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Crystallization and preliminary X-ray diffraction analysis of the 1,3-1,4-β-D-glucanase from *Fibrobacter succinogenes*

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The truncated 1,3-1,4-β-glucanase (1,3-1,4-β-D-glucan 4-glucanohydrolase; E.C. 3.2.1.73) from *Fibrobacter succinogenes* was crystallized in four different forms by the vapour-diffusion method. Form *A* crystals have the largest trigonal *P*321 unit cell, diffracting to 3.0 Å resolution with four to six molecules per asymmetric unit. Form *B* and *C* crystals belong to the same monoclinic space group $P2_1$, but the form *B* unit cell is twice as large as the unit cell of form *C*. Form *B* crystals diffract to 2.5 Å resolution and contain four molecules per asymmetric unit. Form *C* crystals diffract to 2.1 Å resolution and contain two molecules per asymmetric unit. Form *D* crystals have the smallest orthorhombic $P2_12_12_1$ unit cell, containing only one molecule per asymmetric unit, and diffract beyond 2.1 Å resolution. The crystallization conditions for form *B* and *C* crystals are almost identical, except that form *C* crystals were grown in the presence of 2 m*M* Ca^{2+} ions. It is likely that Ca^{2+} directly binds to the glucanase, leading to unit-cell shrinkage as observed in other *Bacillus glucanase* crystals. A self-rotation search identified non-crystallographic twofold axes that combine with the crystallographic twofold dyads to give 222 symmetry for both form *A* and form *B* crystals, indicating that the glucanase has a tendency to pack in 222 symmetry.

1. Introduction

1,3-1,4-β-D-Glucanases (1,3-1,4-β-D-glucan 4-glucanohydrolases; E.C. 3.2.1.73) belong to the family 16 endoglucanases, which hydrolyse mixed-linked glucans containing β-1,3- and β-1,4-glycosidic linkages such as β-glucans from grain endosperm cell walls or lichenan from Icelandic moss (Anderson & Stone, 1975). Cleavage of the natural substrates occurs specifically at β-1,4-glycosidic linkages on 3-O-substituted glucose units in β-glucans (Anderson & Stone, 1975; Buliga *et al.*, 1986; Parrish *et al.*, 1960; Woodward *et al.*, 1983). 1,3-1,4-β-D-Glucanases have been identified from bacteria and plants, including different *Bacillus* species (Borriss *et al.*, 1988; Bueno *et al.*, 1990; Gosalbes *et al.*, 1991; Hofemeister *et al.*, 1986; Lloberas *et al.*, 1991; Louw *et al.*, 1993; Murphy *et al.*, 1984; Tzuka *et al.*, 1989), *F. succinogenes* (Teather & Erfle, 1990), *Ruminococcus flavefaciens* (Flint *et al.*, 1993), *Clostridium thermocellum* (Schimming *et al.*, 1992) and barley (Fincher *et al.*, 1986; Litts *et al.*, 1990). Bacterial β-glucanases share high sequence identity (50–70%) with each other, but show little or no homology to the plant counterpart from barley (Chen *et al.*, 1993).

Crystal structures of several bacterial 1,3-1,4-β-D-glucanases have been reported, including native enzymes from *B. macerans* (Hahn, Olsen *et al.*, 1995) and *B. licheniformis* (Hahn, Pons *et al.*, 1995) and several engineered enzymes, including a *Bacillus* hybrid enzyme H(A16-M) (Keitel *et al.*, 1993) and the circularly permuted enzymes derived from H(A16-M) (Hahn *et al.*, 1994). All of these *Bacillus* enzymes bear a similar 'jelly-roll' β-barrel structure containing two seven-stranded antiparallel β-sheets; the active site is located at the cleft on the concave side of the β-sheet. In contrast, the crystal structure of the barley 1,3-1,4-β-D-glucanase is folded into an α/β-barrel structure (Muller *et al.*, 1998; Varghese *et al.*, 1994) which shows little resemblance to the β-barrel structure of the bacterial enzymes.

