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Crystallization and preliminary X-ray study of alkaline β-mannanase from the alkaliphilic *Bacillus* sp. N16-5

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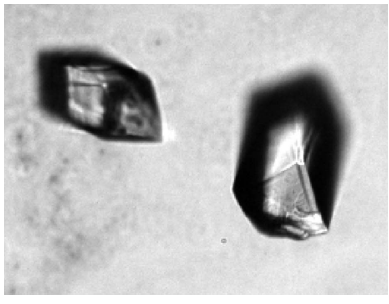
The catalytic domain of an alkaline β-mannanase from the alkaliphilic *Bacillus* sp. N16-5 has been expressed and purified. The recombinant enzyme was crystallized using the hanging-drop vapour-diffusion method at 298 K. X-ray diffraction data were collected to 1.6 Å resolution. The crystal belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 59.03$, $b = 63.31$, $c = 83.34$ Å. Initial phasing was carried out by molecular replacement using the three-dimensional structure of a mannanase from the alkaliphilic *Bacillus* sp. JAMB602 as a search model.

1. Introduction

β-Mannanases (EC 3.2.1.78) are widely distributed in bacteria, fungi and plants (Braithwaite *et al.*, 1995; Duffaud *et al.*, 1997; Reese & Shibata, 1965; Shimahara *et al.*, 1975; Ethier *et al.*, 1998). They randomly hydrolyze β-1,4-mannosidic linkages in mannan and heteromannan (McCleary, 1983; Singh *et al.*, 2003) and have great potential uses in the feed/food, pulp/paper and detergent industries (Dekker & Richards, 1976; Kansoh & Nagieb, 2004). Alkaline β-mannanases offer the obvious advantage of functioning under alkaline conditions during processes in the manufacture of kraft pulp and in the detergent industry. Research on the structure and function of alkaline mannanases will not only deepen our understanding of the adaptation mechanism of proteins to alkaline conditions, but will also help to optimize the pH-dependent characteristics of enzymes for a broad range of applications. Several alkaline β-mannanases from the alkaliphilic *Bacillus* spp. AM001, JAMB602, JAMB750, I633, N16-5 and *B. agaradhaerens* (Akino *et al.*, 1987; Akita *et al.*, 2004; Ma *et al.*, 2004; Hatada *et al.*, 2005; Bettiol & Showell, 2002) have been characterized, although the tertiary structure has only been determined for the β-mannanase from the alkaliphilic *Bacillus* sp. JAMB602 (Akita *et al.*, 2004).

Of the characterized alkaline β-mannanases, that from *Bacillus* sp. N16-5 was of great interest because it not only shows a pH optimum as high as 9.5 but also has the highest specific activity of the mannanases reported to date. These properties, combined with its characteristics of stability under alkaline conditions and insensitivity to some surfactants, would qualify the mannanase as a candidate in the manufacture of kraft pulp and in the detergent industry. Study of the three-dimensional structure of this mannanase will be very helpful in directing improvements in the alkaline adaptation of other mannanases without loss of activity.

The mannanase gene (*man5A*) from *Bacillus* sp. N16-5 (GenBank accession No. AY534912) has been reported to consist of 1479 bp and to encode a 493-amino-acid protein, Man5A, which includes a catalytic domain and a C-terminal region of unknown function (Ma *et al.*, 2004). As part of our ongoing studies on the relationship between structure and function, we have expressed and purified Man5A330 (Ser33–Thr352), which contains only the catalytic domain of Man5A. The biochemical characterization of this partial Man5A showed the same pH-activity profile and specific activity as the original Man5A. That means that the C-terminal region of the protein, previously thought to possibly be a critical region for enzyme alkaliphilicity (Akita *et al.*, 2004; Ma *et al.*, 2004), plays a minor or indirect role in



the pH-dependent activity. In order to elucidate the molecular basis of its alkaline adaptation and high catalytic efficiency, it is of great importance to crystallize Man5A330 and to solve its structure. Here, we describe the expression, purification, crystallization and preliminary X-ray data analysis of Man5A330. The tertiary structure of this domain is currently being determined.

