<http://www.diva-portal.org>

Postprint

This is the accepted version of a paper published in *Plant Molecular Biology*. This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Citation for the original published paper (version of record):

Ubhayasekera, W., Rawat, R., Ho, S., Wiweger, M., von Arnold, S. et al. (2009)
The first crystal structures of a family 19 class IV chitinase: the enzyme from Norway spruce.
Plant Molecular Biology, 71(3): 277-289
<http://dx.doi.org/10.1007/s11103-009-9523-9>

Access to the published version may require subscription.

N.B. When citing this work, cite the original published paper.

Permanent link to this version:

<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-142125>

The first crystal structures of a family 19 class IV chitinase: the enzyme from Norway spruce

Wimal Ubhayasekera^{1*}, Reetika Rawat², Sharon Wing Tak Ho², Malgorzata Wiweger³, Sara Von Arnold⁴, Mee-Len Chye² and Sherry L. Mowbray¹

¹ Department of Molecular Biology, Box 590, Biomedical Center, Swedish University of Agricultural Sciences, SE-751 24, Uppsala, Sweden

² School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, China

³ Leiden University Medical Center, Department of Pathology, L1-Q, Albinusdreef 2, P.O. Box 9600, 2300 RC Leiden, The Netherlands

⁴ Department of Plant Biology and Forest Genetics, Swedish University of Agricultural Sciences, Box 7080, SE-75007, Uppsala, Sweden

* Corresponding author: Tel.: 46-18-471-4007; Fax: 46-18-53-69-71; E-mail: wimal@xray.bmc.uu.se
Department website: <http://xray.bmc.uu.se/>

Running title: Class IV chitinase structures from *Picea abies*

Abstract

Chitinases help plants defend themselves against fungal attack, and play roles in other processes, including development. The catalytic modules of most plant chitinases belong to glycoside hydrolase family 19. We report here x-ray structures of such a module from a Norway spruce enzyme, the first for any family 19 class IV chitinase. The bi-lobed structure has a wide cleft lined by conserved residues; the most interesting for catalysis are Glu113, the proton donor, and Glu122, believed to be a general base that activate a catalytic water molecule. Comparisons to class I and II enzymes show that loop deletions in the class IV proteins make the catalytic cleft shorter and wider; from modeling studies, it is predicted that only three N-acetylglucosamine-binding subsites exist in class IV. Further, the structural comparisons suggest that the family 19 enzymes become more closed on substrate binding. Attempts to solve the structure of the complete protein including the associated chitin-binding module failed, however, modeling studies based on close relatives indicate that the binding module recognizes at most three N-acetylglucosamine units. The combined results suggest that the class IV enzymes are optimized for shorter substrates than the class I and II enzymes, or alternatively, that they are better suited for action on substrates where only small regions of chitin chain are accessible. Intact spruce chitinase is shown to possess antifungal activity, which requires the binding module; removing this module had no effect on measured chitinase activity.

Keywords: chitinase; family 19; *Picea abies*; Norway spruce; conformational changes; Class IV

Introduction

Chitin is a rigid linear polymer composed of β -(1 $\rightarrow$ 4) linked N-acetylglucosamine units (GlcNAc). It is the major component of insect and crustacean exoskeletons, and of fungal cell walls. Chitinases (EC 3.2.1.14, reviewed elsewhere (Patil et al 2000)) hydrolyze the glycosidic bonds in chitin. These enzymes are found in many species, ranging from viruses and bacteria to plants and animals. Depending on the origin, the enzymes have different functions, which include roles in nutrition, growth, development and pathogenesis. As a result, they vary widely in spatial and temporal localization, substrate specificity and structure.

