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Purification, crystallization and preliminary crystallographic analysis of the catalytic domain of the extracellular cellulase CBHI from *Trichoderma harzianum*

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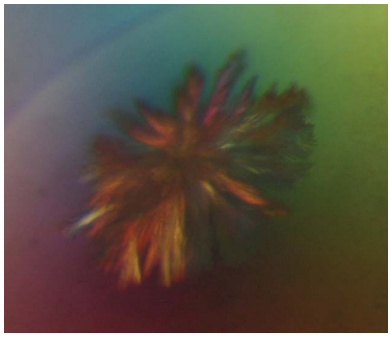
The filamentous fungus *Trichoderma harzianum* has a considerable cellulolytic activity that is mediated by a complex of enzymes which are essential for the hydrolysis of microcrystalline cellulose. These enzymes were produced by the induction of *T. harzianum* with microcrystalline cellulose (Avicel) under submerged fermentation in a bioreactor. The catalytic core domain (CCD) of cellobiohydrolase I (CBHI) was purified from the extracellular extracts and submitted to robotic crystallization. Diffraction-quality CBHI CCD crystals were grown and an X-ray diffraction data set was collected under cryogenic conditions using a synchrotron-radiation source.

1. Introduction

A variety of microorganisms degrade cellulose in nature. The enzymatic process that is involved in the hydrolysis of cellulosic materials has been studied in depth for several filamentous fungi, such as, for example, *Trichoderma reesei*. It is widely accepted that this hydrolytic process is accomplished by several cellulases through a series of reactions that are mediated by exoglucanases (cellobiohydrolases I and II), endoglucanases and β-glucosidases (Kubicek *et al.*, 1993). These enzymes are of considerable biotechnological interest, while the reaction conditions and the production costs of these enzymes significantly influence their range of application (Zhou *et al.*, 2008). Cellulases have been used in waste recycling and in the processing of cellulose-rich raw materials in the food, detergent, paper and textile industries (Bhat, 2000). Recently, the application of cellulases that have potential in the production of biofuels has gained significant importance.

Of the cellulases secreted by *T. reesei*, cellobiohydrolases (CBHs) are the most abundant enzymes, comprising roughly 60% of the cellulases produced by this fungus. These are key enzymes for the degradation of crystalline cellulose and the absence of CBHs reduces the rate of cellulose hydrolysis by as much as 70% (Lynd *et al.*, 2002). In general, the isoforms CBHI and CBHII act on the reducing and nonreducing ends of the cellulose chain, respectively, cleaving the internal glycoside bonds to release the disaccharide cellobiose (Hui *et al.*, 2002). These enzymes are known to be glycosylated proteins with acidic pIs (Medve *et al.*, 1998). Structurally, CBHI is a two-domain enzyme with a tertiary structure arrangement that is characteristic of glycosyl hydrolases (GHs) of family 7 (Coutinho & Henrissat, 1999). CBHs consist of a carbohydrate-binding module (CBM) and a catalytic core domain (CCD) separated by a heavily glycosylated peptide linker that is rich in prolines, serines and threonines (Gruno *et al.*, 2004). Cellulose-binding modules (CBMs) are found in most cellulases (Linder & Teeri, 1996) and play an important role in the interaction of GHs with the insoluble cellulose (Palonen *et al.*, 1999), which is crucial for the initiation and processivity of exoglucanases (Teeri *et al.*, 1998). The activity of the enzyme towards insoluble polymeric substrate in the absence of the CBM is frequently considerably lower than that of the full-length protein (Mattinen *et al.*, 1997). Structural studies have demonstrated that the active site of the CBHI from *T. reesei* is centrally located and is formed mainly by a



large antiparallel β-sandwich followed by a 50 Å long cellulose-binding tunnel composed of four surface loops. As the cellulose chain passes through the tunnel the hydrolytic product cellobiose is formed (Divne *et al.*, 1994).

