Characterization and crystal structure of a novel zearalenone hydrolase from *Cladophialophora bantiana*

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Zearalenone (ZEN) is a mycotoxin which causes huge economic losses in the food and animal feed industries. The lactonase ZHD101 from *Clonostachys rosea*, which catalyzes the hydrolytic degradation of ZEN, is the only known ZEN-detoxifying enzyme. Here, a protein homologous to ZHD101, denoted CbZHD, from *Cladophialophora bantiana* was expressed and characterized. Sequence alignment indicates that CbZHD possesses the same catalytic triad and ZEN-interacting residues as found in ZHD101. CbZHD exhibits optimal enzyme activity at 35°C and pH 8, and is sensitive to heat treatment. The crystal structure of apo CbZHD was determined to 1.75 Å resolution. The active-site compositions of CbZHD and ZHD101 were analyzed.

1. Introduction

Zearalenone (ZEN) is a secondary metabolite of *Fusarium* species, which are widely detected in 'musty' grains (Marin *et al.*, 2013). ZEN is a resorcylic acid lactone with oestrogenic activity as it can bind to and activate oestrogen receptors. Exposure to ZEN-contaminated food or animal feed leads to disruption of the reproductive and endocrine systems, leading to huge economic losses in domestic animal farming and posing a serious threat to human health (Escrivá *et al.*, 2015; Kowalska *et al.*, 2016). Therefore, the development of detoxifying strategies is an important task in the animal feed and food industries. Physical adsorption and chemical treatment are popular methods for mycotoxin removal at present. However, these methods are nonselective and might reduce the nutritional elements in the feedstock. In comparison, the use of enzymes that specifically transform ZEN into nontoxic substances is an attractive alternative to remove ZEN contamination.

The only known ZEN-degrading enzyme is ZHD101 from the ZEN-detoxifying fungus *Clonostachys rosea*, which can specifically hydrolyze ZEN into a nontoxic alkylresorcinol product (Takahashi-Ando *et al.*, 2002). To obtain insight into the molecular mechanism of ZHD101, we solved the structure of this enzyme in complex with ZEN (Peng *et al.*, 2014). The enzyme displays a novel α/β -hydrolase fold, which consists of a core domain that harbours the catalytic centre and an α -helical cap domain that contains the majority of the substrate-interacting residues. ZEN is found to bind into a

deep cleft between the two domains, adjacent to the catalytic triad Ser102–His242–Glu126. Residues that are essential for substrate binding and enzymatic reaction were identified by mutagenesis experiments. More recently, we solved the structure of a complex of ZHD101 and α -zearalenol (α -ZOL), the hydroxylated derivative of ZEN, which is 92-fold more oestrogenic than ZEN but is less sensitive to ZHD101 hydrolysis (Marin *et al.*, 2011; Shier *et al.*, 2001). Structural analyses indicated that the His242 side chain was pushed aside by the lactone ring of α -ZOL, prohibiting the formation of the catalytic triad. We thus modified Val153 to His to form a hydrogen bond to the hydroxyl group of the α -ZOL lactone ring to elevate the ring and yield a space for His242 to rotate into the correct position for catalytic triad formation. The resulting V153H mutant exhibits a 3.7-fold higher activity compared with the wild-type enzyme and retains the same activity towards ZEN (Xu *et al.*, 2016).

In order to expand our understanding of mycotoxin-detoxifying enzymes and to facilitate further protein engineering, the characterization of further ZEN-degrading

enzymes is essential. Isolating mycotoxin-degrading microorganisms and identifying the detoxifying enzyme is a time-consuming process. Instead, searching gene banks for proteins that are homologous to ZHD101 and which might possess ZEN-hydrolytic activity provides a more effective alternative. We have found a hypothetical protein from *Cladophialophora bantiana* which shares 61% protein sequence identity with ZHD101. It possesses a conserved catalytic triad and several ZEN-interacting residues (Fig. 1). The protein has been confirmed to exhibit ZEN-hydrolytic activity and is denoted CbZHD. The structure of the complex of CbZHD with a mycotoxin will provide important information for further studies regarding the engineering of ZEN-degrading enzymes.

2. Materials and methods

2.1. Cloning and protein preparation

The gene encoding the 265-residue protein CbZHD from *Cladophialophora bantiana* CBS 173.52 (NCBI reference

Figure 1

Sequence alignment of ZHD101 and CbZHD. This figure was produced using *ESPrpt* (Gouet *et al.*, 2003). Catalytic triads and substrate-interacting residues are labelled with pink and blue dots, respectively.

