Crystal structure and mutagenesis analysis of chitinase CrChi1 from the nematophagous fungus *Clonostachys rosea* in complex with the inhibitor caffeine

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Chitinases are a group of enzymes capable of hydrolysing the β-(1,4)-glycosidic bonds of chitin, an essential component of the fungal cell wall, the shells of nematode eggs, and arthropod exoskeletons. Chitinases from pathogenic fungi have been shown to be putative virulence factors, and can play important roles in infecting hosts. However, very limited information is available on the structure of chitinases from nematophagous fungi. Here, we present the 1.8 Å resolution of the first structure of a Family 18 chitinase from this group of fungi, that of *Clonostachys rosea* CrChi1, and the 1.6 Å resolution of CrChi1 in complex with a potent inhibitor, caffeine. Like other Family 18 chitinases, CrChi1 has the DXDXE motif at the end of strand \( b_5 \), with Glu174 as the catalytic residue in the middle of the open end of the \((β/α)\)8 barrel. Two caffeine molecules were shown to bind to CrChi1 in subsites \(-1\) to \(+1\) in the substrate-binding domain. Moreover, site-directed mutagenesis of the amino acid residues forming hydrogen bonds with caffeine molecules suggests that these residues are important for substrate binding and the hydrolytic process. Our results provide a foundation for elucidating the catalytic mechanism of chitinases from nematophagous fungi and for improving the pathogenicity of nematophagous fungi against agricultural pest hosts.

INTRODUCTION

Chitin, a polymer of β-(1,4)-linked N-acetylglucosamine (GlcNAc), is an essential structural component of fungal cell walls, the shells of nematode eggs, and the exoskeletons of arthropods. Family 18 chitinases (CAZY GH 18), which degrade this polymer, play key roles in the life cycles of pathogenic fungi (Lorito et al., 1996). Fungi can produce chitinases throughout their growth cycle, and these enzymes are believed to contribute to morphogenetic and pathogenic processes, including spore germination, hyphal branching and mycoparasitic interaction (Gooday et al., 1992; Kuranda & Robbins, 1991; Seidl et al., 2005). For many pathogenic fungi, their chitinases are important virulence factors and promising antifungal targets.

Structural studies of chitinase–inhibitor complexes have provided crucial information on the modes of binding, the specificity of chitinase inhibitors, and the mechanism of the hydrolysis reaction (Terwisscha van Scheltinga et al., 1995; van Aalten et al., 2001). Several chitinase inhibitors have been identified, including allosamidin (Bortone et al., 2002), the cyclic pentapeptides argifin and argadin (Arai et al., 2000; Omura et al., 2000), and 8-chlorotheophylline, kinetin and acetazolamide (Hurtado-Guerrero & van Aalten, 2007). However, due to their high molecular

Template 1

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The filamentous fungus *Clonostachys rosea* (syn. *Gliocladium roseum*) is a potential biocontrol agent with a worldwide distribution. Many isolates of *C. rosea* have been studied as biocontrol agents against diverse fungal plant pathogens (Sutton *et al.*, 1997; Xue, 2003). Recently, a chitinase gene, *Crchi1*, was cloned from *C. rosea*, and its expression level was found to be strongly upregulated by solubilized components (e.g., chitosan components) of the cell wall from the plant-pathogenic fungus *Rhizoctonia solani* (Gan *et al.*, 2007a). Moreover, three endochitinase-encoding genes, *cr-ech58*, *cr-ech37*, and *cr-ech42* (orthologous to *Crchi1* analysed here) and *cr-ech37*, were characterized from *C. rosea* strain IK726 (Mamarabadi *et al.*, 2008). The endochitinase activity was specifically induced in media containing chitin or *Fusarium culmorum* cell walls as sole carbon source. The expression of *cr-ech42* and *cr-ech37* was induced by *F. culmorum* cell walls and chitin whereas that of *cr-ech58* was not affected (Mamarabadi *et al.*, 2008). *CrChi1* showed a high degree of similarity to LpChi1 in primary amino acid sequence. LpChi1 was identified from the nematophagous fungus *Lecanicillium psalliota* (syn. *Verticillium psalliota*) and it could degrade the eggs of the root-knot nematode *Meloidogyne incognita* (Gan *et al.*, 2007b). These results suggest that chitinase *CrChi1* play a key role during the infection of different hosts by *C. rosea* (Gan *et al.*, 2007a; Li *et al.*, 2006). In this study, we describe, we believe for the first time, the crystal structure of a chitinase, *CrChi1*, from the nematophagous fungus *C. rosea*. Furthermore, site-directed mutagenesis was carried out to define the crystal structure of *CrChi1* in complex with the inhibitor caffeine.