T. harzianum is a filamentous fungus that secrets a cellulolytic complex that is able to efficiently hydrolyze a range of substrates to their reducing sugars (Saddler *et al.*, 1985). With respect to the three major groups of cellulases, *T. harzianum* displays well balanced β-glucosidase, endoglucanase and exoglucanase activities, efficiently hydrolyzing cellulolytic substrates into monomeric glucose (de Castro *et al.*, 2010). Sequencing of the gene for *T. harzianum* exoglucanase type I (CBHI) has shown that the enzyme is closely related to *T. reesei* CBHI (81% sequence identity; Guilfoile *et al.*, 1999). To date, little is known about the three-dimensional structure of CBHI from *T. harzianum*. In order to structurally characterize orthologues of *T. reesei* CBHI and to better understand possible differences in the mechanisms of action of cellobiohydrolases, we have purified and crystallized the catalytic domain of CBHI from *T. harzianum* for X-ray diffraction analysis.

2. Experimental methods

2.1. Protein production

The enzymes of the cellulolytic complex were produced by submerged fermentation of *T. harzianum* in a 7.5 l BioFlo 110 bioreactor (New Brunswick Scientific) with an operating volume of 5 l. The culture was maintained under 50% oxygen saturation by aeration and agitation at a temperature of 298 K for 120 h. The fermentation medium was prepared as described previously (Mandels *et al.*, 1976), with microcrystalline cellulose (Avicel) as the carbon source to induce the expression of cellulases.

2.2. Protein isolation and purification

Full-length CBHI (amino acids 1–505, with a theoretical molecular weight of 53 kDa) was purified from a 5 l culture of *T. harzianum*. On the fifth day the culture was harvested for 20 min at 8000*g* and 277 K and the clarified extract was submitted to filtration and concentration using hollow-fibre technology. The extract was then applied onto a Q-Sepharose (GE Healthcare) column equilibrated with 50 m*M* Tris buffer pH 7.0 and eluted with a linear gradient of 0–1 *M* sodium chloride in the same buffer. The CBHI-containing fractions were concentrated with a Vivaspin concentrator and further purified by hydrophobic interaction chromatography on a Phenyl Sepharose column (GE Healthcare) equilibrated with 50 m*M* Tris buffer pH 7.0 and eluted with a linear gradient of 0–1 *M* ammonium sulfate in the same buffer. The protein fractions were monitored for exoglucanase activity using 1%(*w*/*v*) Avicel in 50 m*M* sodium citrate pH 5.0 as a substrate (Ghose, 1987) and the 3,5-dinitrosalicylic acid (DNS) method for quantification of the produced reducing sugars (Tomme *et al.*, 1988). Finally, the fractions containing CBHI activity were pooled and concentrated. The Avicelase activity of pure CBHI was determined according to the procedures of the IUPAC Commission on Biotechnology (Ghose, 1987) using cellobiose as a standard curve.

As the next step, the CBHI was submitted to photolytic cleavage by the addition of papain at a protein:enzyme ratio of 50:1(*w*:*w*). The reaction was carried out in 40 m*M* potassium phosphate buffer pH 6.0 at room temperature for 30 min. Size-exclusion chromatography was applied for further purification of the catalytic core domain using a Superdex 75 10/30 column (GE Healthcare) equilibrated with 50 m*M* Tris–HCl pH 7.0 and 300 m*M* sodium chloride. The eluted protein was dialyzed overnight against 50 m*M* Tris–HCl pH 7.0 and concentrated prior to crystallization. Protein quantification was carried out by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.3. Crystallization

The purified catalytic core domain of CBHI (46 kDa; amino acids 18–449) at a concentration of 8 mg ml^{-1} was submitted to crystallization screening using the sitting-drop vapour-diffusion technique. Drops of 2 µl final volume (1:1 ratio of protein and screen solutions) were set up automatically with a Honeybee 931 crystallization robot (Genomic Solutions Inc.) using a variety of commercially available screens (Qiagen) and maintained at a temperature of 291 K. Needles grew using 35% PEG 4000 as a precipitant. Crystal-growth optimization trials were carried out in both hanging-drop and sitting-drop plates (24-well Linbro plates).