Table 1

Data-collection and refinement statistics for CbZHD.

Values in parentheses are for the highest resolution shell.

Data collection	
Space group	$P2_12_12$
Unit-cell parameters (Å)	$a = 120.86$, $b = 104.40$, $c = 116.83$
Resolution range (Å)	25–1.75 (1.81–1.75)
No. of unique reflections	148988 (14719)
Multiplicity	4.2 (4.1)
Completeness (%)	99.9 (99.9)
Average $I/\sigma(I)$	32.1 (3.3)
$R_{\text{merge}}^\dagger$ (%)	4.8 (48.0)
Refinement	
No. of reflections	141307 (10836)
R_{work} (95% of data)	0.154 (0.237)
R_{free} (5% of data)	0.180 (0.255)
R.m.s.d., bond lengths (Å)	0.011
R.m.s.d., angles (°)	1.48
Dihedral angles	
Most favoured (%)	96.5
Allowed (%)	3.0
Disallowed (%)	0.5
No. of non-H atoms	
Protein	8096
Water	1061
Ligand	32
Ion	4
Average B factor (Å ²)	
Protein	27.42
Water	37.13
Ligand	52.14
Ion	30.77
PDB code	5xwz

$$^\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

sequence XP_016613277.1) was synthesized chemically, amplified by polymerase chain reaction (PCR) with forward primer 5'-GACGACGACAAGATGGCTCCAGAGAGATTGAGATC-3' and reverse primer 5'-GAGGAGAAGCCCGGTTACAGGTACTTTCTGGTGGTCT-3', and then cloned into the pET-46 Ek/LIC vector. The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) cells, which

were grown in LB broth at 37°C until the OD₆₀₀ reached 0.6. The cultures were then induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 16°C for 20 h. The cells were harvested by centrifugation at 5000g and 4°C for 20 min. The cell pellets were then resuspended and disrupted using a French press (Guangzhou JuNeng Biology and Technology Co. Ltd). The cell lysate was clarified by centrifugation at 5000g for 20 min and the resulting supernatant was loaded onto a Ni-NTA column equilibrated with a buffer consisting of 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM imidazole. The His-tagged protein was eluted using an imidazole gradient (20–250 mM). The protein solution was then dialyzed against a buffer consisting of 20 mM Tris-HCl pH 7.5 and was further purified by FPLC using a DEAE column with a 0–500 mM NaCl gradient in 20 mM Tris-HCl pH 7.5. The protein eluted at ~200 mM NaCl. The purified protein was then concentrated to 14 mg ml⁻¹ and stored in 25 mM Tris-HCl pH 7.5, 150 mM NaCl.

2.2. Enzyme-activity measurement

The enzyme activity was analyzed by HPLC and was calculated based on the substrate-degradation method described previously (Peng *et al.*, 2014; Xu *et al.*, 2016) with minor modifications. In brief, a 210 μ l mixture containing 5 μ l enzyme (1 mg ml⁻¹) and substrate (2.5 mg ml⁻¹) in reaction buffer (150 mM NaCl, 25 mM Tris-HCl pH 7.5) was incubated at 30°C for 10 min. The reaction was terminated by adding 50 μ l 1 N HCl and 300 μ l methanol, and 20 μ l of the mixture was filtered and analyzed by HPLC (Agilent 1200) using a Welch Ultimate XB-C15 column. One unit was defined as the amount of enzyme that is required to decompose 1 μ mol of substrate per minute. The optimal pH was determined at 30°C in different buffers in the pH range 6.0–10.6. The optimal temperature was determined at pH 7.5 using temperatures ranging from 25 to 45°C.

Figure 2

The effect of pH/temperature and the thermostability of CbZHD. (a) The ZEN-hydrolytic activity of recombinant CbZHD was measured at various pH values (left) and temperatures (right). The relative activity of each sample is presented as a percentage of the maximal value. (b) CbZHD was treated at various temperatures for 1, 2 or 10 min prior to activity measurement. The relative activity of each sample is presented as a percentage of that of the untreated sample.

