Expression, purification and crystallization of a family 55 β -1,3-glucanase from *Chaetomium thermophilum*

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A β -1,3-glucanase from the thermophilic fungus *Chaetomium thermophilum* was overexpressed in *Pichia pastoris*, purified and crystallized in the presence of 1.8 M sodium/potassium phosphate pH 6.8 as a precipitant. Data to 2.0 Å resolution were collected in-house at 293 K from a single crystal. The crystal was found to belong to space group $P2_1$, with unit-cell parameters $a = 64.1$, $b = 85.8$, $c = 68.5$ Å, $\beta = 93.1^\circ$ and one molecule in the asymmetric unit.

1. Introduction

β -1,3-Glucanases are enzymes that catalyze the hydrolysis of β -1,3-glucans. Based on their mode of hydrolytic activity, they are generally classified into two major categories: exo- β -1,3-glucanases (EC 3.2.1.58) and endo- β -1,3-glucanases (EC 3.2.1.6 and EC 3.2.1.39). β -1,3-Glucanases are widely distributed among various organisms such as viruses, bacteria, filamentous fungi, actinomycetes, yeasts, molluscs and higher plants (Pitson *et al.*, 1993). In recent years, β -1,3-glucanases have attracted considerable attention owing to their potential use in various biotechnological, agricultural and pharmaceutical applications, including vinification (Blättel *et al.*, 2011), medical applications (Miyanishi *et al.*, 2003), animal feeds and defence against parasites (Shi *et al.*, 2010).

Fungi are able to produce various β -1,3-glucanases, possibly owing to the availability of β -1,3-glucans in their cell wall (Bielecki & Galas, 1991). Importantly, thermophilic fungi are considered to be new sources of thermophilic enzymes, especially cellulolytic enzymes (Li *et al.*, 2011; Juturu & Wu, 2014). *Chaetomium thermophilum* is a thermophilic fungus with growth temperatures of up to 333 K. The enzymes produced by *C. thermophilum* are characterized by high activity and stability at high temperatures. During growth in an induced medium containing wheat bran, *C. thermophilum* produces various β -1,3-glucanases at 323 K (Li *et al.*, 2007). Here, we report the expression, purification and crystallization of a β -1,3-glucanase from *C. thermophilum* (CtGluN). In accordance with other fungal glucanases (Donzelli *et al.*, 2001; Nobe *et al.*, 2004), CtGluN also belongs to family 55 of the glycoside hydrolases (GH55). Only one crystal structure of a β -1,3-glucanase from the GH55 family has been reported to date (Ishida *et al.*, 2009). The enzyme consists of two helical domains connected by a linker. The active site is located at the interface of the two domains. The catalytic acid has been identified (Glu633), but no catalytic base was reported.

2. Materials and methods

2.1. Macromolecule production

2.1.1. Construction of the expression plasmid. Based on a reported *C. thermophilum* β -1,3-glucanase gene (*glu-n*; GenBank accession No. GQ149728), special primers (5'-GCG-TACGTAGCTCCAGCTCGGCGGCAG-3' and 5'-GGCCT-AGGCTAAGCAGTCGACTGGAACCACGC-3'; restriction sites for SnaBI and AvrII are underlined) were designed and the full-length genomic cDNA of *glu-n* was cloned using RT-PCR and thermal asymmetric interlaced PCR methods. The full length of the cDNA is 2765 bp, which contains an ORF of 2358 bp encoding 785 amino acids including 23 amino acids as a signal peptide (Table 1).

The *glu-n* gene and the expression vector pPIC9K were digested with SnaBI and AvrII, and ligation was carried out *in vitro*. The recombinant expression plasmid pPIC9K/*glu-n* was linearized with the restriction enzyme SacI (insertion 5' to *AOX1*), transformed into *Pichia pastoris* GS115 competent cells by electroporation methods and screened for His⁺Mut⁺ transformants on minimal dextrose (MD) and minimal methanol (MM) plates according to the Pichia Expression System Kit (Invitrogen). PCR analysis of *P. pastoris* integrants and G418 antibiotic screening determined the multicopy integrants to be induced by methanol. These integrants were used to analyze the expression levels of the protein of interest every 24 h. The engineering strain with the highest expression level was called 4-60. Activity assays were carried out as previously reported (Li *et al.*, 2007). The transformed yeast strain 4-60 exhibited the highest activity 7 d after induction.

2.1.2. Purification. The expressed β -1,3-glucanase was concentrated by fractional ammonium sulfate precipitation at 90% saturation. The precipitated proteins were collected by centrifugation (10 000g, 15 min), dissolved in 50 mM Tris-HCl pH 8.0, dialyzed overnight against three changes of the same

Table 1

Macromolecule-production information.

