Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray crystallographic studies of an exo-β-D-glucosaminidase from *Trichoderma reesei*

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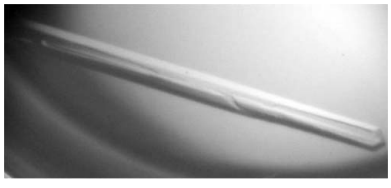
Received 29 August 2009
Accepted 6 January 2010



Chitosan is degraded to glucosamine (GlcN) by chitosanase and exo-β-D-glucosaminidase (GlcNase). GlcNase from *Trichoderma reesei* (Gls93) is a 93 kDa extracellular protein composed of 892 amino acids. The enzyme liberates GlcN from the nonreducing end of the chitosan chain in an exo-type manner and belongs to glycoside hydrolase family 2. For crystallographic investigations, Gls93 was overexpressed in *Pichia pastoris* cells. The recombinant Gls93 had two molecular forms of ∼105 kDa (Gls93-F1) and ∼100 kDa (Gls93-F2), with the difference between them being caused by N-glycosylation. Both forms were crystallized by the hanging-drop vapour-diffusion method. Crystals of Gls93-F1 belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 98.27$, $b = 98.42$, $c = 108.28$ Å, and diffracted to 1.8 Å resolution. Crystals of Gls93-F2 belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 67.84$, $b = 81.62$, $c = 183.14$ Å, and diffracted to 2.4 Å resolution. Both crystal forms were suitable for X-ray structure analysis at high resolution.

1. Introduction

Chitin and chitosan consist of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN), respectively. Chitin is found in the exoskeletons of arthropods, the shells of crustaceans, the cell walls of many fungi and algae and in nematodes and is degraded to GlcNAc by chitinase (EC 3.2.1.14) and *N*-acetylhexosaminidase (EC 3.2.1.52). Chitosan is found in the cell walls of a limited number of fungi and is degraded to GlcN by chitosanase (EC 3.2.1.132) and exo-β-D-glucosaminidase (EC 3.2.1.165; GlcNase).

Trichoderma reesei (*Hypocrea jecorina*) is a mesophilic soft-rot ascomycete fungus that is the main industrial source of the cellulases and hemicellulases used to depolymerize biomass to simple sugars, which are converted to chemical intermediates and biofuels such as ethanol. We have shown that one of the three chitosan 10B-degrading enzyme activities in the culture filtrate of *T. reesei* PC-3-7 grown on GlcNAc was a GlcNase (Gls93), which liberates GlcN from the non-reducing end of the chitosan chain in an exo-type manner (Nogawa *et al.*, 1998). In a previous paper (Ike *et al.*, 2006), we reported the primary structure of Gls93 (GenPept accession No. BAD99604) and identified that this enzyme belonged to a new subgroup of glycoside hydrolase family 2 (GHF2).

The phylogenetic tree of GHF2 is clearly divided into four subgroups: the β-galactosidase, β-glucuronidase, β-mannnosidase and GlcNase subgroups (Ike *et al.*, 2006). Crystal structures of GHF2 enzymes have been determined for *Escherichia coli* β-galactosidase (LacZ; Jacobson *et al.*, 1994; Juers *et al.*, 2001), human β-glucuronidase (GusB; Jain *et al.*, 1996) and *Bacteroides thetaiotaomicron* β-mannnosidase (BtMan2A; Tailford *et al.*, 2007, 2008). Recently, the crystal structure of a bacterial GHF2 GlcNase, *Amycolatopsis orientalis* GlcNase (CsxA), has been reported (van Bueren *et al.*, 2009). However, the crystal structure of a eukaryotic GHF2 GlcNase has not yet been reported. In order to provide structure–function relationship data for the GlcNase subgroup of GHF2, we initiated crystallographic studies of Gls93. Here, we report the crystallization and preliminary X-ray crystallographic studies of the eukaryotic GHF2 GlcNase Gls93.

2. Materials and methods

2.1. Expression and purification

The expression and purification of Gls93 were performed as described previously (Ike *et al.*, 2006). Briefly, a *Pichia pastoris* transformant possessing the full-length *gls93* gene (GenBank accession No. AB218755) under control of the AOX1 promoter was used for the production of recombinant Gls93. Recombinant Gls93 in the methanol-induced culture filtrate was precipitated by the addition of ammonium sulfate (70% saturation). After an initial separation using Q-Sepharose (Pharmacia) column chromatography, Gls93-containing fractions were applied onto a Phenyl Sepharose (Pharmacia) column equilibrated with buffer containing a 30% saturation of ammonium sulfate. After washing, the bound proteins were eluted with a linear gradient of buffer containing a 30–0% saturation of ammonium sulfate. The GlcNase activity of the eluate was separated into two peaks, a non-adsorbed fraction and a gradient fraction, which were named Gls93-F1 and Gls93-F2, respectively. The two peaks were separately pooled, concentrated and purified by gel filtration using Sephacryl S-100 (Pharmacia).