## METHODS

### Crystallization and data collection.
Cloning, expression and purification of chitinase *CrChi1* from *C. rosea* was described in our recent report (Gan *et al.*, 2009). Purified *CrChi1* was concentrated to 20 mg ml⁻¹ in 20 mM Tris/HCl (pH 8.0) using an Amicon centrifugal filter device (Millipore). Crystallization was performed by the hanging-drop vapour-diffusion method at 16 °C. Drops consisting of 0.5 μl protein solution and 0.5 μl reservoir solution were equilibrated against 0.2 ml reservoir solution consisting of 0.2 M ammonium dihydrogen phosphate and 15 % (w/v) PEG3350. Crystals of *CrChi1* complexed with the inhibitor caffeine were obtained by adding 1 μl caffeine (1.0 mM) to the drops which contained the crystals and incubating overnight.

After its formation, the crystal was mounted on a nylon loop and flash-cooled in a stream of nitrogen gas at 100 K using an Oxford Cryosystems cryostream. Diffraction data were collected on a MAR345dbt (MAR Research) image-plate detector at 100 K using a Rigaku MM-007 rotating-anode home X-ray generator operating at 40 kV and 20 mA (λ=1.5418 Å, 0.154128 nm). All intensity data were indexed, integrated and scaled with the HKL2000 package (Otwonowski & Minor, 1997).

### Structure determination and refinement.
The structure of apo-*CrChi1* belonged to the P2₁ space group and was solved by molecular replacement to 1.8 Å employing the crystal structure of a chitinase [Protein Data Bank (PDB) code: 1W9P] from *Aspergillus fumigatus* (Rao *et al.*, 2005a) as an initial search model, using the program Phaser (McCoy *et al.*, 2007). The clear solutions in both the rotation and translation functions indicated the presence of one molecule in the asymmetrical unit, which was also suggested by the Matthews coefficient and solvent content (Matthews, 1968). Residues that differed between *CrChi1* and the search model were manually rebuilt using the program Coot (Emsley & Cowtan, 2004) under the guidance of *E₁₋F₁* and *2Fo−Fc* electron-density maps. After the refinement of the model using simulated annealing, energy minimization, restrained individual B factors and the addition of 502 solvent molecules in CNS (Brünger *et al.*, 1998), the respective working R factor and *R*merge dropped from 0.42 and 0.45 to 0.20 and 0.25 for all data from 50.0 to 1.8 Å. Model refinements were monitored by calculating *Rmerge* based on a subset containing 10% of the total reflections. The crystal structure of *CrChi1* complexed with caffeine was solved by molecular replacement using the apo-*CrChi1* structure as the initial search model in Phaser (McCoy *et al.*, 2007), to 1.6 Å. The same refinement procedure was performed as for the apo-*CrChi1* structure with manually built bound caffeine into a complex structure using unambiguous *E₁₋F₁* and *2Fo−Fc* electron-density maps. Model geometry was verified using the program PROCHECK (Laskowski *et al.*, 1993). Data collection and refinement statistics are detailed in Table 1. All structure figures in this paper were generated by **Pymol** (Delano, 2002), **Molscript** (Esnouf, 1997) and **Raster3D** (Merritt & Murphy, 1994).

### Enzymology and mutagenesis.
Chitinase activity was measured using the fluorogenic substrate 4-methylumbelliferyl β-D-N,N',N'-triacetylcysteinitoside (Sigma) in a standard assay, as by Rao *et al.* (2005a). Briefly, in a final volume of 100 μl, 2 nM enzyme was incubated with 5–25 μM substrate in McIlvain buffer (100 mM citric acid, 200 mM sodium phosphate pH 5.5) containing 0.1 mg BSA ml⁻¹ for 10 min at 37 °C. After the addition of 50 μl 3 M glycine/NaOH (pH 10.3), the fluorescence of the liberated 4-methylumbelliferone was quantified using a SPECTRAMAX GEMINI XS instrument (Molecular Devices), with excitation and emission wavelengths of 360 nm and 455 nm, respectively. Experiments were performed in triplicate. Production of 4-methylumbelliferone was linear with time for the tested incubation period, and less than 10% of the available substrate was hydrolysed.