2.4. Data collection and processing

The cluster of needle-like CBHI CCD crystals was manipulated using an acupuncture needle and transferred to a cryosolution containing a mixture of 35% PEG 4000 and 20% PEG 400. One single needle-like protein crystal was mounted in a cryoloop and directly flash-frozen in a nitrogen stream prior to X-ray data analysis. The diffraction data were collected on beamline MX2 at the Synchrotron Light Source Laboratory (LNLS), Campinas, Brazil using a MAR charge-coupled device detector (Guimarães *et al.*, 2009). Data covering 180° were collected using the oscillation method at a wavelength of 1.46 Å. Data integration was carried out using the program *XDS* (Kabsch, 2010) and data were scaled with the program *SCALA* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

3.1. Enzyme purification and crystallization

As expected, CBHI comprised the majority of the protein that was produced in the extracellular extract of *T. harzianum* (Fig. 1, lane 1). Highly pure full-length CBHI could be obtained after the second purification step *via* hydrophobic interaction chromatography (Fig. 1, lane 3). Its enzymatic activity was determined to be 0.3 U mg^{-1} on Avicel substrate at pH 5.0 and 323 K. This value is at the upper limit or just slightly above the reported Avicelase activity values obtained for *T. reesei* CBHI, which range from 0.014 to 0.26 U mg^{-1} depending

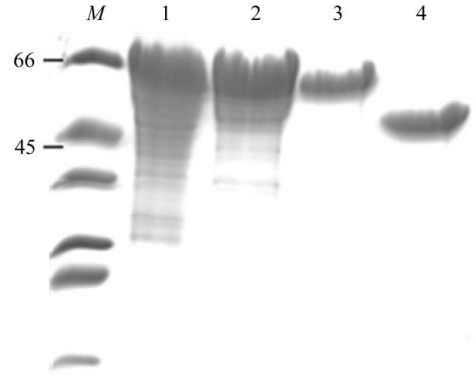


Figure 1
SDS–PAGE gel. Lane 1, supernatant of *T. harzianum* culture broth; lane 2, full-length CBH1 eluted from the anion-exchange column; lane 3, full-length CBH1 eluted from the hydrophobic interaction column; lane 4, CBHI CCD after cleavage with papain and size-exclusion chromatography purification. Lane *M* contains protein markers (labelled in kDa).

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Beamline	MX2, LNLS
Wavelength (Å)	1.46
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 56.9, b = 69.3, c = 90.7
Resolution range (Å)	55.1–2.9 (3.0–2.9)
No. of unique reflections	8493 (1176)
Mosaicity (°)	0.5
Redundancy	7.0 (6.7)
Completeness (%)	99.6 (99.6)
$R_{\rm merge}$† (%)	11.9 (29.5)
$\langle I/\sigma(I)\rangle$	6.5 (2.8)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity of an individual reflection and $\langle I(hkl)\rangle$ is the average intensity of that reflection.

on the initial substrate concentration and the experimental conditions (Tomme *et al.*, 1988; Elgogary *et al.*, 1989; Vehmaanpera *et al.*, 2009). Following protein cleavage with papain, the CCD of CBHI was successfully separated from the CBM by size-exclusion chromatography (Fig. 1, lane 4). According to the size-exclusion chromatography elution volume and SDS–PAGE, the CBHI CCD exhibits a molecular weight of about 50 kDa, which is close to the sequence-based theoretical molecular weight of the CBHI CCD (46 kDa).

The CBHI CCD was crystallized using a crystallization robot and several commercial crystallizations kits covering more than 650 different conditions. Crystals only grew in only one condition, which contained 35% PEG 4000 as a precipitant. Small needles appeared very rapidly using this crystallization condition, so that the needle cluster was completely formed after 10 h of incubation time (Fig. 2). Various attempts were carried out to attempt to reduce the number of nuclei being formed and to favour single-crystal growth. Variations in the pH and the protein and precipitant concentrations and tests of different additives were ineffective, resulting exclusively in a shower of microcrystals or clusters of needles. Needles grown in clusters were tested for X-ray diffraction quality. Surprisingly, a small single needle-like crystal of approximate dimensions 10 × 5 × 5 µm showed a diffraction pattern that extended to a resolution of about 2.9 Å (Fig. 3). A complete native data set was collected on the dedicated wiggler beamline MX2 (LNLS, Brazil; Guimarães *et al.*, 2009). The data set was reduced in the orthorhombic space group *P*222, with unit-cell parameters a = 56.9, b = 69.3, c = 90.7 Å. Analysis of the systematic absences revealed the presence of twofold screw symmetry along all three unit-cell axes. According to the Matthews coefficient of 1.77 Å^3 Da^{-1} (Matthews, 1968), there is only one molecule in the asymmetric unit and the solvent content of the crystal is 31%. Data-collection and processing statistics are shown in Table 1.