Source organism	<i>C. thermophilum</i>
DNA source	<i>C. thermophilum</i>
Forward primer	GCGTACGTAGCTCCAGCTCGGCGGCAG
Reverse primer	GGCCTAGGCTAAGCAGTCGACTGGAACCACGC
Cloning vector	pPIC9K
Expression vector	pPIC9K/ <i>glu-n</i>
Expression host	<i>P. pastoris</i> GS115
Complete amino-acid sequence of the construct produced†	MGFNKLTTCICLLALNLFSPMALAAPARRQAPSGW-WLANIARQGRPAFNPDPNYKIFRNVDYGAVG-DGVTDDTAANAISDGNRCGQGCSSQTTPA-LVYFPPGTYLVSKEPIISYYTQLVGDAISPPT-LKAAANFEGMAVIDADPYDENGNNWNTQNNF-FRQVRNFVIDLTAMPFEVGSIGHWQAQATSL-QNIVFNMRDGGDDNRQGGIFMDNGSGGLMVD-LVFNNGRYGAFFGNQQTTRNLTFNNCKTAIF-MNNWNAWTFQDVKINNCEVGDMSNGGPDGQT-VGSVLLLDSHITNTGIGIKTAYDPAQPHNTGT-LILDNVEMTGTPIAVQNDATGTTIVDGNQKIA-FFAQGRITYGSGIGTSGKAVQTTEQAIKPNV-LDPATGKVFTRSRPQYEDVPVSFVSVKANG-AKGDGVTDDTDAIQAFDSVTPEQIVYFDHGA-YIITKTVRVPPNIRITGEALPLILAGDSSFFK-DQANPKPVFQVQGPGERGRVMSDLIFGTAGP-QPGAIMEWNVAGMEPGAAGLWDVHTRIGGYA-GTQLELEQCAKNPNTNPIKPECFGSFLMLHV-TPGGSAYLENTWYVADHALEPEARDQQIDVF-NGRVLIEGDGPVWGWGTSSEHSLHNYQFNN-ARNVFLALIQTTETPYFQGNPDATQFFTVPNF-ADPDFATSCNTSPNPEQCKRAWGVRAINSTDV-FIYGAGLYSFFDNYDQECCLKTQSCQTNMVSLE-GNSQVHLFGLSTKASVNMLTVDGNAVALDADN-RNNFCATVAWFQSTA

† The signal peptide is underlined. The mature protein contains 762 amino acids.

buffer and purified using ion-exchange chromatography on DEAE Sepharose (1 × 20 cm). The enzyme was eluted with 0.3 M NaCl in 50 mM Tris-HCl pH 8.0. SDS-PAGE analysis revealed a single band corresponding to CtGluN (Fig. 1).

2.2. Crystallization

The purified enzyme was concentrated using Amicon filters (10 000 Da cutoff) and the buffer was exchanged to 10 mM HEPES-NaOH pH 7.0. Initial crystallization screening was carried out with the Index screen (Hampton Research) at 289 K in low-profile 96-well Greiner CrystalQuick crystallization plates using 0.75 μ l protein solution (12 mg ml⁻¹) mixed with an equal amount of crystallization condition. The drops were equilibrated against 75 μ l well solution. No crystals were found during regular inspections of the plate for a few weeks. Small crystals (<0.1 mm in each dimension) were first spotted in condition No. 18 (sodium phosphate monobasic monohydrate/potassium phosphate dibasic pH 6.9) during inspection of an old crystallization plate 18 months after the screen had been set up.

2.2.1. *In situ* crystal characterization. Initial characterization of the crystals produced in condition No. 18 of the Index screen was carried out using the *in situ* diffraction setup on station I04-1 at the Diamond Light Source (DLS), England. Each of the crystals grown in the drop was exposed to the synchrotron X-ray beam (Fig. 2) and diffraction was recorded on a Pilatus 2M detector. The arm of the robot was used as the

Figure 1

SDS-PAGE analysis of purified recombinant CtGluN. Left lane, molecular-weight marker (labelled in kDa); middle lane, purified recombinant CtGluN after ammonium sulfate precipitation; right lane, purified recombinant CtGluN after ion-exchange chromatography.

goniometer and 50 images were collected from each crystal with 0.1 s exposure per image. The total rotation range was 5°. Of the ten crystals present in the drop, only three gave processable data. The best resolution was 1.84 Å.

2.2.2. Crystal optimization and seeding. Attempts to reproduce the crystals were carried out with the hanging-drop vapour-diffusion method using 0.8 ml reservoir solution and

Figure 2
A crystal used for *in situ* characterization. The circle depicts the size and the centre of the X-ray beam. The scale is shown on the right.

(a)

(b)

Figure 3
(a) Irregularly shaped crystals of CtGluN grown using 2.0 M sodium/potassium phosphate pH 6.8. (b) Crystals of CtGluN grown using 1.8 M sodium/potassium phosphate pH 6.8 after seeding. The typical dimensions of these crystals were 0.3 × 0.1 × 0.1 mm.