To determine the action mode of caffeine on *CrChi1*, the inhibitor affinity was first estimated by determining IC₅₀ values in a standard assay, as described above. The Kᵢ values were approximated by
Table 1. Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Crystallographic data</th>
<th>Apoenzyme</th>
<th>Complexed with caffeine</th>
</tr>
</thead>
<tbody>
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<td>P2₁</td>
<td>P2₁</td>
</tr>
<tr>
<td>Cell parameters</td>
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<td>a=44.2 Å, b=72.0 Å, c=59.3 Å, α=γ=90°, β=91.2°</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>50.0–1.8 (1.9–1.8)*</td>
<td>50.0–1.6 (1.7–1.6)*</td>
</tr>
<tr>
<td>Total reflections</td>
<td>126 163</td>
<td>133 236</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>34 080</td>
<td>40 969</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>97.7 (92.3)*</td>
<td>88.2 (96.1)*</td>
</tr>
<tr>
<td>Rmerge (%)†</td>
<td>8.1 (48.0)*</td>
<td>4.3 (20.4)*</td>
</tr>
<tr>
<td>Rmerge (%)‡</td>
<td>17.7 (2.3)*</td>
<td>18.9 (5.1)*</td>
</tr>
</tbody>
</table>

Reefinement statistics

| No. of reflections used | 30 889 | 38 880 |
| No. of reflections in test set | 1561 | 3046 |
| R factor (%)             | 20.3 | 18.0 |
| Rfree (%)§               | 26.0 | 23.1 |
| RMS deviation            |       |        |
| Bonds (Å)                | 0.017 | 0.012 |
| Angles (°)               | 1.225 | 1.349 |
| Mean B factor            | 21.2  | 13.8  |

Ramachandran plot

| Residues in most favoured regions | 95.4 | 90.2 |
| Residues in additional allowed regions | 4.6  | 9.5  |

*Numbers in parentheses correspond to the highest-resolution shell.
†Rmerge = Σ[Ih,Ih]−<Ih>/|Σ[Ih,Ih]<Ih>, where <Ih> is the mean intensity of the observations Ih of reflection h.
‡R factor = Σ(|Iobs|−|Icalc|)/Σ|Iobs|; Rfree is the R factor for a subset (5%) of reflections that was selected for prior refinement calculations and not included in the structure refinement.

Determining the kinetic parameter K_m in the presence of the inhibitor at a concentration close to the IC_50. The mode of action was determined by plotting the data as Lineweaver–Burk plots, and by fitting all data to the standard competitive inhibition equation.

CrChi1 mutants were constructed using the MutantBEST kit from Takara (Japan). The DNA sequences of mutated gene fragments were confirmed using an ABI PRISM 377 DNA sequencer (Applied Biosystems), and mutant genes were cloned and expressed in Escherichia coli BL21(DE3) following the protocols described above. Mutant proteins were purified in the same manner as the wild-type. The renaturation of inclusion bodies followed the procedure described in a previous report (Meng et al., 2006).

Accession numbers. Crystallographic coordinates and structure factors have been deposited in the RCSB Protein Data Bank with accession codes 3G6L (apo-CrChi1) and 3G6M (complex).

RESULTS AND DISCUSSION

Overall structure of CrChi1

The structures of the 44 kDa form of apo-CrChi1 and in complex with its inhibitor caffeine were solved by molecular replacement and refined against 1.8 Å and 1.6 Å diffraction data to R factors of 20% and 18% (Rfree=26% and 23%), respectively. The structure of apo-CrChi1 consists of two parts: eight strands of parallel β-barrels are surrounded by eight α-helices to form the core domain (Fig. 1). The core domain, which was named as a (β/α)_8 TIM barrel, has been observed in other Family 18 chitinases from A. fumigatus (afChi) (PDB code: 1W9P) (Rao et al., 2005a), Coccidioides immitis (cmChi) (PDB code: 1D2K) (Hollis et al., 2000), human chitotriosidase (huChi) (PDB code: 1LG1) (Fusetti et al., 2002), and chitinases A (chiA) (PDB code: 1EDQ) (Papanikolau et al., 2003) and B (chiB) (PDB code: 1O6I) (Houston et al., 2002) from the bacterium Serratia marcescens. An additional α/β domain, composed of five antiparallel β-strands and two α-helices, is inserted in the loop between strand β9 and helix α8, and this domain might give the active site a groove character. Like all other Family 18 chitinases, CrChi1 has the DXDXE motif at the end of strand β5 with Glu174 as the catalytic residue in the middle of the open end of the (β/α)_8 barrel (Fig. 2a). Chitinase CrChi1 showed overall sequence similarities ranging from 24.2% to 54.8% to five other chitinases (afChi, cmChi, huChi, ChiA and ChiB). However, the amino acid residues around the substrate-binding site and the catalytic centre were very conserved (Fig. 2a). When the structures afChi, cmChi, huChi, ChiA and ChiB were superimposed onto CrChi1 using the SSM method (Fig. 2b), we found that the core parts of all these structures had similar folding patterns, with the RMS deviation all between 0.6 and 1.3 Å (Fig. 2b). Their main differences were in the N- and C-terminal domains (Fig. 2a, b). Together, these results showed that...
while the chitinases from different organisms shared low sequence similarity (Fig. 2a), their structures were very conserved (Fig. 2b), especially for the amino acid residues corresponding to the substrate-binding domain and the catalytic domain (Fig. 2a). Our analyses suggest that these chitinases probably share a common catalytic mechanism.

