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Crystallization and preliminary crystallographic analysis of endo-1,3-β-glucanase from alkaliphilic *Nocardiopsis* sp. strain F96

Guntur Fibriansah,[a] Sumiko Masuda,[b] Raita Hirose,[c] Kensaku Hamada,[c] Nobuo Tanaka,[a] Satoshi Nakamura[b] and Takashi Kumasaka[a]*

[a]Department of Life Science, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan, [b]Department of Bioengineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan, and [c]PharmAxess Inc., 3-1-1 Kouto, Kamigori-cho, Ako-gun, Hyogo 678-1205, Japan

Correspondence e-mail: tkumasak@bio.titech.ac.jp

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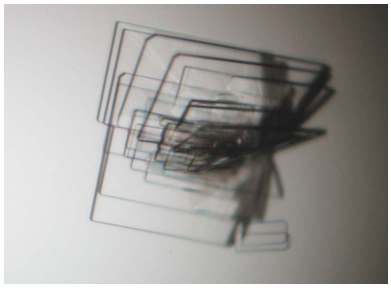

Endo-1,3-β-glucanase, an enzyme that hydrolyzes the 1,3-β-glycosyl linkages of β-glucan, belongs to the family 16 glycosyl hydrolases, which are widely distributed among bacteria, fungi and higher plants. Crystals of a family 16 endo-1,3-β-glucanase from the alkaliphilic *Nocardiopsis* sp. strain F96 were obtained by the hanging-drop vapour-diffusion method. The crystals belonged to space group $P2_1$, with unit-cell parameters $a = 34.59$, $b = 71.84$, $c = 39.67$ Å, $\beta = 90.21^\circ$, and contained one molecule per asymmetric unit. The Matthews coefficient (V_M) and solvent content were 1.8 Å^3 Da^{-1} and 31.8%, respectively. Diffraction data were collected to a resolution of 1.3 Å and gave a data set with an overall R_{merge} of 6.4% and a completeness of 99.3%.

1. Introduction

1,3-β-glucan, a polymer of 1,3-β-linked glucose, is the main constituent of plant and fungal cell walls and is also a major structural and storage polysaccharide of marine macroalga. 1,3-β-Glucanase hydrolyzes the 1,3-β-bonds of 1,3-β-glucan. The class of enzymes known as 1,3-β-glucanases are well characterized in plants, fungi, viruses and bacteria. Based on the hydrolysis reactions catalyzed by the glucanase, 1,3-β-glucanases are classified into exo-1,3-β-glucanases (EC 3.2.1.58) and endo-1,3-β-glucanases (EC 3.2.1.6 and EC 3.2.1.39). The 1,3-β-glucanases play various physiological roles. In plants, 1,3-β-glucanases have been implicated in protection against fungal pathogens through their ability to hydrolyze 1,3-β-glucan, a major cell-wall component of plants, and in plant cell differentiation (Chen *et al.*, 1993; Coutinho *et al.*, 2003). 1,3-β-Glucanase expression in plant seeds plays important roles in the regulation of seed germination and dormancy and in defence against seed pathogens (Metzger, 2003). In fungi, 1,3-β-glucanases are important in morphogenetic processes, β-glucan mobilization and fungal pathogen–plant interactions (Bachman & McClay, 1996). Viral 1,3-β-glucanases are involved in degrading the host cell wall during virus release or are packaged in the virion particle and involved in virus entry (Sun *et al.*, 2000). In bacteria, metabolic functions have been reported for endo-1,3-β-glucanase and endo-1,3–1,4-β-glucanase (Gueguen *et al.*, 1997; Fuchs *et al.*, 2003). Endo-1,3-β-glucanases hydrolyze internal 1,3-β-glucosyl linkages, while endo-1,3–1,4-β-glucanases only hydrolyze internal 1,4-β-glucosyl linkages when the glucosyl residue itself is linked at the O-3 position. Despite these functional differences, bacterial endo-1,3-β-glucanases share sequence similarity with endo-1,3–1,4-β-glucanases (Gueguen *et al.*, 1997) and both belong to glycosyl hydrolase family 16 (Coutinho & Henrissat, 1999). The application of 1,3-β-glucanases is well established in the preparation of protoplasts, in the treatment of fungal diseases and for use in biotechnological processes such as cell fusion, transformation and extraction of protein products (Ballou, 1982; Parrado *et al.*, 1996).