It is believed that plant chitinases play their most significant roles in defense against pathogenic attacks (Brunner et al 1998; Frettinger et al 2006), stress responses (Kim et al 2003; Melchers et al 1994), growth and development, e.g. in zygotic and somatic embryogenesis (Wiweger et al 2003). It has been reported that the enzymes are expressed in all organs and tissues of plants in both the vacuole and the apoplast (Kasprzewska 2003). Consistent with their diverse functions, chitinases can act on various substrates, including chitin (Brunner et al 1998), lipochitooligosaccharides (Nod factors) produced by nitrogen-fixing bacteria (Schultze et al 1998), bacterial peptidoglycan (a polymer of β -1,4 linked GlcNAc and N-acetylmuramic acid residues (Brunner et al 1998)) and arabinogalactan proteins (van Hengel et al 2001). It has been speculated that other GlcNAc-containing glycoproteins present in cell walls can also be endogenous substrates for plant chitinases (Berger et al 1995). Chitinases have been identified as potential bio-control agents, and the enzyme from yam (*Dioscorea opposita* Thunb) has been found to control powdery mildew infections in strawberries (Karasuda et al 2003). Transgenic plants overexpressing such enzymes show enhanced protection against fungal disease (Broglie et al 1991).

Chitinases have been classified into two glycoside hydrolase (GH) families, 18 and 19 (Henrissat and Bairoch 1993), which exhibit different structures and catalytic mechanisms. GH family 18 chitinases having an eight-stranded α/β barrel (TIM-barrel) architecture show two general types; i.e. a 'plant type' with endo-activity that generates products of varying length, and a 'bacterial type' with exo-activity releasing chitobiose or chitotriose from the non-reducing end of chitin (Andersen et al 2005; van Aalten et al 2001). By contrast, GH family 19 chitinases are inverting endochitinases with bi-lobed, highly α -helical structures; they produce variously sized chito-oligomers as products (Robertus and Monzingo 1999). The inverting enzymes act *via* a single/direct displacement step that

replaces the leaving group with a molecule of water. Most plant enzymes belong to GH family 19.

Many chitinases from both families possess one or two chitin-binding modules (CtBM) that have been suggested to anchor the catalytic module (CM) near the substrate and so promote activity.

According to a common alternative nomenclature, plant chitinases have been classified based on sequence similarities in the CM, as well as the presence or absence of an N-terminal CtBM (Meins et al 1994; Neuhaus et al 1996). In this system, most GH family 19 enzymes fall into classes I, II and IV. As outlined in Fig. 1, class I and II enzymes have strongly related CMs, and are distinguished only by the fact that the latter lack a CtBM. Plant chitinases were originally assigned to class IV if their CMs had more than 50% sequence identity to *Phaseolus vulgaris* PR (pathogenesis related) 4 chitinase and possessed deletions relative to classes I and II: one in the CtBM, several in the CM (~22 amino acids deleted) and one at the C-terminal end (Meins et al 1994). There are bacterial examples within family 19, as well, which are not covered by the class system. These enzymes also have several deletions, but in different regions of the sequence.

Family 19 CM structures were first solved for the class II chitinase from barley (Protein Data Bank (PDB) (<http://www.pdb.org>) entries 2BAA (Hart et al 1995) and 1CNS (Song 1996)). Later work supplied structures of the CMs of class II enzymes from jack bean (PDB entry 1DXJ (Hahn et al 2000)) and rice (PDB entry 2DKV (Mizuno et al 2008)) and the class I-like enzyme from *Brassica juncea* (leaf mustard; PDB entries 2Z37, 2Z38, 2Z39 (Ubhayasekera et al 2007)). The last differs from typical class I enzymes in that it possesses two CtBMs rather than one; however, the CM has 55-60% amino acid sequence identity to enzymes of class I and II. Family 19 bacterial chitinase structures have been presented for chitinase C from *Streptomyces griseus* HUT6037 (PDB entries 1WVU, 1WVV and 2DBT (Kezuka et al 2006)) and chitinase G from *Streptomyces coelicolor* A3(2) (PDB entry 2CJL (Hoell et al 2006)).

In contrast to the situation for GH family 18 chitinases, no ligand-bound structure was reported for family 19 until very recently, largely due to the lack of known inhibitors. The location of the active site was deduced on the basis of conserved residues that line the large cleft seen in the apo structures, which appeared to be suitable for binding and hydrolysis of the chitin chain. In such inverting enzymes, two widely separated (~9Å) acidic residues were thought to be required for catalysis. One was proposed to act as a general acid that donates a proton in the reaction, and the other as a general base that activates a nucleophilic water molecule and stabilizes a charged intermediate (Brameld and

Goddard 1998; Fukamizo 2000; Hahn et al 2000). The very recent publication of the structure of class I or II chitinase from papaya bound to two GlcNAc molecules (Huet et al 2008) provided a basis for modeling the binding of four GlcNAc units in the active site, and supported the proposed reaction mechanism.