Inhibitor binding site

The sequence alignment (Fig. 2a) and structure comparison among the chitinases (Fig. 2b) suggested that CrChi1 might have the same active site as the other five homologues. Therefore, caffeine, a novel Family 18 chitinase inhibitor (Rao et al., 2005b; SchütteKopf et al., 2006), was selected for a co-crystallization experiment to investigate the active site of the enzyme and the potential inhibitory mechanism of caffeine on CrChi1.

The effectiveness of caffeine as an inhibitor of CrChi1 was initially approximated by measuring the activity of 2 nM enzyme with 20 mM substrate in the presence of different concentrations of caffeine. The results indicated that 1 mM caffeine reduced the activity by about 50 % (Fig. 3a). Complete steady-state kinetic parameters were then measured for the enzyme in the presence of caffeine using the modified fluorescence assay, and the results are shown in Fig. 3(b). The $K_m$ value of CrChi1 for the fluorescent oligosaccharide substrate 4-methylumbelliferyl $\beta$-D-$N,N',N''$-triacyctylchitotrioside was $9.5 \pm 0.3$ mM. The nature of the inhibition is shown in Fig. 3(b); the double reciprocal plot suggests that caffeine behaves as a competitive inhibitor. The $K_i$ of wild-type CrChi1 for caffeine was $19.7 \pm 0.3$ mM.

A soaking experiment of CrChi1 with caffeine was carried out, followed by collection of diffraction data. The structure of the CrChi1–caffeine complex was solved and refined against 1.6 Å resolution of the X-ray diffraction data (Table 1). The structure of CrChi1 in complex with caffeine defined the active centre of CrChi1 (Fig. 4a). In general, family 18 chitinases bind to their substrates in an extended recognition site. By convention, the sugars on the non-reducing end of the substrate are given negative numbers, and those on the reducing side are given positive numbers (Fig. 4a). In this convention, the scissile glycosidic bond lies between the $-1$ and $+1$ subsites. Previous structural studies of afChi, cmChi, huChi, ChiA and ChiB and their substrate–inhibitor complexes defined the GlcNAc subsites in the enzyme and they were found to form a deep groove on the surface of the protein. Similar properties were also observed in the CrChi1 structure (Fig. 4a). The groove consists of six sugar-binding subsites, numbered from $-4$ (non-reducing end) to $+2$ (reducing end), with hydrolysis taking place on the glycosidic bond between the $-1$ and $+1$ subsites (van Aalten et al., 2001; Fusetti et al., 2002). The subsites $-2$ to $+2$ formed a deep groove, lined by side chains that are highly conserved in Family 18 chitinases, including the conserved DXDXE motif. In the $-1$ subsite, Asp172/Glu174 in CrChi1 are equivalent to Asp155/Glu157 in ScCTS1 (Saccharomyces cerevisiae) (Hurtado-Guerrero & van Aalten, 2007), and to Asp125/Glu127 in hevammin (Terwisscha van Scheltinga et al., 1995), respectively. These are located at the end of $\beta5$ and form the conserved DXDXE motif in Family 18 chitinases. As in other Family 18 chitinases, Glu174 is the catalytic residue, with Asp172 stabilizing the oxazolinium ring of the reaction intermediate. Trp381 is the most conserved residues in all Family 18 chitinases, forming the bottom of the $-1$ subsite, with some conserved residues forming the sidewall.

