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Structure of jack bean chitinase

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The structure of jack bean chitinase was solved at 1.8 Å resolution by molecular replacement. It is an α-helical protein with three disulfide bridges. The active site is related in structure to animal and viral lysozymes. However, unlike in lysozyme, the architecture of the active site suggests a single-step cleavage. According to this mechanism, Glu68 is the proton donor and Glu90 assists in the reaction by moving towards the substrate and recruiting a water molecule that acts as the nucleophile. In this model, a water molecule was found in contact with Glu90 $O^{\varepsilon 1}$ and Thr119 O^{γ} at a distance of 3.0 and 2.8 Å, respectively. The model is in accordance with the observed inversion mechanism.

1. Introduction

Chitinases hydrolyze the β-1,4-glycosidic bond between *N*-acetylglucosamines in chitin. Chitin is a fibrous, insoluble polymer and is the second most abundant biomolecule on earth. The expression of chitinases forms part of the defence strategy of plants against fungal attack. Chitinases belong to either family 18 or family 19 of glycosyl hydrolases (Perrakis *et al.*, 1993). The family 18 chitinases, of which the structure is known, are TIM-barrel proteins (Terwisscha van Scheltinga *et al.*, 1994; Perrakis *et al.*, 1994), the known family 19 chitinases are plant endo-chitinases that are found in various species and are highly homologous in sequence (Perrakis *et al.*, 1993). Two crystal structures of endo-chitinase from barley have been reported (Hart *et al.*, 1995; Song & Suh, 1996).

Modelling of a barley chitinase–substrate complex revealed that the distance between the proton donor and the putative nucleophile is too large to allow direct interaction of both residues with the substrate (Brameld & Godard, 1998). It is therefore more likely that a water molecule acts as the nucleophile in a single-step mechanism with inversion of the configuration at the anomeric carbon. Glu89 and Ser120 in barley chitinase could position the water molecule in a productive way for cleavage (these residues are equivalent to Glu90 and Thr119 in jack bean chitinase). However, in the structures from barley chitinase reported thus far, no water molecule was found at this position (Hart *et al.*, 1995; Song & Suh, 1996).

In this work, the structure of chitinase from jack bean is presented. Differences to the barley enzyme are described. A water molecule was found at a hydrogen-bonding distance from Glu90 and Thr119.

2. Materials and methods

2.1. Purification

The chitinase was isolated from mature seeds of jack bean (*Canavalia ensiformis*). After extraction of flour with 0.1 *M* Tris–HCl pH 9.5, canavalin and concanavalin A were removed by precipitation at pH 5.0 and by binding to Sephadex G75, respectively. The chitinase was further purified by ion-exchange chromatography as described by Schlesier *et al.* (1998).

2.2. Crystallization and data acquisition

The protein was stored at 15 mg ml^{-1} in 0.1 *M* Tris pH 9.5. Crystals were obtained by mixing equal volumes of the protein solution with 1.6 *M* ammonium sulfate solution in sitting-drop setups at room temperature. Crystals grew as long needles in approximately a week. The data were collected at room temperature on a MAR imaging plate (X-ray Research, Hamburg, Germany) mounted on a Nonius FR591 rotating-anode generator (Enraf–Nonius, Delft, Netherlands) and processed with *XDS* (Kabsch, 1993). The crystals diffract to 1.8 Å and belong to the space group $P6_1$ (a = 67.69, c = 110.21 Å). The $R_{\rm sym}$ for 24 446 reflections was 5.9% with an $\langle I/\sigma(I)\rangle$ of 9.8. The data are 92.2% complete with a redundancy of 2.9. In the highest resolution shell (1.8–1.9 Å), the $R_{\rm sym}$ is 26.8%, $\langle I/\sigma(I)\rangle$ is 2.8 and the completeness is 84.6%.