Another important gap in our understanding of chitinases has been the lack of any class IV structure. In *Picea abies* (Norway spruce), for example, class I, II and IV chitinases are all secreted in response to the plant pathogen *Heterobasidion annosum* (the causative agent of root and butt rot, the most devastating disease of conifer trees in boreal forests (Hietala et al 2004)), yet how insertions/deletions in the sequences/structures were related to particular biochemical or biological properties remained unknown. Given that coniferous species have such a wide range of commercial uses e.g. as timber for construction, raw material for pulp and paper production, and as an energy source, a better understanding of the properties of the different types of plant enzymes would be useful in future genetic manipulation. In this paper, we present the first structures of a CM of a GH family 19 class IV chitinase, the enzyme from Norway spruce (PaChi). The structures show how modifications in the sequence can alter substrate preferences, specifically leading to the prediction that class IV enzymes will recognize fewer GlcNAc units than class I/II enzymes. Further, comparisons with previous structures suggest that large-scale conformational changes are associated with substrate binding and activity. We demonstrate, and investigate the basis of, the enzyme's anti-fungal activity against *H. annosum*, showing that it requires the presence of the associated CtBM.

Materials and methods

Construction of yeast expression plasmids

DNA templates that originated from earlier work (Wiweger et al 2003) were used to prepare wild type and Y158C mutant proteins. Polymerase chain reaction (PCR)-generated DNA fragments encoding the various constructs were cloned into the *Pichia pastoris* expression vector pPIC9K (Invitrogen, San Diego, CA, USA), in-frame with the pPIC9K N-terminal secretory signal peptide, to produce recombinant fusion proteins that would be secreted into the growth media.

The yeast plasmid pPa1 for recombinant PaChi(I)-Y158C expression was constructed by cloning a DNA fragment encoding amino acids 1-251 of PaChi. This 0.7-kb fragment was PCR-amplified using primer pair CHB(MLC)/CH2(MLC), and a template sequence containing the full-

length *PaChi1* cDNA found in GenBank accession **FJ423771**. The forward primer CHB(MLC) (5'-CAGAATTC***GCT***CAAACTGTGGCTGTG-3') has an *EcoRI* site (underlined) adjacent to codon 26 (bold italics). Reverse primer CH2(MLC) (5'-ATGCGGCCGCTTAGCAGGAGACATTGGCT-3') contains a *NotI* site (underlined). The *EcoRI* and *NotI* sites were incorporated into the forward and reverse primers, respectively, so that the fragment could be subsequently cleaved and inserted into the *EcoRI* and *NotI* sites of vector pPIC9K. Plasmid pPa2 contains PaChi(s)-Y158C, and includes only the catalytic domain of the mutant enzyme (residues 48-251). A 0.6-kb DNA fragment was PCR-amplified using primer pair Cat(MLC)/CH2(MLC) and **FJ423771** as template. The forward primer Cat(MLC) (5'-CAGAATTC***AGCGT***GGGGGGCATAATTTC-3') has an *EcoRI* site (underlined) adjacent to codon 72 (bold italics).

Similarly, plasmid pPa3 coded for the longer version of the wild type protein. A 0.7-kb DNA fragment was PCR-amplified using primer pair CHB(MLC)/CH2(MLC) and sequence **FJ423770** containing the full-length *PaChi2* cDNA as template. Plasmid pPa4 encodes only the CM of the wild type protein. A 0.6-kb DNA fragment was PCR-amplified using primer pair Cat(MLC)/CH2(MLC) and **FJ423770** as template.

PCR amplification was performed using 100 pg of plasmid DNA in a 100-μl reaction mixture, containing 2.5 U *Pfu* DNA polymerase (Life Technologies), 100 μM of each dNTP and 100 pM of primers. Reactions were incubated at 95 °C for 4 min, followed by 29 cycles of denaturation (95 °C for 15 s), annealing (50 °C for 15 s) and extension (72 °C for 90 s), with a final extension at 72 °C for 10 min. Following PCR, the DNA was cleaved with *EcoRI* and *NotI*, before cloning into corresponding sites in plasmid pPIC9K. *Escherichia coli* Top10F' (Invitrogen) cells were transformed with the pPIC9K derivatives. PCR-derived DNA in the plasmids was analyzed by DNA sequencing before transformation of *P. pastoris*.

