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Crystallization and preliminary X-ray study of alkaline mannanase from an alkaliphilic *Bacillus* isolate

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An alkaline mannanase (EC 3.2.1.78) from the alkaliphilic *Bacillus* sp. strain JAMB-602 was cloned and sequenced. The deduced amino-acid sequence of the enzyme suggested that the enzyme consists of a catalytic and unknown additional domains. The recombinant enzyme expressed by *B. subtilis* was crystallized using the hanging-drop vapour-diffusion method at 277 K. X-ray diffraction data were collected to 1.65 Å. The crystal belongs to space group $P2_12_12_1$, with unit-cell parameters $a = 70.7$, $b = 79.5$, $c = 80.4$ Å. The asymmetric unit contains one protein molecule, with a corresponding V_M of 2.26 Å^3 Da^{-1} and a solvent content of 45.6%. Molecular replacement for initial phasing was carried out using the three-dimensional structure of a mannanase from *Thermomonospora fusca* as a search model, which corresponds to the catalytic domain of the alkaline mannanase. It gave sufficient phases to build the unknown domain.

1. Introduction

Mannan is a major component of the cell walls of angiosperms and is a polysaccharide consisting of a backbone of β-1,4-linked mannose units. Depending on their origin, mannans are substituted with galactose and/or acetate residues and sometimes include glucose in their backbone (Timell, 1967). Mannanase (endo-β-1,4-mannanase; EC 3.2.1.78) is an enzyme that randomly hydrolyses the β-1,4-mannosidic linkages in mannans (McCleary, 1983). The structures of mannanases from *Thermomonospora fusca* (Hilge *et al.*, 2001) and *Tricoderma reesi* (Sabini *et al.*, 2000) are known. The optimal pH of the mannanase from *Thermomonospora fusca* is below 8 (Hilge *et al.*, 1996).

Bacillus sp. strain JAMB-602 is an alkaliphilic isolate that exoproduces an alkaline mannanase. The enzyme has a pH optimum around 9. The activity remains more than 80% even if the enzyme is treated in a highly alkaline solution (pH 11) for 24 h at 277 K. The optimal temperature is 338 K. The nucleotide sequence of the alkaline mannanase has been determined (DDBJ code AB119999). The open reading frame of the enzyme comprised of 1470 base pairs encoding 490 amino-acid residues. The mature enzyme comprised of 468 amino-acid residues, corresponding to a molecular weight of approximately 50 kDa.

Amino-acid sequence analysis using a BLAST database (Altschul *et al.*, 1997; http://www.ncbi.nlm.nih.gov/BLAST) search showed that the alkaline mannanase was similar to a mannanase from *B. circulans* (Swiss-Prot code O66185) with 58% sequence identity. According to PFAM (Bateman *et al.*, 1999), the alkaline mannanase contains the $(\beta/\alpha)_8$-barrel structure classified into glycoside hydrolase family 5 (GH-5; Henrissat, 1991; http://afmb.cnrs-mrs.fr/CAZY/index.html). GH-5 includes not only mannanases but also cellulases. The catalytic domain of enzymes in GH-5 is composed of the $(\beta/\alpha)_8$-barrel structure. Of the cellulases, this family contains the structurally known alkaline cellulase K from *Bacillus* sp. strain KSM-635 (Shirai *et al.*, 2001). It is possible that the alkaline mannanase has adapted to alkaline environments, as in the cases of cellulase K (Shirai *et al.*, 2001) and high-alkaline protease (Shirai *et al.*, 1997).

The catalytic domain of the alkaline mannanase is located in the first 300 residues of the N-terminal region. The C-terminal region (160 residues) was similar to that (residues 358–512) of β-mannanase from *Bacillus* sp. strain AM-001 (Akino *et al.*, 1989; Swiss-Prot code P16699) with 46% identity using a BLAST search. The β-mannanase from *Bacillus* sp. strain AM-001 also acts in an alkaline environment (pH optimum 8.5–9.0) and is classified into glycoside hydrolase family 26. The classification into this family is based primarily on the similarity of the catalytic domains. Therefore, these two mannanases are classified into different families, even though the similarity of their C-terminal regions is moderately high. According to structure prediction using the threading program 123*D*+ (Alexandrov *et al.*, 1995; http://123d.ncifcrf.gov/123D+.html), the C-terminal region of the alkaline mannanase assumes a galactose-

binding domain-like structure. It is possible that the C-terminal region (domain) may be key to clarifing how enzymes act in an alkaline environment. Because of this, revealing the function and structure of the C-terminal domain is of great importance.