2.2. Crystallization

The purified and lyophilized Gls93s (Gls93-F1 and Gls93-F2) were dissolved in pure water to a concentration of 4.5 mg ml^{-1}. Initial sparse-matrix crystal screening (Jancarik & Kim, 1991) was conducted using Crystal Screen I (Hampton Research, USA), Wizard I, II and III, Ozma PEG 4K and 8K and Cryo I and II (Emerald BioSystems, USA). Crystallization was carried out by the hanging-drop vapour-diffusion method, in which 1 µl protein solution was mixed with the same volume of crystallization buffer and incubated at 293 K. The drops were suspended over 200 µl reservoir solution in 48-well plates.

2.3. X-ray data collection

For data collection under cryogenic conditions, the crystals in a droplet were directly transferred to harvesting solution [20%(*v*/*v*) glycerol in reservoir solution] for 30 s. Crystals were then mounted in nylon loops and flash-cooled in a cold nitrogen-gas stream at 100 K just prior to data collection. Data collections were performed by the rotation method at 100 K using an ADSC Q210 CCD detector with synchrotron radiation [λ = 1.000 Å on beamline NW12A of the Photon Factory Advanced Ring (PF-AR), Tsukuba, Japan] or using an ADSC Q270 CCD detector with synchrotron radiation [λ = 1.000 Å on beamline BL17A of the Photon Factory (PF), Tsukuba, Japan]. The Laue group and unit-cell parameters were determined using the *HKL*-2000 package (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Expression, purification and crystallization

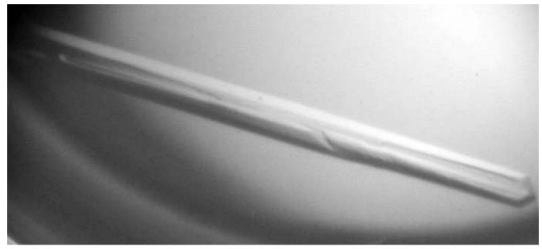
Both of the recombinant Gls93s (Gls93-F1 and Gls93-F2) were successfully expressed and purified to homogeneity. SDS–PAGE of the purified enzymes revealed a single 105 kDa protein band for Gls93-F1 and a single 100 kDa protein band for Gls93-F2 on Coomassie Brilliant Blue staining. It has been reported that the molecular masses of both Gls93-F1 and Gls93-F2 are reduced to 97 kDa by treatment with glycopeptidase F (Ike *et al.*, 2006). This indicates that the discrepancies in molecular mass between Gls93-F1 and Gls93-F2 may be explained by differences in the N-glycosylation pattern.

Initial crystal screening for Gls93-F1 produced several microcrystals in a week. Rectangular parallelepiped-shaped crystals grew from condition Nos. 18 and 46 of Crystal Screen I [No. 18, 20%(*w*/*v*) PEG 8000 and 0.2 *M* magnesium acetate in 0.1 *M* cacodylate buffer pH 6.5; No. 46, 18%(*w*/*v*) PEG 8000 and 0.2 *M* calcium acetate in cacodylate buffer pH 6.5]. Trials to improve the crystallization conditions were performed by varying the pH, the buffer system and the precipitant concentration. To obtain crystals suitable for X-ray analysis, a droplet was prepared by mixing equal volumes (2 µl + 2 µl) of the working solution described above and reservoir solution [14%(*w*/*v*) PEG 8000 and 0.2 *M* ammonium acetate in 0.1 *M* cacodylate buffer pH 7.0] and was suspended over 500 µl reservoir solution in a 24-well plate. Rectangular parallelepiped-shaped crystals with typical dimensions of approximately 0.05 × 0.05 × 0.5 mm were grown in one month (Fig. 1*a*).