2.3. Structure determination and refinement

A V_m of 2.8 Å^3 Da^{-1} indicated one molecule in the asymmetric unit (Matthews, 1968). The chitinase from barley was used as a search model (Song & Suh, 1996; PDB entry 1cns; 67% sequence identity to jack bean chitinase). Structure solution with *AMoRe* (Navaza, 1994) gave an R factor of 38.5% after rigid-body refinement from 12 to 4 Å. Further refinement was performed with *X-PLOR* (Brünger, 1992*a*) using a test data set (10% of the data) for cross validation (Brünger, 1992*b*). The amino-acid sequence was changed to the jack bean chitinase sequence (TrEMBL accession number 081934). A simulated-annealing calculation followed by *B*-factor refinement was calculated to 1.8 Å resolution and decreased the $R_{\rm free}$ to 30.3%. Model correction and addition of solvent molecules was peformed with *O* (Jones, 1978). Alternating runs of positional and *B*-factor refinement converged with an $R_{\rm free}$ of 22.2%. The 20 final cycles with 156 water molecules (average B, 33.6 Å^2) and a sulfate ion added were calculated against the complete data set and gave an R factor of 18.2%.

3. Results and discussion

3.1. Protein structure and comparison with barley chitinase

Refinement with *X-PLOR* led to a model with deviations from ideal bond lengths and bond angles of 0.009 Å and 1.45°, respectively. Average temperature factors for main-chain and side-chain atoms are 13.1 and 16.1 Å^2, with σ values of 6.5 and 9.1, respectively. The N-terminal aspartate was omitted from the model because of its lack of electron density. With the exception of His120, all residues are in the allowed regions of the Ramachandran plot (most favoured regions, 87.2%; additionally allowed regions, 12.3%). This was also reported for the corresponding histidine in barley chitinase.

Jack bean chitinase is a monomeric enzyme of 243 amino acids. It has the same fold and topology as its barley homologue. Ten helices of 7–18 amino acids in length were found. The three disulfide bonds are conserved and connect residues 24 to 86, 98 to 104 and 203 to 235 (Fig. 1). There is a two-residue deletion in jack bean chitinase compared with the barley enzyme (Gln102, Trp103 in the barley sequence), an additional aspartate at the N-terminus and an additional glycine residue at the C-terminus of jack bean chitinase.

Superposition of jack bean chitinase and the barley chitinase model used for molecular replacement (published at 2.0 Å) yields an r.m.s. deviation of 0.98 Å for all C$^\alpha$ positions. As a comparison, the superposition of jack bean chitinase and the second published barley chitinase model (at 1.8 Å; PDB entry 2baa) gives 1.0 Å r.m.s. deviation and the comparison of both barley chitinase models yields a value of 0.78 Å. In Fig. 2(*a*) the sequence alignment of jack bean and barley chitinase is shown and in Fig. 2(*b*) the superposition of the three-dimensional structures is shown in stereo. The coordinates were superimposed with *LSQKAB* (Kabsch, 1976).

The residues in secondary structures have a smaller r.m.s. deviation than those in loop regions (0.60 compared with 1.33 Å). Identical residues show a smaller r.m.s deviation than those that differ (0.87 compared with 1.14 Å). This is in accordance with the observation that the frequency of iden-

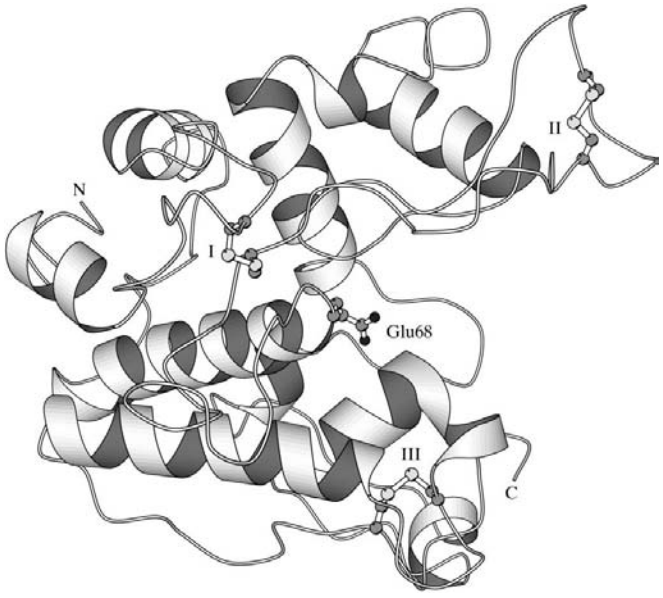

Figure 1
Model of jack bean chitinase at 1.8 Å resolution. Helices and termini are indicated, disulfide bridges are identified by roman numerals (I, Cys24/Cys86; II, Cys98/Cys104; III, Cys203/Cys235). Glu68 is the catalytic residue in the cleavage reaction. Figs. 1 and 3 were drawn with *MOLSCRIPT* (Kraulis, 1991).