Transformation of P. pastoris

Following linearization with *SacI*, each plasmid was used in transformation of *P. pastoris* strain KM71 by electroporation (Scorer et al 1994). His⁺ transformants were selected on RDB agar (1 M sorbitol, 2% dextrose, 1.34% yeast nitrogen base, 4x10⁻⁵% biotin) and were subsequently screened for G418 resistance at a final concentration of 1.0 mg/ml G418, according to the instructions in the Multi-copy

Pichia Expression Kit (Invitrogen). PCR and Southern blot analyses were used to confirm that target DNA had recombined into the chromosomes of the yeast before use in protein expression.

Production and purification of secreted chitinases expressed in P. pastoris

P. pastoris strain KM71 was used for expression of recombinant chitinases. Each yeast strain was maintained on YPD agar (1% (w/v) yeast extract, 2% (w/v) peptone, 2% dextrose, 2% agar). Minimal medium, consisting of 1.34% (w/v) yeast nitrogen base, 4×10^{-5} % (w/v) biotin and 2% (w/v) dextrose, was used in selection.

To initiate protein production in *Pichia*, 10 ml of BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base, 4×10^{-5} % (w/v) biotin, 1% (v/v) glycerol) was inoculated with a single colony and incubated overnight at 30 °C. Subsequently, the overnight culture was added to 800 ml of BMGY medium and further incubated until the absorbance at 600 nm reached at least 2.0. The cells were harvested by centrifugation at 5,000 x g at room temperature for 5 min. To induce recombinant protein expression, the cell pellet was resuspended in 80 ml of BMMY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base, 4×10^{-5} % (w/v) biotin, 0.5% (v/v) methanol) and grown at 30 °C for a further two days, during which methanol was added to a final concentration of 0.5% (v/v) every 24 h. Cells were then harvested by centrifugation (5,000 x g at 4 °C for 15 min). The *Pichia*-expressed protein in the supernatant was precipitated overnight using 65% (w/v) ammonium sulfate. The precipitated protein was resuspended in 10 mM sodium phosphate buffer, pH 7.2, then dialyzed against the same buffer. The dialyzed sample was centrifuged at 5,000 x g at 4 °C for 15 min, passed through a membrane filter (0.2 µM, Nalgene) and concentrated using a Centricon concentrator (10 kDa MW cut-off, Millipore). The protein was then loaded on a gel filtration (Superdex HiLoad 16/60) column in an FPLC system (GE Healthcare, Uppsala, Sweden). The column was pre-equilibrated with 10 mM sodium phosphate buffer, pH 7.2. Fractions of 2 ml were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was stained with Coomassie Brilliant Blue to check purity. The purest fractions containing the desired band were pooled and stored at -80 °C.

Purified proteins were shipped as ammonium sulfate precipitates by surface mail from Hong Kong to Sweden for structural and antifungal activity studies.

Chitinase assays

Colorimetric chitinase assays (Wirth and Wolf 1990) were carried out using the substrate carboxymethylchitin-Remazol Brilliant Violet 5R (Loewe Biochemica GmbH, Sauerlac, Germany). Samples from crude cell extracts (2-10 µg) were made up to 250 µL with water and incubated with 250 µL 0.1 M sodium acetate buffer, pH 5 and 250 µL CM-RBV-chitin at 37 °C for 1 h. The reaction was stopped with 250 µL 1 M HCl and placed on ice for 15 min to precipitate the non-degraded chitin. The reaction mixture was then centrifuged at 20,000 x g at 4 °C for 5 min. absorbance at 550 nm was read and values were corrected for background (using controls with protein that had been heated for 10 min at 100 °C to destroy activity). Three replicates were performed and average values were used.

Antifungal activity

Antifungal activity against *Heterobasidion annosum* (strain FP5) was tested using the agar diffusion method. Hagem agar plates (0.5 % glucose, 0.05 % NH₄NO₃, 0.05 % KH₂PO₄, 0.05 % MgSO₄·7H₂O, 0.5 % malt extract, 2.0 % agar, pH 5.5) were inoculated with an agar plug containing the hyphae of the fungus, then incubated at room temperature in the dark. When fungal growth was observed, three wells were created using a sterile pipette tip. After approximately four days, mycelial growth had reached the wells, after which addition of enzyme was initiated: 30 µl of protein (0.25 mg/ml) or buffer control was added twice a day for 5 days.

