

Crystal Structure of an Endochitinase from *Hordeum vulgare* L. Seeds

P. John Hart, Arthur F. Monzingo, Michael P. Ready
Stephen R. Ernst and Jon D. Robertus†

Department of Chemistry and Biochemistry
University of Texas
Austin, TX 78712, U.S.A.

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Higher plants contain multiple constitutively expressed proteins for defense against infection by viruses, bacteria, and fungi. One such class of proteins, the chitinases, are effective antifungal agents because they hydrolyze the insoluble β -1,4-linked polymer of *N*-acetylglucosamine (chitin), which is the major component of the mycelial cell wall of many fungi. We report here the three-dimensional, 2.8 Å, crystal structure of a 26 kDa endochitinase from barley (*Hordeum vulgare* L.) seeds. The 243 amino acid residue molecule is rich in α -helices and has three disulfide bonds. A prominent elongated cleft runs the length of the molecule, and is presumably the region responsible for substrate binding and catalysis. Endochitinases from various species of plants show a high degree of similarity in their amino acid sequences. It is therefore likely that the barley endochitinase structure can serve as a model for other plant endochitinases and that catalytic models based on that structure will be applicable to the entire enzyme family.

Keywords: crystal structure; X-ray analysis; endochitinase; barley

1. Introduction

Higher plants contain multiple constitutively expressed proteins for defense against infection by viruses, bacteria, and fungi. The mechanisms by which they accomplish this include: (1) the manufacture of metabolites (phytoalexins) that are toxic to bacteria and fungi (Albersheim & Valent, 1978); (2) the production of proteinase inhibitors (Walker-Simmons *et al.*, 1983); (3) the production of enzymes that produce physical barriers to fungal invasions by altering the cell wall (Esquerre-Tugaye *et al.*, 1985); (4) the manufacture of ribosome inactivating proteins (Roberts & Selitrennikoff, 1986); and (5) the manufacture of lytic enzymes such as β -1,3 glucanase and chitinase (Abeles *et al.*, 1970; Boller *et al.*, 1983). Chitin is an insoluble linear β -1,4-linked polymer of *N*-acetylglucosamine commonly found in the exoskeletons of arthropods and is a primary component of many fungal cell walls. Exochitinases, usually found in bacteria, hydrolyze the disaccharide diacetylchitobiose from the non-reducing end of chitin in a stepwise manner (Roberts & Cabib, 1982). Endochitinases, commonly

found in grains, bean and tobacco plants can hydrolyze the chitin polymer internally, releasing polysaccharides (Molano *et al.*, 1979; Boller *et al.*, 1983). In hyphal extension-inhibition assays, endochitinases (but not exochitinases) have been shown to be very effective in preventing the invasion of fungal mycelia into areas where an endochitinase is present (Roberts & Selitrennikoff, 1986, 1988; Leah *et al.*, 1991). Typically, endochitinase activity is found at low or basal levels in healthy plants, but its expression can be increased in response to pathogen attack (Boller, 1988). Recently, experiments *in vivo* have shown that transgenic tobacco plants constitutively expressing a bean endochitinase gene were better able to resist fungal infection than corresponding non-transformed plants (Broglie *et al.*, 1991).

Endochitinases from various species of plants show a high degree of similarity in their amino acid sequences; endochitinases from bean, barley, tobacco, corn, rice, potato, pea, and *Arabidopsis* are homologous (Swegle *et al.*, 1989; Nishizawa & Hibi, 1991; Zhu & Lamb, 1991; Gaynor, 1988; Huynh *et al.*, 1992; Verburg & Huynh, 1991). Increased knowledge about the structure/function relationships of these proteins may have implications for plant engineering.

† Author to whom all correspondence should be addressed.