tical amino acids is higher in the helices (72.5%) than in the loops (61.4%). As to be expected, sequence comparison reveals that the conserved amino acids are mostly located in the interior of the protein and in the binding groove, whereas mutated positions are more often found on the protein surface.

Almost all stacking interactions of aromatic residues that were observed in the barley enzyme are also found in jack bean chitinase. The high degree of conservation of stacking interactions in proteins has been reported previously (Burley & Petsko, 1985). There is a conserved stacking interaction between Phe190 and Tyr218 and there are several examples of conserved perpendicular ring interactions. These involve two (Tyr85 and Phe150) or three side chains (Tyr81, Phe217 and Tyr221; Phe45, Phe61 and Trp157) and even in one case seven side chains (Phe12, Phe29, Tyr30, Tyr32, Phe35, Tyr85 and Phe150). The only hydrophobic interaction reported for the barley chitinase that is not found in the jack bean homologue is that of Trp72 with Trp83, because Trp72 is mutated to alanine in jack bean chitinase. However, the side chain of His18 is at a reasonable distance for stacking with Trp83.

Roughly a third of all water molecules are conserved in the chitinase models. This is judged from their distances from each other after superposition of corresponding C^{α} atoms and from the existence of at least one common hydrogen-bonding partner. A small subset of these conserved water molecules is located in cavities (molecules 301, 302, 303, 304, 305, 306, 308, 314, 322; 301 and 308 as well as 304 and 306 are found in the same cavity and have a hydrogen-bonding interaction with each other).

3.2. The cleavage mechanism

The structural similarity between animal lysozyme, phage lysozyme and barley chitinase has been reported previously (Holm & Sander, 1994; Monzingo *et al.*, 1996). When the related regions are superimposed, the proton donor in hen egg-white lysozyme, Glu35, superimposes well on Glu67 of barley chitinase. A study of the retention of the reaction products of bean chitinase with HPLC (Iseli *et al.*, 1996) and NMR studies on reaction products of barley chitinase (Hollis *et al.*, 1997) demonstrated that, unlike in hen egg-white lysozyme, a single-step mechanism with inversion of the configuration at C_1 is the likely mechanism for plant chitinases. This is in accordance with modelling studies on barley chitinase (Brameld & Godard, 1998). It was therefore proposed that the nucleophilic water molecule needed for this reaction should be activated by a glutamic acid acting as the general base. In our 1.8 Å structure of jack bean chitinase there is a water molecule (H_2O338) held in place by hydrogen bonds to the carboxyl group of Glu90 and the hydroxyl group of Thr119. The density of this water molecule is well defined, with a *B* factor of 22.8 Å^2 (Fig. 3).

Furthermore, it was proposed that the loop around Glu90 has to move closer to Glu68 by 4–5 Å to take part in the reaction (Brameld & Godard, 1998). There is some indication that the loop following Glu90 is flexible enough to fulfill this requirement. The loop residues have elevated temperature factors in our model. The average *B* factor of residues 91–108 in jack bean chitinase is 24.8 Å^2, whereas the average for all atoms is 14.5 Å^2. In barley chitinase the flexible loop, as indicated by high *B* factors, ranges from residue 89 to 96 (Hart *et al.*, 1995). Additionally, the largest deviations between the barley and jack bean models are found in this loop region, although it is constrained by an internal disulfide bridge (connecting Cys98 to Cys104). The same is true for both