The 1,3-1,4-β-D-glucanase from *F. succinogenes* (referred to as Fsβ-glucanase) was first isolated and characterized by Erfle and coworkers (Erfle *et al.*, 1988; Teather & Erfle, 1990). Recently, the specific amino-acid residues involved in the catalysis and thermal stability of the Fsβ-glucanase were identified and characterized (Chen *et al.*, 2001). Moreover, a truncated form of Fsβ-glucanase with an approximately 10 kDa peptide fragment deleted at the C-teminus was constructed which exhibits a higher thermal stability than that of the wild-type Fsβ-glucanase (Shyur *et*

al., 2000). Kinetic analyses showed that the truncated form of Fsβ-glucanase has a 3.9-fold increase in specific activity and a minor 1.5-fold decrease in binding affinity for lichenan relative to the wild-type enzyme (Shyur *et al.*, 2000). A comparison of all the β-glucanases with similar substrate specificity from bacteria, fungi and plants showed that the 1,3-1,4-β-D-glucanase from *F. succinogenes* was the only naturally occurring enzyme with a circularly permutated sequence, *i.e.* the two conserved catalytic domains are switched in position. In addition, Fsβ-glucanase shares lower sequence homology (~30% identity) with the 1,3-1,4-β-D-glucanases isolated from different origins (Schimming *et al.*, 1992). X-ray diffraction was thus employed in the present study to analyze the protein structure of the truncated form of Fsβ-glucanase in an effort to gain more insight into the stability and catalytic activity of the enzyme. Comparison of the crystal structure of Fsβ-glucanase with other known structures of *Bacilli* enzymes should provide useful information for better understanding the protein folding and molecular evolution of 1,3-1,4-β-D-glucanase. Here, we present the crystallization and preliminary X-ray diffraction data of the truncated form of Fsβ-glucanase.

Table 1
Diffraction statistics for the crystals of the truncated Fsβ-glucanase.

Values in parentheses are for the highest resolution shell.

Crystal form	*A*	*B*	*C*	*D*
Cell parameters				
Space group	*P*321	$P2_1$	$P2_1$	$P2_12_12_1$
Unit-cell parameters				
a (Å)	150.68	87.13	69.94	40.71
b (Å)	150.68	43.93	44.08	73.27
c (Å)	99.18	123.91	80.87	73.65
β (°)	90.0	98.8	110.0	90.0
No. of molecules per a.u	4, 5 or 6	3 or 4	2	1
V_M (Å^3 Da^{-1})	2.9/2.3/1.9	2.7/2.1	2.1	2.0
Diffraction data				
Resolution	3.0 (3.11–3.00)	2.5 (2.54–2.50)	2.1 (2.14–2.10)	2.1 (2.14–2.10)
Observed reflections	152429	86928	146349	136929
Unique reflections	26298	30607	26880	13397
Completeness (%)	99.7	94.3	98.6	98.8
$I/\sigma(I)$	18.0 (6.3)	18.6 (6.98)	37.9 (10.1)	56.1 (17.5)
R_{merge}†	8.7 (19.1)	6.2 (15.5)	4.4 (9.3)	4.1 (7.0)

† $R_{merge} = \sum_h \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i I_{h,i}$, where $\langle I_h \rangle$ is the mean intensity of i observations for a given reflection h.

2. Materials and methods

2.1. Expression and purification of Fsβ-glucanase

The DNA coding sequence of the truncated form of *F. succinogenes* 1,3-1,4-β-D-glucanase was obtained by using a PCR-based method with a pair of specific primers and the full-length cDNA of Fsβ-glucanase in pJI10 as a template (Teather & Erfle, 1990). The amplified DNA fragment was ligated with a pET26b(+) vector (Novagen, USA) and then transformed into BL21(DE3) host cells. The truncated form of Fsβ-glucanase was effectively expressed and secreted into LB culture medium as a soluble protein at 306 K after 1 m*M* IPTG induction for 16 h. The culture supernatant containing approximately 85% of the expressed truncated form of Fsβ-glucanase was collected by centrifugation at 8000*g* for 15 min at 277 K and concentrated to one-tenth of its volume using a Pellicon Cassette concentrator (Millipore, Bedford, MA) with an M_r = 10 000 cutoff membrane. The concentrated supernatant was then dialyzed against 50 m*M* Tris–HCl buffer pH 7.8 (buffer *A*) and loaded onto a Sepharose Q FF column (Pharmacia, Sweden) pre-equilibrated with the same buffer. Fsβ-glucanase proteins were collected from eluants of the column using a 0–1 *M* NaCl salt gradient in buffer *A*. A second and third Ni-NTA affinity column equilibrated with 50 m*M* sodium phosphate pH 8.0, 0.3 *M* NaCl and 10 m*M* imidazole buffer (buffer *B*) were then employed for further purification of the truncated enzymes. From a 10–300 m*M* imidazole gradient eluant, a homogeneous enzyme preparation was obtained as verified by SDS–PAGE. The truncated form of Fsβ-glucanase has a molecular mass of 28 524 Da (258 amino acids) as determined by mass spectrometry.