The endochitinase from barley is a 26,000 dalton protein that exists as a monomer in solution (Leah *et al.*, 1987, 1991). We previously reported the crystallization of this protein in space group $P4_12_12$ ($P4_32_12$) (Hart *et al.*, 1992) but in the process of searching for heavy-metal isomorphous derivatives another crystal form was obtained, which was superior for X-ray diffraction studies. In this paper we report the 2.8 Å (1 Å = 0.1 nm) structure of the barley endochitinase obtained using these new crystals

2. Materials and Methods

Crystals of barley seed endochitinase were obtained by the hanging drop vapour diffusion method using protein at a concentration of 25 to 30 mg ml⁻¹ in 100 mM Tris-HCl (pH 8.5). The reservoir solution, 30% saturated ammonium sulfate, was mixed with an equal volume of protein solution, generally 5 µl. The drop was seeded using a microseeding technique, and large block-like crystals grew within 4 days. Three-dimensional diffraction data were collected on a SDMS multi-wire area detector using the method of Xuong *et al.* (1985). Crystal alignment was facilitated by the program INDEX, written by Dr Stephen Ernst in our laboratory, which allows rapid orientation angle determination from a random crystal mounting. Crystals were rotated about ω , collecting 0.1° frames for 30 s each. The X-ray source was an Elliot GX-20 rotating anode generator operating at 40 kV, 40 ma with a graphite monochromator. Data were both collected to 2.3 Å resolution and evaluated using the University of California, San Diego (UCSD) software system (Howard *et al.*, 1985).

Suitable endochitinase crystals were soaked for 2 weeks in artificial mother liquor containing 5 mM-K₂PtCl₄. The highest peak on the $\nu = 1/2$ Harker section of the difference Patterson map (site 1) was assigned to a metal site which was given the arbitrary y coordinate of zero. Four additional platinum sites were identified by difference Fouriers and verified by the presence of the appropriate Harker peaks and cross-peaks in the difference Patterson.

There are 7 cysteine residues in the barley endochitinase sequence, indicating that it will contain at least 1 free thiol. A single-site mercury derivative was subsequently prepared by soaking a suitable crystal in 1 mM-CH₃Hg(C₂H₃O₂) in artificial mother liquor for 1 week. Heavy-atom positions were determined *via* Patterson methods using *in-house* programs.

Phasing and heavy-atom parameter refinement for the 2 isomorphous derivatives were performed using the programs PHASEPROG and REFINER obtained from Dr Gregory Petsko. Chain tracing and model-building was done using the FRODO graphics package (Jones, 1982). Ribbon diagrams were made using the Priestle series of programs (Priestle, 1988).

3. Results and Discussion

The new crystallization conditions produce large block-like crystals, as shown in Figure 1(a). They have space group $P2_1$, with $a = 41.5$ Å, $b = 64.5$ Å, $c = 43.1$ Å, and $\beta = 108.0^\circ$. The crystals diffract to 1.7 Å on still photographs. Figure 1(b) shows a 16° precession picture of the $hk0$ zone. The nominal resolution at the picture edge is 2.5 Å. The monoclinic crystals also lacked the decay problems asso-