In summary, the determination of the three-dimensional structure of the alkaline mannanase is important for the following two reasons: (i) to clarify the mechanism(s) of alkaline adaptation in mannanase and (ii) to solve the structure of the C-terminal domain of the alkaline mannanase. Here, we report the crystallization and X-ray diffraction analysis of the alkaline mannanase.

2. Materials and methods

2.1. Expression and purification of recombinant alkaline mannanase

The alkaline mannanase gene (designated *amn5A*) was cloned and sequenced from the genomic DNA of *Bacillus* sp. strain JAMB-602. The genomic DNA was digested with *Eco*RI and the digests were then inserted into the corresponding cloning site of plasmid pUC18 (TaKaRa Bio, Kyoto, Japan). Transformants of *Eschericha coli* HB101 harbouring the plasmid were selected and one of the positive clones was sequenced. In order to produce the alkaline mannanase for crystallization, the *amn5A* gene in the plasmid was amplified by PCR using LA *Taq* DNA polymerase (TaKaRa Bio) and then inserted into the cloning site of the expression vector pHSP64 (Sumitomo *et al.*, 1995). The recombinant plasmid was introduced into *B. subtilis* ISW1214 cells. 20 batches of *B. subtilis* ISW1214 cells harbouring the plasmid were cultured at 303 K for 72 h with shaking without any inducers in a liquid medium composed of 10%(*w*/*v*) corn steep liquor (Nihon Syokuhin Kako, Shizuoka, Japan), 1.0%(*w*/*v*) fish meat extract (Wako Pure Chemical, Osaka, Japan), 0.1%(*w*/*v*) yeast extract (Difco, MD, USA), 0.1%(*w*/*v*) KH_2PO_4, 0.02%(*w*/*v*) $MgSO_4{\cdot}7H_2O$, 0.05%(*w*/*v*) $CaCl_2$, 7%(*w*/*v*) maltose and 15 µg ml^{-1} tetracycline. From each batch, cells and culture supernatants were separated by centrifugation at 12 000*g* for 10 min. Each supernatant obtained was checked for mannanase activity and production of target protein by SDS–PAGE and then selected for an appropriate batch. The selected batch was dialyzed against 10 m*M* Tris–HCl buffer pH 7.5 and used for enzyme purification. The dialyzed solution was loaded onto a DEAE-Toyopearl 650M column (Tosoh, Tokyo, Japan) equilibrated with 10 m*M* Tris–HCl pH 7.5 and proteins were eluted with a 50–150 m*M* NaCl linear gradient in 10 m*M* Tris–HCl pH 7.5. The active fractions were combined and directly loaded onto a hydroxyapatite column (Seikagaku Kogyo, Tokyo, Japan) equilibrated with 10 m*M* sodium phosphate buffer pH 7.0 and proteins were eluted with a 10–150 m*M* linear gradient of sodium phosphate. The fractions containing the recombinant alkaline mannanase were then concentrated and loaded onto a DEAE-Toyopearl 650M column equilibrated with 10 m*M* Tris–HCl pH 7.5 and eluted with an 80–140 m*M* linear gradient of NaCl in 10 m*M* Tris–HCl pH 7.5. The purified enzyme was dialyzed against 5 m*M* Tris–HCl pH 7.5 and the retentate was concentrated to 14 mg ml^{-1}. The protein concentration was measured using a Bio-Rad protein assay kit with bovine serum albumin as a standard (Hercules, CA, USA).

Table 1
Summary of typical purification of alkaline mannanase.

The volume of culture supernatant was 31.9 ml.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Culture supernatant	599	14000	23	100	1.0
DEAE-Toyopearl, first	49	9220	188	66	8.2
Hydroxyapatite	31	6811	220	49	9.6
DEAE-Toyopearl, second	16	4595	287	33	12.5

2.2. Crystallization and data collection

Crystallization of the alkaline mannanase was performed at 277 K using the hanging-drop vapour-diffusion method. Initial crystal screening of the enzyme was carried out using Hampton Research (Aliso Viejo, CA, USA) crystallization screens: Crystal Screens (I, II, Lite, Cryo), Grid Screens (PEG 6000, ammonium sulfate, sodium chloride and MPD) and PEG/Ion screen. 1 µl protein solution, prepared as above, was mixed with an equal volume of reservoir solution and equilibrated against 800 µl reservoir solution. To obtain a larger crystal, the conditions were varied based on the initial screenings.