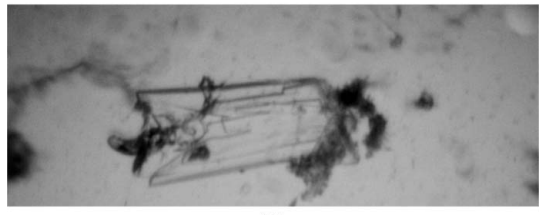
Similarly, Gls93-F2 crystals were initially obtained in condition Nos. 11 and 34 of Ozma PEG 8K [No. 11, 20%(*w*/*v*) PEG 8000 and 0.2 *M* calcium acetate; No. 34, 20%(*w*/*v*) PEG 8000 and 0.2 *M* potassium/sodium tartrate]. Again, several trials were required to improve the initial crystallization conditions. As a result, rectangular parallelepiped-shaped crystals of Gls93-F2 with typical dimensions of approximately 0.15 × 0.05 × 0.5 mm were obtained by mixing equal volumes (2 µl + 2 µl) of the working solution described above and reservoir solution [20%(*w*/*v*) PEG 4000 and 0.2 *M* sodium isothiocyanate] and suspending the droplet over 500 µl reservoir solution in a 24-well plate. Crystals appeared after two weeks (Fig. 1*b*).

3.2. Data collection

The Laue group of both Gls93-F1 and Gls93-F2 crystals was found to be *mmm* and only reflections with $h = 2n$, $k = 2n$ and $l = 2n$ were observed along the [*h*00], [0*k*0] and [00*l*] axes, respectively, indicating the orthorhombic space group $P2_12_12_1$. The unit-cell parameters were $a = 98.27$, $b = 98.42$, $c = 108.28$ Å and $a = 67.84$, $b = 81.62$, $c = 183.14$ Å for the Gls93-F1 and Gls93-F2 crystals, respectively. Assuming the presence of one molecule per crystallographic asymmetric unit led to empirically acceptable V_M values of 2.69 and 2.63 Å^3 Da^{-1}, corresponding to solvent contents of 54.3 and 53.2% (Matthews, 1968), for Gls93-F1 and Gls93-F2, respectively. The current best diffraction data



(*a*)



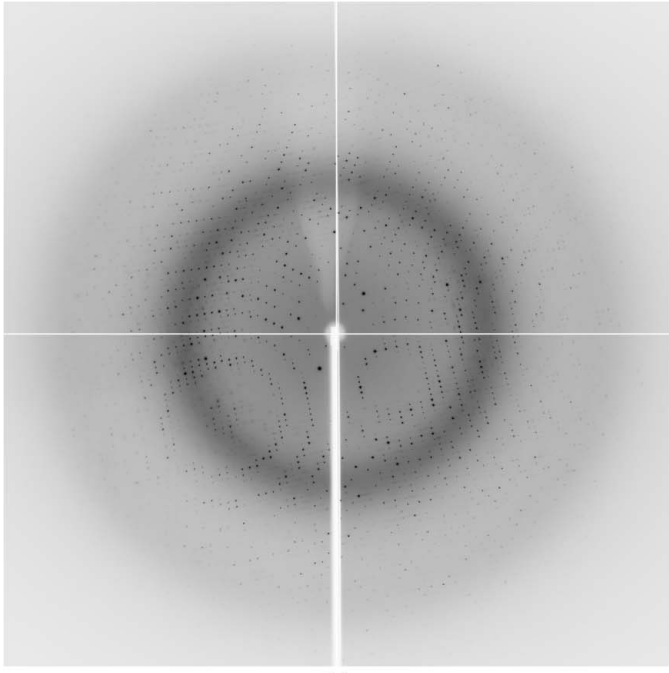
(*b*)

Figure 1
Orthorhombic crystals of Gls93. (*a*) Gls93-F1. (*b*) Gls93-F2.

from Gls93-F1 and Gls93-F2 crystals were collected to 1.8 and 2.4 Å resolution, respectively (Fig. 2). Data-collection statistics are summarized in Table 1.

3.3. Initial phase determination

Initial phase determination for the Gls93-F1 crystal was performed by the molecular-replacement (MR) technique using the coordinate set of a bacterial GHF2 GlcNase, *A. orientalis* CsxA (PDB code 2vzs; van Bueren *et al.*, 2009), which has approximately 37% amino-acid sequence identity to Gls93, as a search model. Bound water molecules were removed from the search model. Cross-rotation and translation functions were calculated using the programs *MrBUMP* (Keegan & Winn, 2008) and *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The results showed a clear solution [correlation coefficient of 0.441 (the first noise solution was 0.254) and *R* factor of 0.544 (the first noise solution was 0.605) in the resolution range 40.0–1.8 Å] and a reasonable molecular arrangement of Gls93-F1 in the asymmetric unit. Automatic model building and refinement using the programs *ARP*/*wARP* (Lamzin & Wilson, 1993) and *REFMAC*5 (Murshudov *et al.*, 1997) and further iterative manual model building and refinement with the programs *Coot* (Emsley & Cowtan, 2004) and *REFMAC*5 are currently in progress.



