments had been set up, microcrystals appeared in the condition consisting of 0.2 M NaAc, 0.1 M Tris-HCl pH 8.5, 30%(w/v) PEG 4000. The crystals obtained from this condition were needle-like and diffracted X-rays poorly. The initial conditions were optimized in order to yield crystals that were suitable for X-ray diffraction. After several rounds of refinement, large crystals appeared at 289 K in a condition consisting of 0.2 M NaAc, 0.1 M Tris-HCl pH 8.8, 27%(w/v) PEG 4000. Typical crystal dimensions were 0.10 × 0.10 × 0.25 mm (Fig. 2) and the

Figure 3
X-ray diffraction map obtained from the AmyP crystal.

crystal diffracted to about 2.1 Å resolution (Fig. 3). The crystal belonged to space group $P2_12_12$. The calculated Matthews coefficient (V_M) was likely to be 3.87, 2.58 or 1.94 Å³ Da⁻¹, with a corresponding solvent content of 68.25, 52.38 or 36.50%, suggesting that there may be two, three or four molecules in one asymmetric unit, respectively (Matthews, 1968). Self-rotation function calculation revealed only one NCS peak indicating that there are two molecules in one asymmetric unit. As the size-exclusion chromatography indicated that AmyP was predominantly a monomer in solution, the dimer in one crystal asymmetric unit might be the consequence of crystal packing.

Since in the PDB there is no structure which shares more than 30% sequence identity with AmyP, it was difficult to determine the structure of AmyP by the molecular-replacement method directly. Experimental phasing using the selenomethionine single-wavelength anomalous dispersion (SAD) method is being carried out.

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