The caffeine inhibitor binds to CrChi1 in a position equivalent to the allosamidin allosamizoline moiety (Rao et al., 2005a). Allosamidin is a natural pseudotrisaccharide inhibitor of Family 18 chitinases and binds to subsites $-3$ to $-1$ in complex with chitinases (Rao et al., 2005a; van Aalten et al., 2001). This allosamizoline moiety is known to mimic the oxazolinium ion reaction intermediate formed upon nucleophilic attack of the $N$-acetyl oxygen on the anomeric carbon (van Aalten et al., 2001). The methylxanthine core makes similar interactions with the chitinases. In addition to the methylxanthine in the $-1$ subsite, an additional ordered inhibitor molecule was also observed.

The interactions between the caffeine moiety and the enzyme are similar to those observed in the afChiB1–caffeine complex and the afChiB1-C2–dicaffeine complex (Rao et al., 2005b; SchütteKopf et al., 2006). The active-site Asp172 points down into the catalytic core. The electron-density map shows that the primary caffeine moiety is sandwiched between Trp134 and Trp381, and accepts two hydrogen bonds, one from the backbone amide of Trp134 and the other from the hydroxyl of Tyr242 (Fig. 4b).

Fig. 1. Overall structure of CrChi1. Helices, strands and coil are coloured purple, slate and green, respectively. All secondary structural elements are labelled.
molecular interaction between CrChi1 and caffeine is described in Table 2.

**Mutagenesis and enzymology**

The structure shown in Fig. 4(b) suggests that residues Tyr46, Trp134 and Tyr242 in CrChi1 play important roles in the formation of hydrogen bonds between the enzyme and caffeine. To obtain an insight into how the conserved residues are involved in the binding, we conducted a mutagenesis study of CrChi1. Table 3 shows the kinetic constants and $K_i$ values for the wild-type CrChi1 as well as its mutant derivatives. Caffeine showed competitive inhibitory activity in all cases.

The amino acid substitutions caused a decrease of over 1000-fold in the enzymatic activity. Two mutations, E174Q and W134G, completely inactivated the enzyme, confirm-
Table 2. Molecular interactions between CrChi1 and the inhibitor caffeine

<table>
<thead>
<tr>
<th>Atom in caffeine</th>
<th>Atom in CrChi1</th>
<th>Distance (Å)</th>
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</thead>
<tbody>
<tr>
<td>Caffeine 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td>M240 S 6</td>
<td>3.1</td>
</tr>
<tr>
<td>C6</td>
<td>D172-O 42</td>
<td>3.0</td>
</tr>
<tr>
<td>N3</td>
<td>Y242-0H</td>
<td>2.7</td>
</tr>
<tr>
<td>O1</td>
<td>Wat453-D243-O 62</td>
<td>2.8–2.7</td>
</tr>
<tr>
<td>O2</td>
<td>W134-N</td>
<td>2.9</td>
</tr>
<tr>
<td>Caffeine 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>D243-O 42</td>
<td>3.0</td>
</tr>
<tr>
<td>C2</td>
<td>D243-O 42</td>
<td>2.9</td>
</tr>
<tr>
<td>O1</td>
<td>Wat258-Y242-O</td>
<td>2.7</td>
</tr>
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</table>

Table 3. Mutagenesis of Crchi1 and enzymological data for the wild-type and mutant proteins

All experiments were performed in triplicate. ND, No significant signal.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Protein</th>
<th>(K_m) (μM)</th>
<th>(V_{max}) (μM s(^{-1}))</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>(k_{cat}/K_m) (μM(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Soluble</td>
<td>9.5 ± 0.3</td>
<td>0.005 ± 0.00007</td>
<td>2.59 ± 0.03</td>
<td>0.27</td>
</tr>
<tr>
<td>Y46F</td>
<td>Insoluble</td>
<td>11.5 ± 1.0</td>
<td>0.001 ± 0.00003</td>
<td>0.0062 ± 0.0002</td>
<td>0.0005</td>
</tr>
<tr>
<td>W134G</td>
<td>Soluble</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E174Q</td>
<td>Soluble</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Y242F</td>
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<td>0.0054 ± 0.0002</td>
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<tr>
<td>Y242G</td>
<td>Soluble</td>
<td>6.9 ± 1.0</td>
<td>0.0009 ± 0.0001</td>
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REFERENCES


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