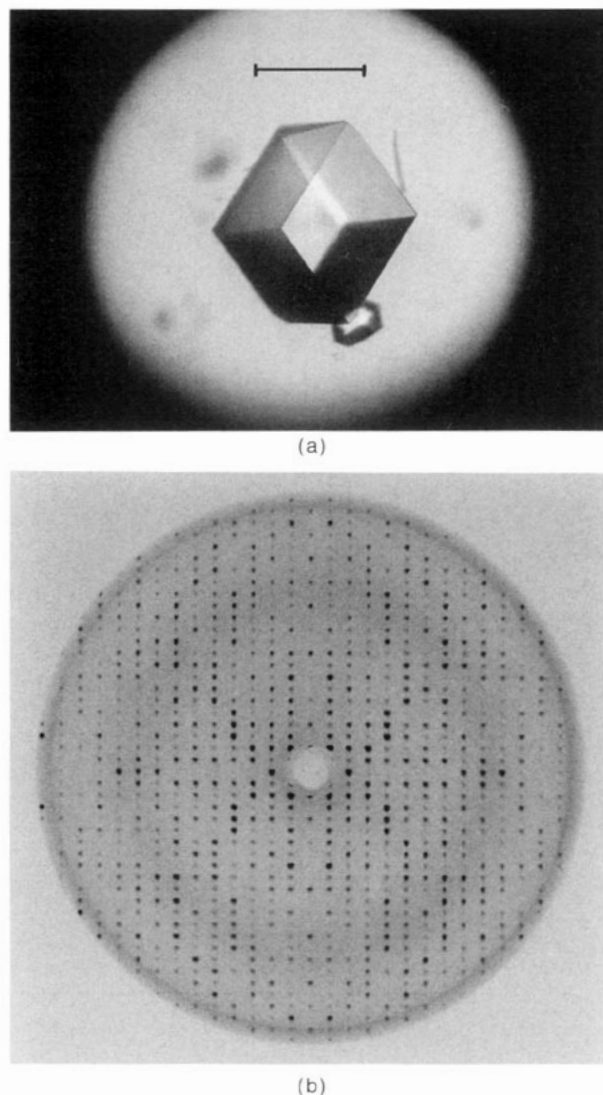


Figure 1. (a) Crystals of the 26 kDa barley endochitinase. The bar represents 0.5 mm. (b) A 16° precession photograph of the barley endochitinase crystals showing the $hk0$ zone. The photograph was exposed to monochromated CuK α radiation from a Rigaku RU-200 rotating anode for about 20 h.

ciated with the tetragonal form, where reflections exhibited a significant decay in intensity after about 30 hours. The new crystals allowed complete data collection for the native and derivatives from single crystals.

X-ray diffraction data collected from crystals of the barley endochitinase and isomorphous derivatives are summarized in Table 1. Isomorphous data from the platinum and mercury derivatives, together with anomalous difference information from the mercury derivative, were used in the generation of a 2.8 Å multiple isomorphous replacement map. A 2.8 Å solvent-flattened multiple isomorphous replacement map was subsequently produced using the Wang algorithm (Wang, 1985). Both maps were of high quality, and the side-chains of the amino acids were clearly visible. The solvent-flattened, multiple isomorphous replacement map

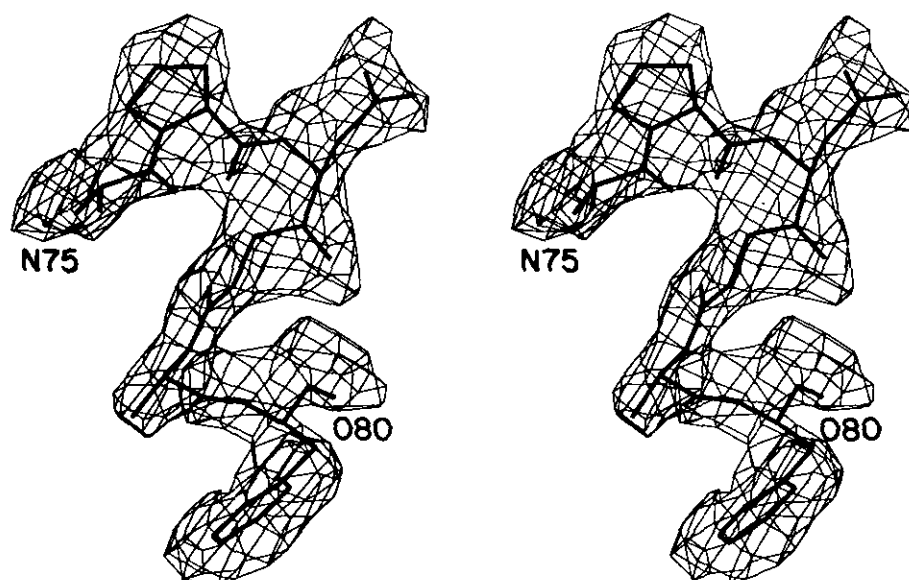


Figure 2. The 2.8 Å multiple isomorphous replacement map of chitinase. The stereo image shows a representative portion of the multiple isomorphous replacement map, without solvent-flattening, around residues 75 to 80. The top 15% of the electron density is shown here.