The gene encoding a family 16 endo-1,3-β-glucanase (BglF) was cloned from the alkaliphilic *Nocardiopsis* sp. strain F96 and expressed in *Escherichia coli* strain BL21(DE3) carrying plasmid pET-BglF (Masuda *et al.*, 2003). BglF was classified into the family 16 endo-1,3-β-glucanases based on both substrate specificity against laminarin and on amino-acid sequence similarities. BglF purified from the clone has a molecular weight of 34.1 kDa. BglF has a broad pH spectrum of

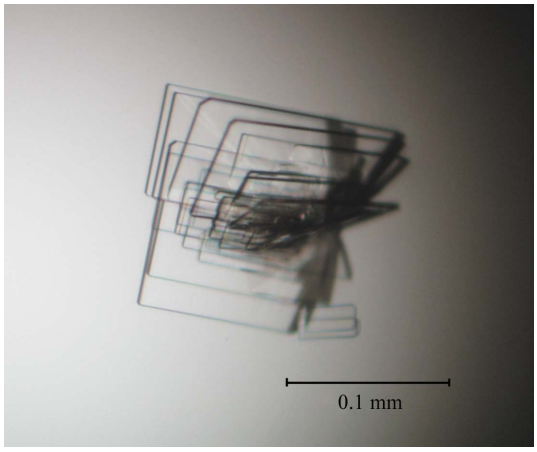

Figure 1
Crystals of BglF.

its activity from pH 4 to 11 and a broad temperature activity from 303 to 353 K. The optimal pH and temperature of the enzyme were pH 9.0 and 343 K, respectively (Masuda *et al.*, 2004). Although crystal structures of bacterial endo-1,3–1,4-β-glucanases have been determined (Keitel *et al.*, 1993; Hahn *et al.*, 1995; Tsai *et al.*, 2003), the structure of a bacterial endo-1,3-β-glucanase has not yet been reported. Although crystal structures of barley family 17 endo-1,3-β-glucanase and endo-1,3–1,4-β-glucanase have been reported, there is neither sequential nor tertiary structural homology between the plant and bacterial enzymes (Varghese *et al.*, 1994; Müller *et al.*, 1998). The crystal structure of another archaeal endo-1,3-β-glucanase has also been reported as being determined (Ilari *et al.*, 2004). The X-ray structure of BglF will provide an understanding of the structure–function relationship of not only this enzyme, but also the bacterial enzymes in general. Moreover, knowledge of the three-dimensional structure of the active site will provide us with information about the possible structural determinants of its unusual stability at high pH. Here, we report the crystallization and preliminary crystallographic analysis of endo-1,3-β-glucanase from the alkaliphilic *Nocardiopsis* sp. strain F96.

2. Purification and crystallization

The recombinant BglF was produced in *E. coli* BL21(DE3) containing the pET-BglF plasmid. The enzyme in crude cell extract was purified from the culture supernatant using the Vivaflow 50 ultrafiltration system (Sartorius AG, Goettingen, Germany) followed by ion-exchange chromatography using a DEAE-Toyopearl 650M (Tosoh, Tokyo, Japan) and BioAssistQ (Tosoh) column chromatography as described previously (Masuda *et al.*, 2004). The purified BglF was screened with Crystal Screens 1 and 2 (Hampton Research, Aliso Viejo, CA, USA) and Wizard Screens 1 and 2 (Emerald Biosystems, Bainbridge Island, USA). Most crystals appeared in conditions that contained ammonium sulfate. Further screening was performed using Grid Screen Ammonium Sulfate and Additive Screen (Hampton Research). For crystallization screening, the protein concentration was 10 mg ml^{-1} and the sitting-drop vapour-diffusion method was used. In the optimal conditions, the reservoir solution contained 0.1 *M* Tris–HCl pH 8.0, 1.3–1.4 *M* $(NH_4)_2SO_4$ and 1% ethanol with protein concentration 5 mg ml^{-1}. Crystallization was achieved at 293 K by the hanging-drop vapour-diffusion method (Jancarik & Kim, 1991). 1 µl protein sample was mixed with an equal amount of the reservoir solution and allowed to equilibrate. The crystals appeared in 3–4 d (Fig. 1).

3. Data collection

A crystal of the enzyme was picked up from a droplet in a nylon loop (Hampton Research, Aliso Viejo, CA, USA), transferred into a cryoprotectant solution (0.1 *M* Tris–HCl pH 8.0, 1.5 *M* ammonium sulfate, 2% ethanol, 20% glycerol) and then placed directly into a cold nitrogen-gas stream at 100 K. X-ray diffraction data were