2.2. Crystallization of Fsβ-glucanase

Crystallization of the truncated Fsβ-glucanase was carried out using the hanging-drop vapour-diffusion method at room temperature. Prior to crystallization, the purified protein was concentrated to 10 mg ml^{-1} in 10 m*M* Tris–HCl buffer pH 7.5. 1 µl drops of the truncated Fsβ-glucanase solution were mixed with 1 µl of various reservoir solutions. Four different crystal forms were obtained using different reservoir solutions. Form *A* crystals were grown from reservoir solution I containing 1.8 *M* $(NH_4)_2SO_4$, 0.2 *M* $LiNO_3$ and 0.2 *M* sodium acetate buffer pH 6.0. Tiny crystals appeared in one to two weeks, reaching maximum dimensions of 0.1 × 0.02 × 0.01 mm after two to three months. The other three crystal forms, *B*, *C* and *D*, were crystallized from similar conditions using the same precipitant. Form *B* crystals were crystallized from reservoir solution II containing 0.2 *M* $CH_3COONa.3H_2O$, 0.1 *M* Tris–HCl buffer pH 8.5 and 30%(*w*/*v*) PEG 4000. Form *C* crystals were grown from the same reservoir solution II, except with the addition of 2 m*M* $CaCl_2$. Form *D* crystals were grown from reservoir solution III containing 2 m*M* $CaCl_2$, 0.1 *M* CH_3COONa, 0.05 *M* Tris–HCl pH 9.0 and 30%(*w*/*v*) PEG 4000. Form *B*, *C* and *D* crystals appeared in about one week.

3. Results and discussion

Crystals were soaked in cryoprotectant consisting of 10% glycerol in the respective reservoir solution for 1 min prior to data collection. All four data sets were collected at low temperature (~100 K) using the synchrotron X-ray radiation source (λ = 1.0 Å) with a MAR Research (Hamburg, Germany) CCD detector at the BL41XU experimental station, SPring-8 (Hyogo, Japan). Indexing and integration of diffraction data were performed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The space groups, unit-cell parameters and statistics for diffraction data are listed in Table 1.

Form *A* crystals have the largest trigonal unit cell, with unit-cell parameters *a* = 150.68, *c* = 99.18 Å. There are four, five or six molecules per asymmetric unit, with corresponding V_M values (Matthews, 1968) of 2.9, 2.3 or 1.9 Å^3 Da^{-1}, respectively, and solvent contents of 58, 47 or 36%, respectively. Form *B* and form *C* crystals have the same monoclinic space group of $P2_1$, but the unit cell for form *B* crystals (*a* = 87.13, *b* = 43.93, *c* = 123.91 Å, β = 98.8°) is twice as large as the unit cell for form *C* crystals (*a* = 69.94, *b* = 44.08, *c* = 80.87 Å, β = 110.0°). There are likely to be four molecules per asymmetric unit for form *B* crystals and two molecules for form *C*, corresponding to a V_M of

2.1 Å^3 Da^{-1} and a solvent content of ~40% for both crystal forms. Form *D* crystals have the smallest orthorhombic $P2_12_12_1$ unit cell, containing only one molecule per asymmetric unit; the solvent content is calculated to be 36.7%, corresponding to a V_M of 2.0 Å^3 Da^{-1}.

The addition of 2 m*M* $CaCl_2$ to the form *B* reservoir solution produced form *C* crystals, whose unit cells are half the size of the unit cells of form *B* crystals. A slight modification of the crystallization conditions resulted in formation of crystals with different space groups, *i.e.* form *C* crystals ($P2_1$) were grown from mother liquor containing twice the buffer concentration of the mother liquor of form *D* ($P2_12_12_1$). It was reported that hybrid *Bacillus* glucanase H(A16-M) crystals grown in the presence of calcium ions led to shrinkage of the unit-cell volume compared with crystals grown in the absence of Ca^{2+}. Moreover, previous crystal structure studies showed that a Ca^{2+} ion directly binds to the *Bacillus* enzyme and that this calcium binding stabilizes the three-dimensional structure of the protein (Hahn, Olsen *et al.*, 1995; Keitel *et al.*, 1994). It is very likely that the Ca^{2+} ion also binds to Fsβ-glucanase.

Self-rotation functions were calculated using the program *GLRF* to reveal non-crystallographic symmetry (NCS) elements (Tong & Rossmann, 1990). In the $\kappa = 180^\circ$ section (Fig. 1*a*) calculated from data from form *A* crystals, there are three crystallographic twofold axes perpendicular to the reciprocal *c* axis at (φ, ψ) = (0, 30), (0, 90) and (180, 30). Interestingly, two NCS twofold axes perpendicular to each crystallographic dyad were observed and as a result six non-crystallographic twofold axes were found. Therefore, the glucanase molecules in the asymmetric unit of the trigonal cell are packed in a 222 symmetry, within which one twofold is the crystallographic twofold and the other two are NCS twofolds. The self-rotation functions for the form *B* crystals also showed 222 symmetry (see Fig. 1*b*), with a crystallographic dyad parallel to the reciprocal *b* axis and two NCS dyads in the *ab* plane perpendicular to the crystallographic twofold axis. All these results indicate that the truncated Fsβ-glucanase has a tendency to pack in 222 symmetry.

