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Crystallization and preliminary X-ray analysis of a chitinase from the fungal pathogen *Coccidioides immitis*

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Abstract

Chitinase is necessary for fungal growth and cell division and, therefore, is an ideal target for the design of inhibitors which may act as antifungal agents. A chitinase from the fungal pathogen *Coccidioides immitis* has been expressed as a fusion protein with gluathione-S-transferase (GST), which aids in purification. After cleavage from GST, chitinase was crystallized from 30% PEG 4000 in 0.1 *M* sodium acetate pH 4.6. The crystals have a tetragonal crystal lattice and belong to space group $P4_12_12$ or $P4_32_12$ and diffract to 2.2 Å resolution. The unit-cell parameters are $a = b = 91.2$, $c = 95.4$ Å; there is only one chitinase molecule in the asymmetric unit.

1. Introduction

The fungus *Coccidioides immitis* poses a major problem in human pathogenesis. It is responsible for the life-threatening lung infection coccidioidemycosis or San Joaquin Valley Fever (Cole & Kirkland, 1991). Fungal infections can be particularly dangerous in immunocompromized patients, such as those with HIV infections. The severity of such fungal infections, particularly from *Coccidioides*, *Candida*, *Cryptococcus* and *Histoplasmosis*, has recently been extensively reviewed (Minamoto & Rosenberg, 1997).

Fungal infections are usually difficult to treat because of the lack of effective antifungal agents, and this has spurred a search for enzymatic targets which may be suitable for the design of antifungal agents. Fungi are eukaryotes, and many of their enzymes are sufficiently similar to human analogs that drugs aimed at them will also adversely affect human tissue. Identifying enzymes which are unique to fungi and necessary for growth, therefore, allows the possible targeting of drugs which would minimize effects on the recipients. One significant difference between fungi and mammals is that fungi contain a cell wall, and enzymes responsible for cell-wall maintenance qualify as possible candidates for drug design. The polysaccharide chitin is a major structural component in the cell walls of many fungi, and chitin metabolism is required to assure proper growth and cell division. The enzyme chitinase catalyzes the hydrolysis of chitin, a β-1,4-linked polymer of *N*-acetylglucosamine, and has been shown to be necessary for cell separation during the growth of *Saccharomyces cerevisiae* (Kuranda & Robbins, 1991).

C. immitis contains two chitinases of 41 and 91 kDa called CTS1 and CTS2, respectively (Pishko *et al.*, 1995). The former protein is the primary diagnostic complement fixing antigen for the fungus and a monoclonal antibody has been raised against it (Johnson & Pappagianis, 1992; Dolan & Cox, 1991). As a result, the chitinase has also been referred to as the CF/chitinase protein (Yang *et al.*, 1996). The *C. immitis* chitinase shows significant amino-acid similarities to chitinases from other fungi such as *Aphanocladium album* and *Trichoderma harzianum* and from the bacteria *Serratia marcescens* (Pishko *et al.*, 1995). It has been suggested that this enzyme class plays a role in remodeling fungal cell walls and in endospore release (Johnson & Pappagianis, 1992). The structure of the *C. immitis* protein will be important to our understanding of the mechanism of this important class of enzymes and possibly to the design of chitinase inhibitors which may act as fungal-specific antibiotics.

Extracts of *E. coli* which had been transformed with the GST-chitinase fusion plasmid yielded a single electrophoretic band of 67 kDa when passed over a glutathione Sepharose affinity column. After cleavage with thrombin and adsorption of GST on the column, a single band of 45 kDa was obtained. The fusion and the cleaved protein had chitinase activity as judged by the hydrolysis of 4-methylumbelliferyl β-*N*,*N*′,*N*″-triacetylchitotrioside.

Long thin rod-like crystals of the fusion protein at 20 mg ml^{-1} appeared in 12% PEG 20000, 0.1 *M* MES, pH 6.5, 0.1 *M* cadmium chloride. These crystals grew to almost 1 mm in length, but never to more than 0.01 mm in diameter. Identical-looking crystals of the chitinase alone appeared in 8% PEG 8000, 0.1 *M* Tris–HCl, pH 8.5. Diffraction experiments with both crystal forms failed to show any detectable diffraction.

In an effort to improve crystal quality and diffraction, screens of various molecular-weight PEGs were tried with varying pH. After several weeks, small crystals of the chitinase appeared in 30% PEG 4000, 0.1 *M* sodium acetate, pH 4.6. It was found that these block-shaped crystals could be grown to dimensions of 0.3 mm in less than a week by microseeding.