(*a*)

(*b*)

Figure 2
X-ray diffraction images from (*a*) a Gls93-F1 crystal and (*b*) a Gls93-F2 crystal. The edge of the detector corresponds to a resolution of 1.8 Å in (*a*) and 2.2 Å in (*b*).

Table 1
Data-collection statistics for Gls93.

Values in parentheses are for the outer shell.

	Gls93-F1	Gls93-F2
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 98.27, b = 98.42, c = 108.28	a = 67.84, b = 81.62, c = 183.14
No. of subunits in asymmetric unit	1	1
Solvent content (%)	54.3	53.2
X-ray source	PF-AR NW12A	PF BL17A
Detector	ADSC Q210	ADSC Q270
Wavelength (Å)	1.000	1.000
Resolution (Å)	1.8 (1.9–1.8)	2.4 (2.53–2.4)
No. of observed reflections	562303	352986
No. of unique reflections	97246	40669
Multiplicity	5.8 (5.9)	8.7 (8.8)
Mean $I/\sigma(I)$	19.6 (5.3)	12.6 (2.7)
B factor (Wilson plot) (Å^2)	25.2	43.5
$R_{\rm merge}$† (%)	4.8 (33.7)	5.6 (34.7)
Completeness (%)	99.6 (99.4)	100.0 (100.0)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of $I(hkl)$ and $\langle I(hkl)\rangle$ is the weighted mean of all measurements of $I(hkl)$.

The incompletely refined Gls93-F1 model (R = 0.168 and $R_{\rm free}$ = 0.211 at 1.8 Å resolution) could nevertheless be used for structure determination of the Gls93-F2 crystal by the MR method, which was performed using procedures similar to those described above. The results showed a clear solution [correlation coefficient of 0.712 (the first noise solution was 0.274) and *R* factor of 0.393 (the first noise solution was 0.626) in the resolution range 50–2.6 Å] and a reasonable molecular arrangement of Gls93-F2 in the asymmetric unit. Further model building and refinement at 2.4 Å resolution for the Gls93-F2 crystal are in progress.

We thank Drs Y. Yamada, N. Matsugaki and N. Igarashi of the Photon Factory for their help with data collection at the synchrotron facilities. This work was supported in part by a grant from the Protein 3000 Project (to NT) of the MEXT of Japan.

References

Bueren, A. L. van, Ghinet, M. G., Gregg, K., Fleury, A., Brezinski, R. & Boraston, A. B. (2009). *J. Mol. Biol.* **385**, 131–139.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Emsley, P. & Cowtan, K. (2004). *Acta Cryst.* D**60**, 2126–2132.
Ike, M., Isami, K., Tanabe, Y., Nogawa, M., Ogasawara, W., Okasa, H. & Morikawa, Y. (2006). *Appl. Microbiol. Biotechnol.* **72**, 687–695.
Jacobson, R. H., Zhang, X.-J., DuBose, R. F. & Matthews, B. W. (1994). *Nature (London)*, **369**, 761–766.
Jain, S., Drendel, W. B., Chen, Z., Mathews, F. S., Sly, W. S. & Grubb, J. H. (1996). *Nature Struct. Biol.* **3**, 375–381.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.

Juers, D. H., Heightman, T. D., Vasella, A., McCarter, J. D., Mackenzie, L., Withers, S. G. & Matthews, B. W. (2001). *Biochemistry*, **40**, 14781–14794.
Keegan, R. M. & Winn, M. D. (2008). *Acta Cryst.* D**64**, 119–124.
Lamzin, V. S. & Wilson, K. S. (1993). *Acta Cryst.* D**49**, 129–147.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). *Acta Cryst.* D**53**, 240–255.
Nogawa, M., Takahashi, H., Kashiwagi, A., Ohshima, K., Okada, H. & Morikawa, Y. (1998). *Appl. Environ. Microbiol.* **64**, 890–895.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Tailford, L. E., Money, V. A., Smith, N. L., Dumon, C., Davies, G. J. & Gilbert, H. J. (2007). *J. Biol. Chem.* **282**, 11291–11299.
Tailford, L. E., Offen, W. A., Smith, N. L., Dumon, C., Morland, C., Gratien, J., Heck, M. P., Stick, R. V., Blériot, Y., Vasella, A., Gilbert, H. J. & Davies, G. J. (2008). *Nature Chem. Biol.* **4**, 306–312.
Vagin, A. & Teplyakov, A. (1997). *J. Appl. Cryst.* **30**, 1022–1025.