Table 1
Crystallographic data

Space group: $P2_1$ ($a = 41.5$ Å, $b = 64.5$ Å, $c = 43.1$ Å, $\beta = 108.0^\circ$)					
Barley Endochitinase	Native	K_2PtCl_4	$CH_3Hg(C_2H_3O_2)$		
No. of observations	35,719	40,610	36,885		
No. of unique observations	5428	5416	5167		
Completeness (%)	99.7	99.9	95.7		
Resolution limit (Å)	2.8	2.8	2.8		
R_{merge} (%)†	4.0	9.3	4.4		
Mean fractional isomorphous change (%)		18.4	20.3		
Phasing power‡	d_{min} (Å)			$Anom$	
	7.9	1.1	3.5		
	5.6	1.8	4.0	1.4	
	4.6	1.6	3.1	1.8	
	3.9	1.2	2.3	1.5	
	3.5	1.3	2.1	1.2	
	3.2	1.2	2.3		
	3.0	1.3	2.6		
	2.8	1.3	3.0		
Heavy-atom sites (fractional coordinates)					
K_2PtCl_4	<i>X</i>	<i>Y</i>	<i>Z</i>	Occupancy (arbitrary units)	Temperature factor (Å ²)
1	0.0267	0.0000	0.2735	0.6196	26.0
2	0.7355	0.2921	0.3737	0.4050	27.9
3	0.8665	0.4483	0.0381	0.3476	34.4
4	0.8270	0.2136	0.3911	0.1532	20.5
5	0.3961	−0.0446	0.2915	0.0349	18.2
$CH_3Hg(C_2H_3O_2)$					
1	0.5448	−0.0133	0.9053	0.8155	14.6
Mean figure of merit: 0.79					

† R_{merge} = conventional discrepancy R -factor for scaling symmetry-related intensities, in this case from single crystals.

‡ Phasing power = $f_h/E, f_h''/E''$.

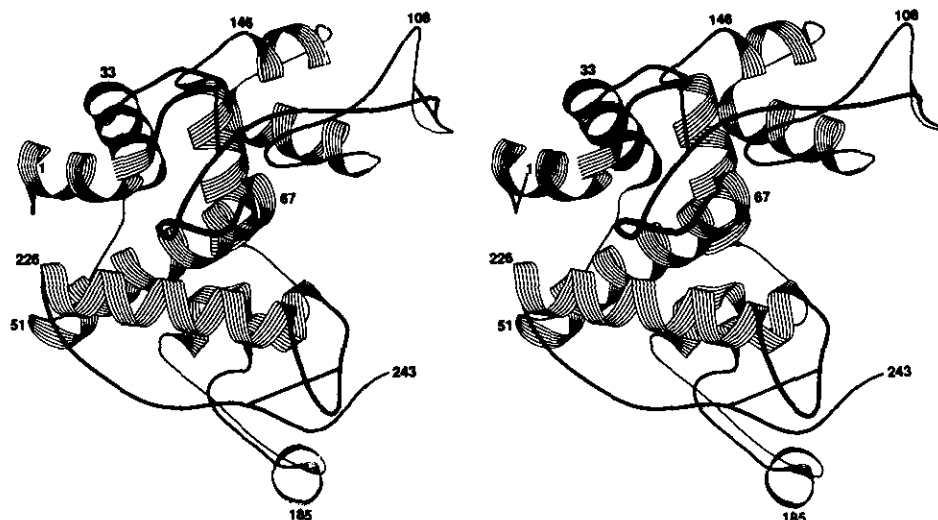


Figure 3. Ribbon drawing of the barley seed endochitinase backbone. The 3 disulfide bonds are represented by heavy lines.

was used to build the core of the molecule while the exterior regions of the molecule were best visualized using the map with no solvent-flattening. Figure 2 shows a representative portion of the latter map. The initial *R*-factor for the model at 2.8 Å resolution was 0.41 and after one round of energy minimization and simulated annealing using XPLOR (Brunger, 1988), the *R*-factor dropped to 0.20.

The overall fold of the barley seed endochitinase is shown in Figure 3. The molecule is a compact globular structure approximately 40 Å × 45 Å × 42 Å. It has three disulfide bonds; the cysteine pairs are 23 to 85, 97 to 105, and 204 to 236. Cysteine 223 is a free thiol that reacted to form the sole mercury derivative site. The protein has ten helical segments, which comprise 47% of the linear sequence. An elongated cleft running the length of the molecule is clearly evident, and is presumed to be the region responsible for substrate binding and catalysis. Amino acid sequence comparisons relate the barley endochitinase only to other plant endochitinases, and these show no obvious relation to any other known class of proteins. An initial inspection of the crystal structure suggests that the barley seed endochitinase fold does not closely resemble that for other known proteins, and thus appears to be a new structure. Crystallographic refinement is underway, as well as difference Fourier analysis of the molecule with substrate analogs bound.

Structural data have been submitted to the Brookhaven protein data bank.

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