Crystallization

Protein samples were dialyzed against 10 mM HEPES buffer, pH 7.0, prior to crystallization experiments, and stored at 4 °C. In the first successful crystallization experiments, 1 µl of the mother-liquor containing 20% w/v polyethylene glycol 3000, 0.1M citrate, pH 5.5, was mixed with 1 µl of 8.2 mg/ml Norway spruce chitinase and then allowed to equilibrate by sitting-drop vapor diffusion at room temperature. Rectangular prisms (0.025 x 0.01 x 0.01 mm³) appeared after 7 months. The precipitant concentration was then optimized at 30%. Crystals were transferred briefly to 35% polyethylene glycol 1500 as the cryoprotectant and then flash-cooled directly in liquid nitrogen.

Data collection, structure solution and refinement

X-ray data were collected at 100 K at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The PaChi(s)-3 data were processed with DENZO and SCALEPACK (Otwinowski and Minor 1997), whereas the other sets were processed with MOSFLM (Leslie 1992) and SCALA (Evans 1997); statistics are presented in [Table 2](#).

Analysis of the unit-cell contents of PaChi(s)-1 suggested that there would be one molecule in the asymmetric unit, consistent with a solvent content of 34% and a V_M of 1.9 (Matthews 1968). Molecular replacement with MOLREP (Vagin and Teplyakov 1997), as implemented in the CCP4 interface (Collaborative Computational Project 1994) (Potterton et al 2003), utilized the homology model of PaChi(s) prepared earlier using the structure of barley seed chitinase as the template (PDB entry 1CNS; (Song 1996) 53% amino acid sequence identity in the matching regions) as a search model. The clear solution was improved with rigid-body and restrained refinement in REFMAC5 (Murshudov et al 1997). The protein was rebuilt as needed in O and refined in a cyclic fashion. Waters were placed using the ARP/wARP-solvent command in CCP4. For PaChi(s)-2 and PaChi(s)-3, the protein structure of PaChi(s)-1 was used as the starting point for refinement, rebuilding and water addition as described above. Statistics for the final refined models are presented in [Table 2](#).

Structural analysis, comparisons and homology modeling

Similar proteins were located using BLAST (Altschul et al 1997). Structures were obtained from the PDB (Berman et al 2000) and compared in O and LSQMAN (Kleywegt and Jones 1997). Pair-wise alignment of the CtBM and wheat germ agglutinin isolectin sequences (PDB entry 2WGC (Wright 1990)) was used to create a homology model with the program SOD (Kleywegt et al 2001). The model was adjusted in O (Jones et al 1991) using rotamers to improve packing as needed. Figures were prepared with the programs O, Molscript (Kraulis 1991) and Molray (Harris and Jones 2001).

PDB accession numbers

Atomic coordinates and structure factors have been deposited in the Protein Data Bank (<http://www.rcsb.org/>), with entry codes 3HBD, 3HBE and 3HBH for the 1.8, 1.55 and 2.25 Å resolution structures, respectively.

Results

Constructs, chitinase and anti-fungal activity

Residue numbering refers to the sequence of the mature protein, which lacks the 26-residue signal sequence, but includes a CtBM, 12-residue linker and CM. The four constructs used (Fig. 2) originated from two different plasmids, one with the wild type sequence, and one with Tyr158 replaced by cysteine (the latter obtained through a fortuitous mutation at the amplification step). Each variant is represented by two constructs. One pair of constructs comprises the complete mature protein, i.e. PaChi(l) and PaChi(l)-Y158C, while the other includes only the catalytic domain (PaChi(s) and PaChi(s)-Y158C).

In each case, the chitinase activity was indistinguishable for the long and short constructs (Table 1) with carboxymethylchitin-Remazol Brilliant Violet 5R as a substrate. The mutated chitinases with Tyr158 replaced by cysteine were inactive within the limits of sensitivity in the assay.

PaChi(l) and PaChi(s) were tested for activity against the pathogen *H. annosum* (Fig. 3). The longer construct containing the CtBM inhibited fungal growth most efficiently. A very low level of inhibition was exhibited by PaChi(s) at micromolar concentrations.