2. Materials and methods

2.1. Cloning, expression and purification

Using the *Bacillus* sp. N16-5 genomic DNA as a template, the catalytic domain of Man5A was amplified using the primers 5′-CTAGCTAGCAGTTCAGGCTTTTATGTTGATGG-3′ and 5′-CGCGGATCCTGTGCTTCCTTCAAAGTCATACAAGG-3′. Engineered unique restriction sites for *Nhe*I and *Bam*HI were introduced. The amplified products were digested with *Nhe*I and *Bam*HI and the digests were then inserted into the corresponding cloning site of plasmid pET28a (Novagen Co., USA). The recombinant plasmid was introduced into *Escherichia coli* BL21 (DE3) (Novagen Co., USA). The transformants of *E. coli* BL21 (DE3) were grown in 400 ml LB containing 40 µg ml^{-1} kanamycin at 310 K until the OD_{600} reached 0.6. Expression of the recombinant protein was induced by adding 1.0 m*M* IPTG and growth of the culture continued for 5 h at 310 K. All subsequent steps were carried out at 277 K. The cells were harvested by centrifugation (6000 rev min^{-1}, 10 min) and the cell pellets were resuspended in 16 ml binding buffer (20 m*M* Tris–HCl, 0.5 *M* NaCl, 5 m*M* imidazole pH 7.9) and disrupted by sonication. Cell debris was removed by centrifugation (18 000 rev min^{-1}, 10 min). The expressed protein was purified using an Ni–IDA His-Bind Superflow column (Novagen Co., USA). The column was washed with 12.5 ml binding buffer after the lysate had been loaded onto the column. The column was rinsed with 12.5 ml washing buffer (20 m*M* Tris–HCl, 0.5 *M* NaCl, 40 m*M* imidazole pH 7.9) and then eluted with 4 ml elution buffer (20 m*M* Tris–HCl, 0.5 *M* NaCl, 500 m*M* imidazole pH 7.9). Subsequent purification was performed on FPLC by gel filtration (10 m*M* Tris–HCl pH 8.0) on Superdex75 10/300GL columns (GE Healthcare, USA; Zhang *et al.*, 2008). SDS–PAGE was performed with 12%(*w*/*v*) polyacrylamide gels and stained with Coomassie Brilliant Blue R-250. The protein concentration was measured using a protein-assay kit (Bio-Rad, USA) with BSA as a standard.

2.2. Crystallization

Crystallization of the catalytic domain of alkaline β-mannanase was performed at 298 K using the hanging-drop vapour-diffusion method. Initial crystal screening of the enzyme was carried out using Crystal Screens I and II from Hampton Research (Aliso Viejo, California, USA). Drops contained equal volumes (1 µl each) of protein solution prepared as above and reservoir crystallization buffer and were equilibrated against 200 µl reservoir solution. To obtain a crystal suitable for X-ray crystallographic analysis, the conditions were optimized based on the initial screenings.

2.3. Data collection and processing

After a brief immersion in reservoir solution containing 15% glycerol, the crystals were mounted in nylon loops and flash-cooled in a cold nitrogen-gas stream at 100 K (Parkin & Hope, 1998). Data collection was performed in-house using a Rigaku MicroMax007 rotating-anode X-ray generator operated at 40 kV and 20 mA (Cu $K\alpha$; λ = 1.5418 Å) equipped with an R-AXIS VII^{++} image-plate detector. Data were indexed and scaled using the *HKL*-2000 software package (Otwinowski & Minor, 1997). The initial phase set was calculated by the molecular-replacement method using *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994). The search model for molecular replacement was the structure of the mannanase from alkaliphilic *Bacillus* sp. JAMB602 (PDB code 1wky; Akita *et al.*, 2004), which showed 77% sequence identity.

3. Results

Recombinant Man5A330 protein was successfully expressed and purified to apparent homogeneity. Its molecular weight was estimated to be 36 kDa on the basis of relative mobility on the SDS–PAGE gel (Fig. 1).

Initial attempts to crystallize the alkaline mannanase were performed using crystallization screening kits with the hanging-drop vapour-diffusion technique at 298 K. Man5A330 crystals were

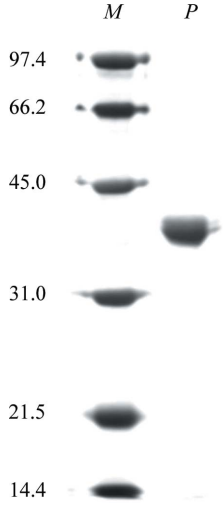


Figure 1
12% SDS–PAGE analysis of purified Man5A330 (lane *P*). Lane *M* contains molecular-weight markers (labelled in kDa).

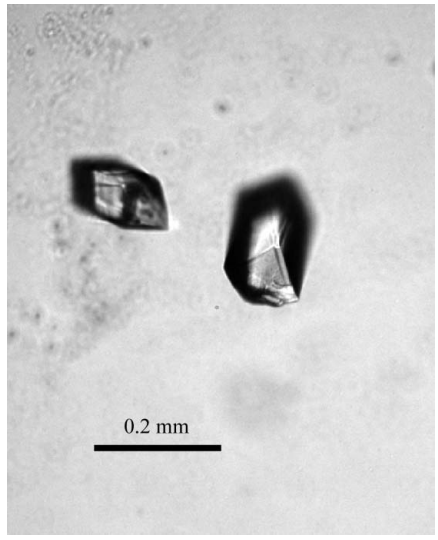


Figure 2
Typical crystal of Man5A330.

observed using several crystallization screening conditions. After several steps that improved the crystallization process, the best crystals were obtained in 2–3 d by setting up 2 µl crystallization drops containing 0.5 *M* $(NH_4)_2SO_4$, 0.1 *M* sodium citrate pH 5.6, 1.2 *M* Li_2SO_4 and 2.0 mg ml^{-1} protein. The crystals grew with good reproducibility and reached average dimensions of 50 × 100 × 200 µm (Fig. 2).