Prior to X-ray analysis, the crystals were soaked in a cryoprotectant consisting of the crystallization solution and 15%(*w*/*v*) glycerol. The crystals were mounted in a cryoloop and flash-frozen by plunging them into liquid nitrogen. X-ray diffraction data were collected using synchrotron radiation at the BL41XU beamline at SPring-8, Hyogo, Japan. The X-ray wavelength was 1.0 Å. The crystal-to-detector distance was 100 mm, the oscillation range was 1° and the exposure time was 5 s per frame. The total oscillation range was 180°. The collected data set was processed with *HKL*2000 (Otwinowski & Minor, 1997). The native data were analyzed by a self-rotation function search using the program *POLARRFN* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

2.3. Phasing trials

We calculated an initial phase set with 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 the molecular replacement was the structure of the mannanase from *Thermomonospora fusca* (PDB code 1bqc; Hilge *et al.*, 2001), which showed the highest sequence identity to the alkaline mannanase (45.7% in a 299 amino-acid overlap) of the structurally known GH-5 proteins.

3. Results and discussion

The purity of the protein solution was estimated by SDS–PAGE to be approximately 95% (Fig. 1), with a yield of approximately

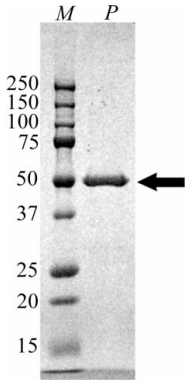

Figure 1
Coomassie-stained SDS–PAGE of the purified alkaline mannanase (lane *P*; 1 µg protein) on a 12.5%(*w*/*v*) polyacrylamide gel. Protein molecular-weight markers (kDa) are indicated on the left (lane *M*).

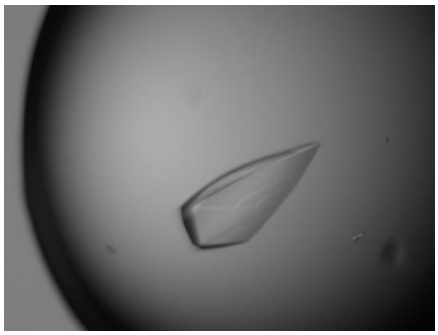

Figure 2
Photograph of a crystal of alkaline mannanase.

33% (Table 1). Initial attempts to crystallize the alkaline mannanase were performed using crystallization screening kits. The crystals were obtained from Grid Screen Sodium Chloride within a week. The best condition was 4 *M* NaCl and 0.1 *M* MES pH 6.0 and gave wedge-shaped crystals. The crystals grew with good reproducibility and reached average dimensions of 100 × 100 × 300 µm (Fig. 2). The crystals were stable for at least one month at 277 K.

X-ray diffraction data for the crystal were collected to a resolution of 1.65 Å. Diffraction data processing showed the crystals to be orthorhombic. From the systematic absences, the space group was determined to be $P2_12_12_1$. The unit-cell parameters were $a = 70.7$, $b = 79.5$, $c = 80.4$ Å. A summary of the data-processing statistics is shown in Table 2. Non-crystallographic symmetry-related peaks were not observed in the Patterson self-rotation function. Therefore, the asymmetric unit of the crystal contained one molecule (50 kDa) with an expected V_M of 2.26 Å^3 Da^{-1}, which corresponds to a solvent content of 45.6% (Matthews, 1968).

Table 2
Crystallographic parameters and data-collection statistics.

Values in parentheses refer to the reflections in the outer shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 70.7$, $b = 79.5$, $c = 80.4$
Resolution limits (Å)	50.0–1.65 (1.71–1.65)
No. observed reflections	406055
No. unique reflections	55268
Redundancy	7.3 (7.3)
Average $I/\sigma(I)$	28.4 (3.2)
Completeness (%)	99.9 (100.0)
Completeness in low-resolution shell (50.0–3.55 Å) (%)	99.3
R_{merge}† (%)	7.1 (38.6)

† $R_{merge} = \sum |I - \langle I \rangle| / \sum I$, where I is the observed intensity and $\langle I \rangle$ is the mean intensity of all symmetry-related reflections.

Phasing of the crystal structure was attempted using the molecular-replacement method. After determination of the rotation and translation parameters, *MOLREP* showed a correlation coefficient and *R* factor of 0.295 and 54.1%, respectively. Checking the solution using the resulting coordinates and 1.65 Å diffraction data, the main-chain atoms almost fitted the $2F_o - F_c$ electron-density map.

The number of residues determined by the molecular replacement was 302 amino acids, which corresponds to the N-terminal catalytic domain of the alkaline mannanase. To clarify the unidentified C-terminal domain, an $F_o - F_c$ residual map was calculated. Although the map was not clear, there was a peptide-like structure near the N-terminal domain, which could be a novel C-terminal extension of the alkaline mannanase. We are now building the C-terminal domain and further refining the N-terminal domain.

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