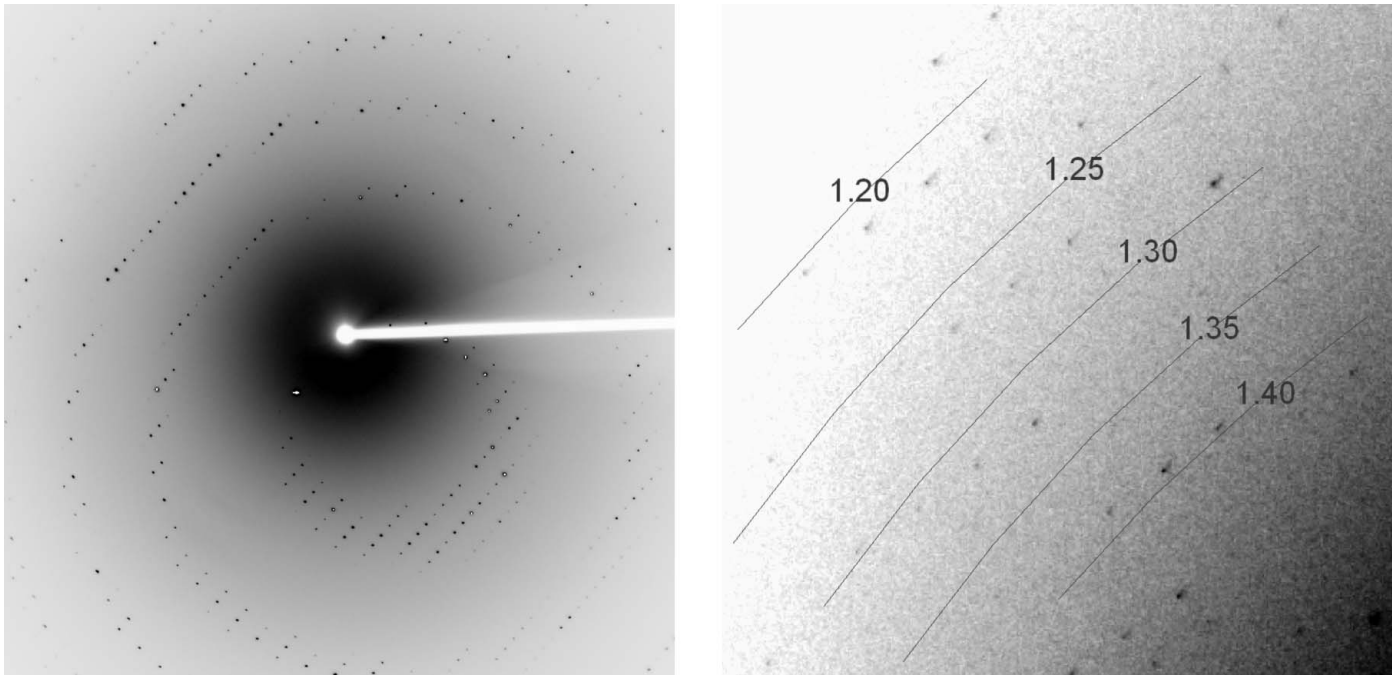

Figure 2
Diffraction pattern of a BglF crystal. The first frame of the data collection (left) and a zoom of the upper left corner with resolution shells labelled (right) are shown.

Table 1
Data-collection statistics for BglF.

Values in parentheses are for the highest resolution shell.

X-ray source	SPring-8, BL41XU
Detector	Quantum 315 CCD
Wavelength (Å)	0.9
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 34.59, b = 71.84, c = 39.67, β = 90.21
Resolution range (Å)	39.65–1.30 (1.35–1.30)
Measured reflections	293219 (21955)
Unique reflections	47558 (4574)
Completeness (%)	99.3 (95.7)
$R_{\rm merge}$† (%)	6.4 (35.0)
$\langle I/\sigma(I)\rangle$	14.2

† $R_{\rm merge} = \sum_{h,i} |I(h)_i - \langle I(h)\rangle| / \sum_{h,i} I(h)_i$, where $I(h)_i$ are the measurements contributing to the mean reflection intensity, $\langle I(h)\rangle$.

collected on a Quantum 315 CCD detector using synchrotron radiation of wavelength 0.9 Å at BL41XU, SPring-8 (Hyogo, Japan). Data were collected by the standard oscillation method with 1° increments using a crystal-to-detector distance of 155 mm. Diffraction data for the crystal were obtained in the resolution range 39.65–1.30 Å (Fig. 2) and were processed using the *HKL*2000 program package (*DENZO* and *SCALEPACK*; Otwinowski & Minor, 1997). X-ray data statistics are summarized in Table 1.

4. Results

The crystals of BglF belonged to space group $P2_1$, with unit-cell parameters a = 34.59, b = 71.84, c = 39.67 Å, β = 90.21°. The crystals grew in 3–4 d and have a stacked plate form. The $V_{\rm M}$ value (Matthews, 1968), the crystal volume per unit of protein molecular weight, was calculated to be 1.8 Å^3 Da^{-1} assuming the presence of one molecule in an asymmetric unit; the solvent content was 31.8%. The $V_{\rm M}$ value and solvent content are lower than those usually observed in protein crystals.

Enzymes that belong to the different subfamilies of the family 16 glycosyl hydrolases show a similar fold and varying substrate specificity. The sequence identity among the members of different subfamilies is 10–25% and the sequence similarity is restricted to a few invariant residues involved in catalysis (Allouch *et al.*, 2003). Based on the BglF primary structure analysis, the molecular architecture of BglF may be envisaged as resembling that of endo-1,3–1,4-β-glucanase from *Paenibacillus macerans* (PDB code 1mac; Hahn *et al.*, 1995) and those of other family 16 glycosyl hydrolases whose three-dimensional structures have been solved (Coutinho & Henrissat, 1999). The molecular-replacement method has been used to solve the structure by using several homologues as models but none of them gave a good solution. Structure determination of BglF by the multiple-wavelength anomalous diffraction (MAD) method using a selenomethionyl derivative of the protein (Doublié, 1997) is currently under way.

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