The molecular-replacement method was applied to solve the structure for the four different crystal forms, as Fsβ-glucanase shares ~30 % sequence identity with several *Bacillus* glucanases of known crystal structure. The *Bacilli* glucanase structures (PDB codes 1byh, 1cpm, 2ayh and 1gbg) were used as search models; however, no clear rotation solution could be identified. This indicates that the structure of Fsβ-glucanase is likely to be significantly different from those of *Bacillus* glucanases. Single/multiple isomorphous replacement (SIR/MIR) and multi-wavelength anomalous dispersion (MAD) methods are currently being used to further explore the structure of Fsβ-glucanase.

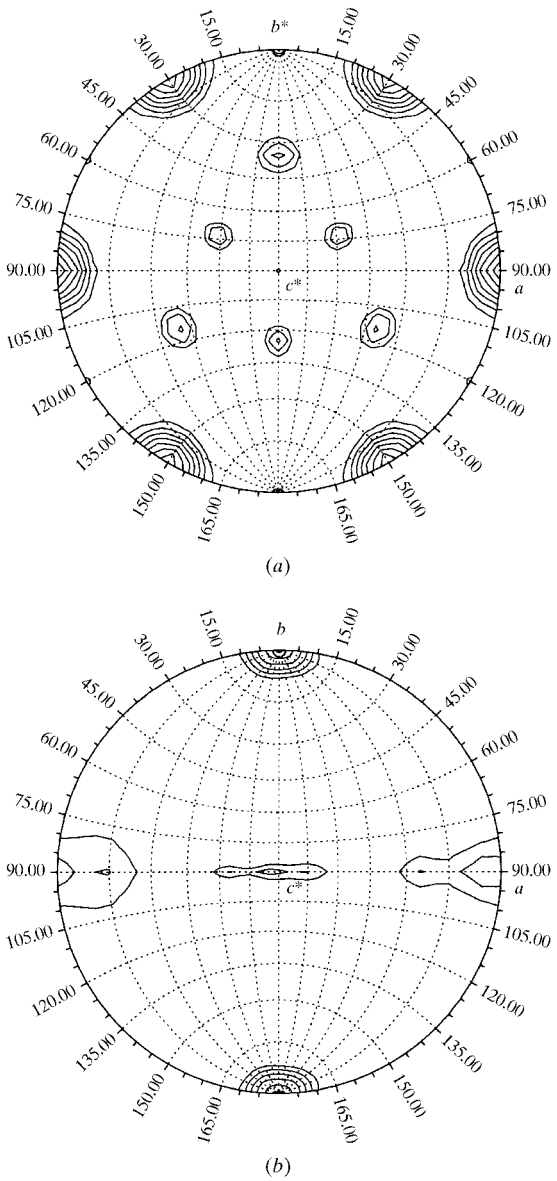

Figure 1
Stereographic projection of the self-rotation function at $\kappa = 180^\circ$ section in spherical polar angles. The rotation function was computed in the resolution range 10.0–3.0 Å with $I > 2\sigma(I)$ and a Patterson integration radii of 10.0 Å. Maps are contoured above 3σ with an increment of 1σ. (*a*) For form *A* crystals (trigonal *P*321), the crystallographic twofolds are located at (φ, ψ) = (0, 30), (0, 90) and (180, 30°), with a peak height of 9.2σ. The non-crystallographic twofold axes have a peak height of ~4.4σ and are each perpendicular to a crystallographic twofold axis, resulting in a 222 symmetry. The NCS dyads at (φ, ψ) = (40, 115) and (120, 75) are perpendicular to the crystallographic dyad at (φ, ψ) = (0, 30°), the NCS dyads at (φ, ψ) = (90, 35) and (90, 125°) are perpendicular to (φ, ψ) = (0, 90°) and the NCS dyads at (φ, ψ) = (60, 75) and (140, 115) are perpendicular to (φ, ψ) = (180, 30°). (*b*) For form *B* crystals, two non-crystallographic symmetric axes located at (φ, ψ) = (5, 90) and (95, 90°) with peak heights of ~4.0σ are both perpendicular to the crystallographic twofold at (φ, ψ) = (0, 0), with a peak height of 7.8σ.

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