Figure 3

Overall structure and catalytic mechanism of CbZHD. (a) The four polypeptide chains in the asymmetric unit of a CbZHD crystal. The upper and lower pairs of monomers form two dimers. (b) The overall structure of CbZHD is shown as a cartoon model. The ZEN was modelled from the structure of the complex of an inactive form of ZHD101 with ZEN (PDB entry 3wzm; Peng *et al.*, 2014) and is shown in stick representation. (c) Superimposition of CbZHD and ZHD101. ZEN from the structure of its complex with inactive ZHD101 was modelled and the residues surrounding ZEN in CbZHD and ZHD101 are shown in green and cyan, respectively. The catalytic triad residues are shown in purple/blue. Dashed lines indicate distances of <2.0 Å. (d) The proposed catalytic mechanism of CbZHD. The attacking water molecule is shown in blue.

2.3. Crystallization, data collection and structure determination

The purified CbZHD protein was first screened for crystallization using the SaltRx Kit (Hampton Research, Laguna Niguel, California, USA) with the sitting-drop vapour-diffusion method in a 24-well plate (Hampton Research). Crystals were grown in reservoir solution No. 35, which consists of 1.4 M sodium malonate, 0.1 M bis-tris propane pH 7.0. All crystals were stored at 22°C for 7 d to reach a suitable size for X-ray diffraction and were cryoprotected using 2.0 M sodium malonate, 0.15 M bis-tris propane pH 7.0, 30% glycerol. An X-ray diffraction data set was collected on beamline BL15A1 at the National Synchrotron Radiation Research Center (NSRRC), Hsinchu, Taiwan. The diffraction images were processed using the *HKL-2000* suite (Otwinowski & Minor, 1997). The crystal structure of wild-type CbZHD was solved by the molecular-replacement method using *Phaser* (McCoy *et al.*, 2007) with ZHD101 as the search model. Further structure refinement was carried out using *REFMAC5* (Murshudov *et al.*, 2011) and *Coot* (Emsley *et al.*, 2010). Prior to refinement, a randomly selected 5% of the reflections were set aside to calculate R_{free} . The data-collection and refinement statistics are summarized in Table 1.

3. Results and discussion

The recombinant CbZHD protein was expressed in *E. coli* and purified. HPLC analysis indicated that the hydrolytic product of CbZHD was the same as that of ZHD101. Based on gel-filtration analysis, CbZHD forms a dimer in solution. CbZHD exhibited optimal activity at 35°C and pH 8 (Fig. 2*a*); the specific activities of the enzyme towards ZEN and α -ZOL were 0.688 and 0.121 U mg⁻¹, respectively. CbZHD was stable at 35°C for at least 10 min, but lost more than 50% of its activity when incubated at 40°C for 2 min (Fig. 2*b*). The enzyme was unstable when incubated at temperatures higher than 45°C.

The CbZHD crystal diffracted to a high resolution of 1.75 Å and belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 120.86$, $b = 104.40$, $c = 116.83$ Å (Table 1). The overall structure was solved by molecular replacement as described above. Four polypeptide chains were found in the asymmetric unit, which form two dimers with the same configuration as that of ZHD101 (Fig. 3*a*). Similar to ZHD101, CbZHD folded into cap and core domains (Fig. 3*b*). A cleft was found between the two domains, which was presumed to accommodate the substrate. By superimposing the structure of CbZHD onto that of ZHD101 complexed with ZEN, the residues surrounding the substrate can be identified (Fig. 3*c*). Although the majority of the active-site residues were identical in both enzymes, some variations are found. In particular, Ser157 and Met160 in CbZHD, which substitute for Met154 and Val158 in ZHD101, respectively, were located at the entry to the substrate-binding pocket. Notably, the distance between the hydrophobic Met160 side chain and the modelled ZHD is

too short to form an interaction (<2.0 Å; Fig. 3*c*). Thus, the substrate-binding pattern of CbZHD might be distinct from that of ZHD101. We are currently working on solving the structures of complexes of CbZHD with various substrates to address this issue. Based on these results, a putative catalytic mechanism for CbZHD that follows the classic serine hydrolyase mechanism is proposed (Fig. 3*d*). Firstly, the nucleophile (Ser105) that was deprotonated by the base (His243) attacks the carbonyl C atom of ZEN to form a covalently bound intermediate. The intermediate collapses back to a carbonyl as the base protonates the first leaving group. The carbonyl C atom of the acyl-enzyme intermediate is then attacked by a water molecule. The newly formed intermediate, which bears a negatively charged O atom, reforms the double bond and the covalent bond between the enzyme and substrate is attacked by the protonated base. As a result, the product at the end of the reaction is released.

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