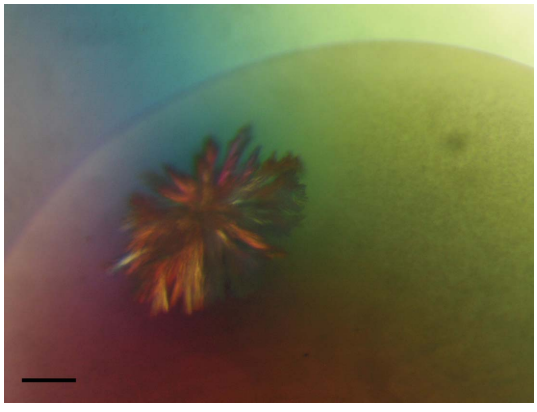


Figure 2
Crystals of CBHI CCD grew as clusters in hanging drops using 35%(*w*/*v*) PEG 4000 as precipitant. The black bar corresponds to 10 µm.

4. Conclusions

The cellulase CBHI was successfully purified from the soluble extracellular fraction of *T. harzianum* cultured in a bioreactor. The catalytic core domain of the enzyme was submitted to crystallization and diffraction-quality crystals were obtained. An X-ray diffraction data set from a single needle-like CBHI catalytic core domain crystal was collected using a synchrotron source.

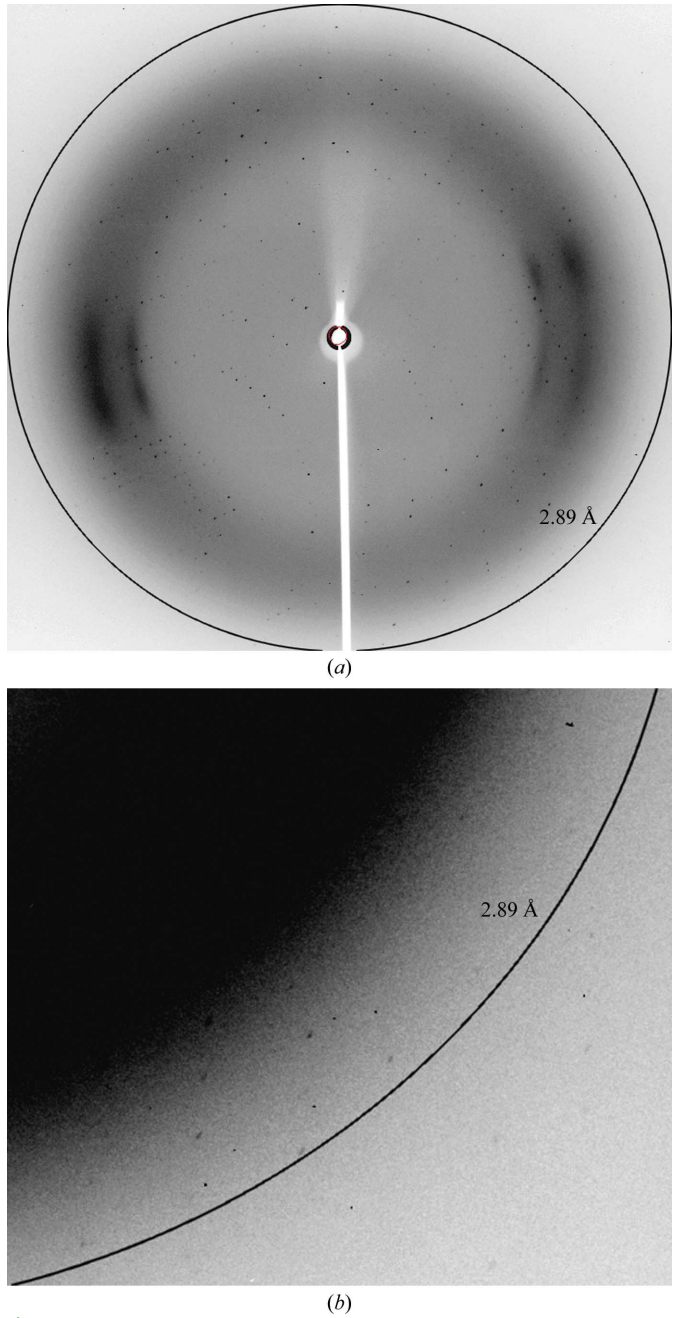


Figure 3
(*a*) Diffraction pattern collected from one single needle-shaped crystal extending to a resolution of 2.9 Å. (*b*) Close-up view of the diffraction image with enhanced contrast.