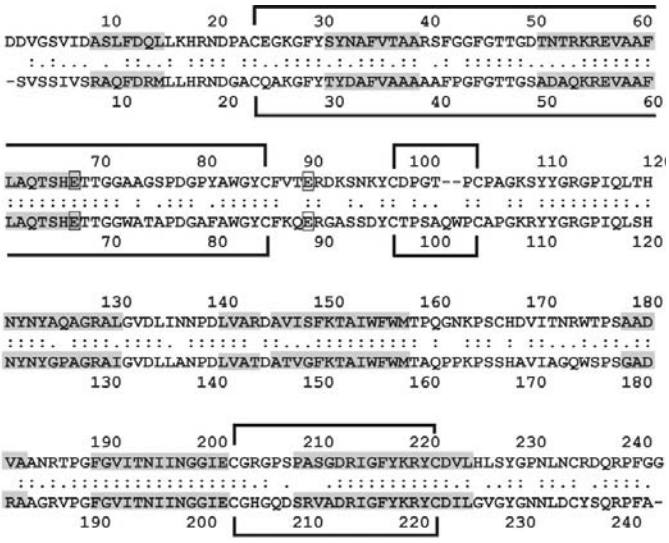

Figure 2
(*a*) Sequence alignment of jack bean (top) and barley chitinase (bottom) with *FASTA* (Pearson & Lipman, 1988). Identical residues are indicated by two dots, conservative mutations by one dot. The homology is 67.2% for 241 amino acids. The helical regions of both enzymes are indicated by grey shading. The active-site glutamic acids are boxed and disulfide bridges are indicated by lines. (*b*) Superposition of barley (light grey) and jack bean chitinase (dark grey) in ribbon representation. Disulfide bridges are drawn in black. The orientation of the molecules is the same as in Fig. 1. The figure was drawn with *SETOR* (Evans, 1993).

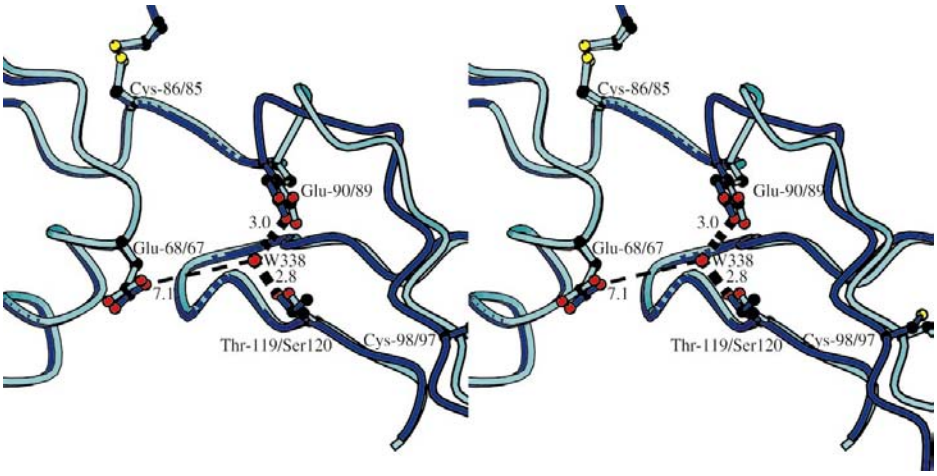

Figure 3
Stereoview into the active site after superposition of jack bean and barley chitinase. The light-blue model is derived from the barley chitinase structure, the dark blue model is from jack bean. The side chains of Glu68, Cys86, Glu90, Cys98 and Thr119 from jack bean chitinase, and Glu67, Cys85, Glu89, Cys97 and Ser120 from barley chitinase are shown. A water molecule is held in place by hydrogen bonds to $O^{\varepsilon 1}$ of Glu90 and $O^{\gamma 1}$ of Thr119 in jack bean chitinase, with the distances given in Å. Glu68/67 is the proton donor.

models of barley chitinase: the model from crystals obtained with ammonium sulfate (Hart *et al.*, 1995) and the two independent molecules in the asymmetric unit in crystals of barley chitinase obtained with PEG 3000 deviate most at this loop (Song & Suh, 1996; Song *et al.*, 1993). This stretch is one of the least conserved among the sequences of family 19 plant chitinases.

The hydrogen bond from O^{γ} of Thr119 seems to help positioning water molecule H_2O338, but it is unclear if Thr119 moves towards the substrate concomitantly with Glu90. The neighboring His120 has an unusual geometry ($\varphi = 81$, $\psi = 135^{\circ}$) that might lower the energy for a shift of these residues.

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