Homology modeling of the chitin-binding module (CtBM)

The CtBM of PaChi has been assigned to carbohydrate binding module family 18 (Boraston et al 2004). Homology modeling using the closest available structure, wheat germ agglutinin isolectin 3 (amino acid sequence identity 65%), as template was carried out as a convenient way of synthesizing the structural and sequence data. The fold consists of a small, globular, irregular β -sheet with three strands (Fig. 4a), which is connected at its C-terminal end to the CM by a (presumably flexible) linker. Three disulfide bridges (Cys6/Cys18, Cys11/Cys25 and Cys29/Cys34) maintain the shape and stability of the fold, and are correspondingly highly conserved in related sequences. Three surface aromatic residues are also well conserved, in this case tyrosines 15, 17 and 24. These and other highly conserved residues concentrated on one face of the protein (shown in Fig. 4b) are likely to be significant for chitin binding. Although the available structures for complexes of family 18 binding modules (e.g. 1EN2, 1ENM, 1K7T and 1T0W) show some variation in the exact positions of binding subsites on this surface, the model, as shown in Fig. 4b, suggests that three GlcNac units can be accommodated.

Structures of PaChi(s)

Crystals were obtained only with the sample of PaChi(l). However, the three structures obtained were found to lack the CtBM, and we will therefore refer to them as PaChi(s). SDS gels indicated that cleavage of the protein had indeed occurred on prolonged storage and in the crystallization drop.

The first structure, PaChi(s)-1, was solved by molecular replacement using a homology model based on the structure of the barley seed CM (53% amino acid sequence identity in the matching regions, PDB entry 1CNS (Song 1996)) as a search model, and refined to 1.8 Å resolution. PaChi(s)-2 and PaChi(s)-3 were solved using the protein portion of PaChi(s)-1 as the starting model, and refined to resolutions of 1.55 and 2.25 Å, respectively. Data collection and refinement statistics are reported in [Table 2](#).

The three structures are generally similar, although some interesting differences are described below. Maps in all cases show clear electron density for residues Ser48 to Cys251, revealing the CM to be a highly α -helical, bi-lobed structure with a small irregular β -sheet ([Fig. 5a](#)). The two lobes are demarcated by a wide cleft that runs along one face of the protein ([Fig. 5b](#)). Disulfide bonds link residues Cys69/Cys118, Cys130/Cys138 and Cys219/Cys251. Comparison of the C α backbones of the various pairs of molecules resulted in overall root-mean-square (r.m.s.) differences in the range of 0.1-0.2 Å. The largest differences (~ 0.4 Å) are in segments designated as loops I and III in [Fig. 6a](#); larger movements in these loops seem to be prevented by crystal packing. The temperature factors for the two loops are not significantly elevated (e.g. average B factors 13 and 14 Å² in PaChi(s)-2, compared to values of ~ 12 Å² in the core of the protein).

Based on sequence comparisons within GH family 19 (Tang et al 2004; Ubhayasekera et al 2007) and by analogy to the structure of the papaya chitinase bound to two molecules of GlcNAc (Huet et al 2008), the active site lies in the wide cleft between the two domains. The general acid, Glu113, is part of a triad that also includes Arg230 and Glu218 ([Fig. 7](#)). Two conformations of Glu113 are clearly substantiated by electron density. That marked as “X” in [Fig. 7a](#) is seen in PaChi(s)-1, while that marked as “Y” is seen in PaChi(s)-3; both conformations are observed in the highest resolution structure, PaChi(s)-2. All of these crystals were grown at pH 5.5, close to the expected pK_a of a glutamate side chain. One carboxylate oxygen of the glutamate (OE2) always makes a salt link to

Arg230, although with different nitrogen atoms in the two conformations. The side-chain density of Glu218 is not as well defined in any of the structures as that of the other two residues of the triad (Fig. 7b); this residue also has higher temperature factors in all of the structures (B factors are on average 9 Å² for Glu113 and Arg230 in PaChi(s)-2, while those of Glu218 average 20 Å²). Glu122 (average B factor 12 Å² in PaChi(s)-2), which is thought to be the general base, is positioned on the opposite side of the binding cleft. The closest distance between Glu113 and Glu122 is ~9Å, compatible with the separation generally observed for inverting GHs (McCarter and Withers 1994). However, one of the Glu113 conformations (X) brings the two residues closer, with a minimum distance of 8.7 Å as compared to 9.7 Å. Three water molecules that are consistently observed close to the catalytic residues are also shown in Fig. 7a.