We collected 121 441 observations of 20 899 reflections. The data were 99.7% complete to 2.2 Å with an overall $R_{\rm merge}$ of 12%; Table 1 contains statistics for the data collection. Chitinase crystals show slight radiation sensitivity, deteriorating after about 15 h in the X-ray beam. The crystal lattice belongs to space group $P4_12_12$ or $P4_32_12$ and the unit-cell parameters are $a = b = 91.2$, $c = 95.4$ Å. One molecule of chitinase per asymmetric unit gives a reasonable volume-to-mass ratio, V_m (Matthews, 1968), of 2.2 Å^3 Da^{-1}, indicating that the crystals contain about 42% solvent.

Soaking of the crystals with heavy metals proved unsuitable because of crystal cracking or to large changes in the crystal parameters. Chitinase which had been crystallized in the presence of heavy metals, however, yielded usable crystals. Harker sections of a difference Patterson map between the *p*-hydroxymercuribenzoate (PHMB) derivative and native data

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Table 1. *Statistics for crystallographic data from native chitinase*

Resolution (Å)	2.2
$R_{\rm merge}$ (%)	12.2
$R_{\rm merge}$ (last shell) (%)	21.3
$\lvert I/\sigma_I\rvert$	10.3
$\lvert I/\sigma_I\rvert$ (last shell)	5.2
Completeness (%)	99.7
Completeness ($I/\sigma_I > 5$) (%)	68.1
Completeness ($I/\sigma_I > 5$) (last shell) (%)	43.1
Unique reflections	20899
Redundancy	6.0

show self-consistent peaks for a single-site derivative. Refinement of the metal position with *MLPHARE* from the *CCP4* program suite (Collaborative Computational Project, Number 4, 1994) gave a figure of merit of 0.35. We plan to phase the data either by a combination of *SIR* with the PHMB derivative and molecular-replacement methods using the structure of a related chitinase from *Serratia marcescens* (Perrakis *et al.*, 1994) as a search model or using molecular replacement alone if the model is sufficient.

2. Materials and methods

The gene for the 47 kDa chitinase from *C. immitis* was fused to the C-terminal region of the gene for glutathione S-transferase (Yang *et al.*, 1996). During construction of the fusion the amino-terminal 20 amino acids of chitinase were removed, since they form a signal peptide which is normally cleaved away during protein processing.

We transformed the fusion plasmid into *E. coli* and grew 0.5 l cultures to stationary phase. The cells were broken in a French pressure cell (6.895×10^6 Pa), the debris was centrifuged out and the supernatant passed over a 3 ml glutathione Sepharose affinity column treated according to the manufacturers directions (Sigma, St Louis, MO, USA). About 30 mg of fusion protein was obtained in a 20 ml volume. The effluent was concentrated to 2 ml, and a 10× thrombin-cleavage buffer added (Novagen, Milwaukee, WI, USA). To remove GST from the fusion protein, 15 units of thrombin were added and allowed to react for 2 h at 288 K. After an overnight dialysis against 6 m*M* phosphate and 300 m*M* NaCl at pH 7.3, the mixture of cleaved proteins was again passed over the affinity column to remove GST and any remaining uncleaved fusion protein. The resultant purified chitinase, about 15 mg, was dialyzed into 50 m*M* Tris buffer pH 7.5.

Chitinase activity was measured by monitoring the production of methylumbelliferone product from the hydrolysis of 4-methylumbelliferyl β-*N*,*N*′,*N*″-triacetylchitotrioside (Hollis *et al.*, 1997). Release of free methylumbelliferone was measured by fluorescence spectrophotometry, using an SLM 8000 fluorescence spectrophotometer, exciting at 360 nm and measuring emission at 450 nm. A standard curve of free methylumbelliferone was constructed and shown to be linear over the range of product measured; this allowed the conversion of fluorescence counts to nanomoles of methylumbelliferone released per hour.

Crystallization trials were performed using both the GST-chitinase fusion protein (20 mg ml^{-1}) and the purified chitinase (4 mg ml^{-1}). Initial screening for crystals was performed with Crystal Screen and Crystal Screen II (Hampton Research) using the sitting-drop vapor-diffusion method. Sitting drops contained equal volumes of protein solution and precipitant solution from the well.

Heavy-metal soaks of methyl mercury, *p*-hydroxymercuribenzoate (PHMB) and platinum tetrachloride were performed by the addition of these compounds, dissolved in well solution, to the drops containing crystals. Final metal concentrations of 1, 2 and 5 m*M* were tried; the protein was also crystallized in the presence of 2 m*M* of these compounds.

All diffraction experiments were performed in quartz capillaries at room temperature. Diffraction data were collected on these crystals to 2.2 Å resolution using an MSC R-AXIS IV detector with a Rigaku RU200 rotating-anode source with focusing mirrors. Data were collected by 20 min exposures of 1.0° oscillations about the φ axis. The diffraction data were processed with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

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