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion, seeding
Plate type	24-well Linbro
Temperature (K)	289
Protein concentration (mg ml ⁻¹)	12
Buffer composition of protein solution	10 mM HEPES–NaOH pH 7.0
Composition of reservoir solution	1.8 M sodium/potassium phosphate pH 6.8
Volume and ratio of drop	4 µl (1:1 ratio of protein:reservoir solution)
Volume of reservoir (µl)	800

drops consisting of 2 µl protein solution mixed with an equal volume of reservoir solution. Irregularly shaped crystals were grown using 2.0 M sodium/potassium phosphate pH 6.8 (Fig. 3a). However, lower concentrations of sodium/potassium phosphate did not produce any crystals. Seeding was therefore employed in an effort to crystallize the enzyme. One of the irregularly shaped crystals was slightly scratched at one end with a needle to generate microseeds in the drop. A small aliquot of the drop (0.2 µl) was taken and diluted with 50 µl 2.0 M sodium/potassium phosphate pH 6.8. From this dilution, 0.2 µl was taken to seed crystallization drops through serial dilution by transferring 0.2 µl from a seeded drop to the next unseeded drop. Four drops in a row were typically used, resulting in a ~10 000-fold final dilution of the initial seeding solution. The crystallization drops were seeded after 1 d of equilibration against a well solution (0.8 ml) consisting of 1.8 M sodium/potassium phosphate pH 6.8. Crystals appeared in the seeded drops after 2 d and continued to grow for about 10 d (Fig. 3b). Crystallization information is summarized in Table 2.

2.3. Data collection and processing

Following crystal optimization as described above, data were collected at room temperature (293 K) to 2.0 Å resolution from a single crystal mounted in a quartz capillary (Table 3). X-rays were produced using an in-house Rigaku MicroMax-007 generator and the diffraction images were recorded on a MAR345 imaging-plate detector. The data were processed with *XDS* (Kabsch, 2010).

3. Results and discussion

3.1. Purification of recombinant CtGluN

A β-1,3-glucanase from *C. thermophilum* was successfully expressed in *P. pastoris* and purified to homogeneity using ammonium sulfate precipitation and ion-exchange chromatography. SDS–PAGE revealed a single band close to the molecular weight of 82.7 kDa calculated according to the gene sequence. The purified enzyme was active as judged by an activity assay.

3.2. Crystallization

Initial crystallization gave one promising condition, which was discovered after a period of 18 months. The appearance of crystals after a long time usually implies either some changes

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Wavelength (Å)	1.5418
X-ray source	MicroMax-007
Temperature (K)	293
Crystal-to-detector distance (mm)	174.8
Detector	MAR345
Rotation range per image (°)	1
Total rotation range (°)	140
Exposure time per image (s)	300
Space group	$P2_1$
a, b, c (Å)	64.1, 85.8, 68.5
α, β, γ (°)	90, 93.1, 90
Mosaicity (°)	0.3
Resolution range (Å)	50–2.0 (2.1–2.0)
Total No. of reflections	214907 (17480)
No. of unique reflections	49315 (5990)
Completeness (%)	98.3 (88.3)
Multiplicity	4.3 (2.9)
$\langle I/\sigma(I) \rangle$	10.4 (2.6)
$R_{\text{meas}}^\dagger$ (%)	13.8 (74.8)
$CC_{1/2}^\ddagger$	99.1 (72.4)
Overall B factor from Wilson plot (Å ²)	12.9

[†] Diederichs & Karplus (1997). [‡] Karplus & Diederichs (2012).

to the protein (for example proteolysis) or difficulties in nucleation. Owing to the small size of the crystals and the uncertainty in reproducing them, initial characterization for diffraction was carried out using *in situ* diffraction. The crystals diffracted to ~ 1.8 Å resolution, but a complete data set was not collected owing to radiation damage and the fact that only three of the ten crystals present in the drop were successfully autoindexed. The crystals belonged to a monoclinic space group, with unit-cell parameters $a = 65.0$, $b = 86.6$, $c = 69.3$ Å, $\beta = 93.1^\circ$. Using fresh protein, several crystallization drops were set up to reproduce the crystals. In the end, a seeding approach was adopted to assist the nucleation. The resultant crystals diffracted to 2.0 Å resolution using an in-house X-ray generator and a full data set was collected from a single crystal at room temperature without the need for cryoprotection. The crystals belonged to space group $P2_1$, with unit-cell parameters $a = 64.1$, $b = 85.8$, $c = 68.5$ Å, $\beta = 93.1^\circ$, similar to those characterized in the *in situ* diffraction. Assuming the presence of one molecule in the asymmetric unit, the Matthews coefficient V_M (Matthews, 1968) is 2.32 Å³ Da^{−1}, corresponding to a solvent content of $\sim 47.0\%$. Structure determination is currently under way using the structure of a β -1,3-glucanase from the basidiomycete *Phanerochaete chrysosporium* (PDB entry 3eqn; Ishida *et al.*, 2009; sequence identity $\sim 49\%$) as the template in molecular-

replacement efforts. The use of synchrotron-radiation sources and crystal cryoprotection are planned in order to collect higher resolution diffraction data. The elucidation of the CrGluN crystal structure in the presence and absence of substrates (e.g. laminarioligosaccharides) combined with site-directed mutagenesis studies will provide insights into the glycoside hydrolases of family 55 and a better understanding of their stabilities and catalytic mechanism for potential use in biotechnology.

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