Crystallographic data statistics are summarized in Table 1. The data set is 92.74% complete to 1.6 Å resolution (Fig. 3), with an R_{merge} of 7.6%. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 59.0, b = 63.3, c = 83.3 Å. The asymmetric unit contains one molecule, resulting in a Matthews coefficient of 2.36 Å^3 Da^{-1} and a solvent content of 47.91% (Matthews, 1968).

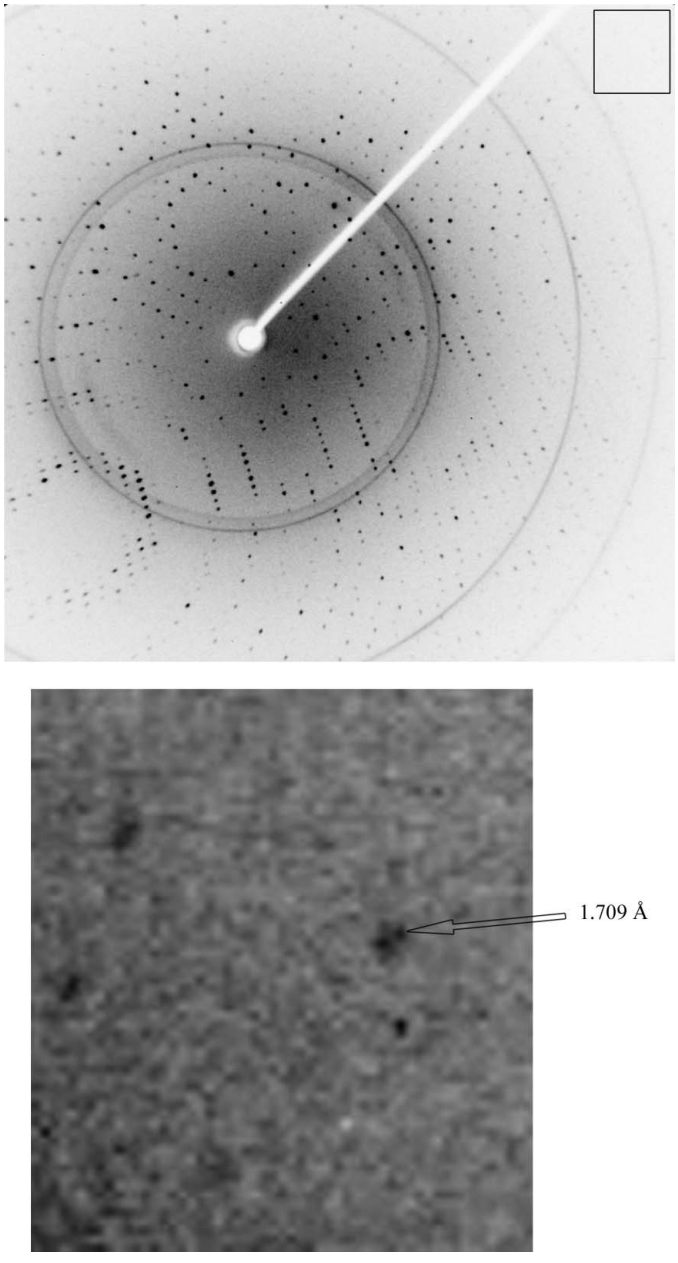


Figure 3
Diffraction pattern of Man5A330 from *Bacillus* sp. N16-5.

Table 1
Diffraction data statistics.

Values in parentheses are for the outer shell.

Space group	$P2_12_12_1$
Unit-cell parameters	
a (Å)	59.03
b (Å)	63.31
c (Å)	83.34
α (°)	90
β (°)	90
γ (°)	90
Resolution range (Å)	63.29–1.60 (1.71–1.60)
Total No. of reflections	182424 (7471)
No. of unique reflections	38945 (4841)
Average redundancy	4.68 (1.54)
Completeness (%)	92.74 (65.44)
R_{merge}† (%)	7.64 (40.66)
Average $I/\sigma(I)$	7.63 (1.95)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl.

Crystallographic model building and structure refinement are in progress. We hope that the three-dimensional structure will provide insight into deciphering the molecular basis of the alkaline adaptation and efficient catalytic mechanism of the alkaline mannanase from *Bacillus* sp. N16-5.

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