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References

Bhat, M. K. (2000). *Biotechnol. Adv.* **18**, 355–383.
Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Castro, A. M. de, Pedro, K. C., Cruz, J. C., Ferreira, M. C., Leite, S. G. & Pereira, N. Jr (2010). *Appl. Biochem. Biotechnol.* doi:10.1007/s12010-010-8986-0.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Coutinho, P. M. & Henrissat, B. (1999). *Recent Advances in Carbohydrate Bioengineering*, edited by H. J. Gilbert, G. J. Davies, B. Henrissat & B. Svensson, pp. 3–12. Cambridge: Royal Society of Chemistry.
Divne, C., Stahlberg, J., Reinikainen, T., Ruohonen, L., Pettersson, G., Knowles, J. K. C., Teeri, T. T. & Jones, T. A. (1994). *Science*, **265**, 524–528.
Elgogary, S., Leite, A., Crivellaro, O., Eveleigh, D. E. & Eldorry, H. (1989). *Proc. Natl Acad. Sci. USA*, **86**, 6138–6141.
Ghose, T. K. (1987). *Pure Appl. Chem.* **59**, 257–268.
Gruno, M., Valjamae, P., Pettersson, G. & Johansson, G. (2004). *Biotechnol. Bioeng.* **86**, 503–511.
Guilfoile, P., Burns, R. G., Zu-Yi, Amundson, M. & Chang, F.-H. (1999). *J. Minn. Acad. Sci.* **64**, 18–22.
Guimarães, B. G., Sanfelici, L., Neuenschwander, R. T., Rodrigues, F., Grizolli, W. C., Raulik, M. A., Piton, J. R., Meyer, B. C., Nascimento, A. S. & Polikarpov, I. (2009). *J. Synchrotron Rad.* **16**, 69–75.
Hui, J. P. M., White, T. C. & Thibault, P. (2002). *Glycobiology*, **12**, 837–849.
Kabsch, W. (2010). *Acta Cryst.* D**66**, 125–132.
Kubicek, C. P., Messner, R., Gruber, F., Mach, R. L. & Kubicekpranz, E. M. (1993). *Enzyme Microb. Technol.* **15**, 90–99.
Linder, M. & Teeri, T. T. (1996). *Proc. Natl Acad. Sci. USA*, **93**, 12251–12255.
Lynd, L. R., Weimer, P. J., van Zyl, W. H. & Pretorius, I. S. (2002). *Microbiol. Mol. Biol. Rev.* **66**, 506–577.
Mandels, M., Andreotti, R. & Roche, C. (1976). *Biotechnol. Bioeng. Symp.* **6**, 21–33.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mattinen, M. L., Kontteli, M., Kerovuo, J., Linder, M., Annila, A., Lindeberg, G., Reinikainen, T. & Drakenberg, T. (1997). *Protein Sci.* **6**, 294–303.
Medve, J., Lee, D. & Tjerneld, F. (1998). *J. Chromatogr. A*, **808**, 153–165.
Palonen, H., Tenkanen, M. & Linder, M. (1999). *Appl. Environ. Microbiol.* **65**, 5229–5233.
Saddler, J. N., Hogan, C. M. & Louisseize, G. (1985). *Appl. Environ. Microbiol.* **22**, 139–145.
Teeri, T. T., Koivula, A., Linder, M., Wohlfahrt, G., Divne, C. & Jones, T. A. (1998). *Biochem. Soc. Trans.* **26**, 173–178.
Tomme, P., Vantilbeurgh, H., Pettersson, G., Vandamme, J., Vandekerckhove, J., Knowles, J., Teeri, T. & Claeyssens, M. (1988). *Eur. J. Biochem.* **170**, 575–581.
Vehmaanpera, J., Alapuranen, M., Puranen, T., Siika-aho, M., Kallio, J., Hooman, S., Voutilainen, S., Halonen, T. & Viikari, L. (2009). US Patent 20090042266.
Zhou, J., Wang, Y.-H., Chu, J., Zhuang, Y.-P., Zhang, S.-L. & Yin, P. (2008). *Bioresour. Technol.* **99**, 6826–6833.