Comparisons to other family 19 chitinases

Comparisons of PaChi to the other available family 19 structures are summarized in Table 3, and related to the sequences in Fig. 1. It can be seen that the PaChi is equally distant from bacterial and class I/II enzymes, with r.m.s. differences ~1 Å, and sequence identity ~50% in the equivalent regions. It was found earlier (Ubhayasekera et al 2007) that some surface loops (designated as loops I-V) of *Brassica juncea* class I-like chitinase were flexible. By this convention, loops II, IV and V are missing from the PaChi structure (Fig. 6b), due to deletions after residues 115, 194 and 203, respectively. As noted above, some plasticity is observed in the two remaining loops of PaChi, i.e. loops I (residues 65-71) and III (residues 123-144), both of which border the active site cleft. All three disulfide bonds are found in equivalent positions in class I, II and IV proteins. Using the same convention, loops I, II and V are missing from the family 19 bacterial chitinases (Fig. 1). The first of the three disulfide bonds is also absent. Comparison of the two molecules in the asymmetric unit in the bacterial structures indicated that loops III and IV show some mobility, comparable to that of loops I and III in PaChi.

The structure of papaya chitinase bound to GlcNAc is significantly more closed than any of the others, as reflected in the distances between pairs of residues in the two lobes (Table 3), and illustrated for the papaya/PaChi comparison in Fig. 6c.

Discussion

Based on strong sequence conservation within GH family 19, two glutamates lining the putative binding cleft were quickly identified as catalytic residues of PaChi. The first, Glu113, is found in a triad that also includes Arg230 and Glu218, and is believed to donate a proton to O1/O4 in the inverting mechanism. It was speculated previously (Tang et al 2004) that the function of the triad could be to change the pKa of the proton donor to activate it in catalysis. In the present structures, Glu113 shows two conformations, indicating that this side chain can move if necessary during the course of the reaction, while still maintaining its interaction with Arg230. For other family 19 enzymes, mutation of either of these two residues caused an essentially complete loss in activity, but mutation of the second glutamate (equivalent to Glu218 here) had much less drastic effects (Tang et al 2004).

Glu122 lies on the other side of the active site cleft, and presumably, the other side of the chitin chain. This glutamate is thought to serve as a general base that activates a water, which subsequently carries out a nucleophilic attack on the anomeric carbon, resulting in cleavage, and inversion of the configuration (Brameld and Goddard 1998). However, it has been observed that this residue is not as critical for activity as the proton donor, based on mutational studies of *Brassica* chitinase (Tang et al 2004) and *S. coelicolor* A3(2) chitinase G (Hoell et al 2006). It seems likely that the correct positioning of a water molecule near Ser153 is sufficient for catalysis to occur.

A fuller understanding of the action of family 19 chitinases was long hampered by the lack of known inhibitors, and so of interesting complex structures. This was thought to arise from the wide catalytic cleft, which is better suited to the binding of large, insoluble molecules rather than of small, soluble compounds. Very recently, the structure of papaya chitinase (Huet et al 2008), crystallized in the presence of very high (molar) concentrations of GlcNAc, provided vital information about chitin binding. For example, the profound effects of a mutation in His211 of the *Brassica* enzyme (Tang et al 2004) (equivalent to His112 of PaChi) are likely to result from problems in binding/positioning of the substrate. Tyr158 (which is shown to be the site of an inactivating mutation here) has a more structural role, being part of a newly described motif that is conserved in most family 19 sequences (Huet et al 2008).

As the first structures of a class IV enzyme, our study of PaChi illuminates other links between structure and catalysis. Five flexible loops bordering the active-site cleft were identified in the class I *Brassica* structure, as defined in Fig. 6. Comparison of the available structures and sequences indicates that loops II, IV and V are missing in class IV enzymes, while loops I, II, V are missing in the bacterial

chitinases. Thus, loop III is the only one present in all enzymes; further it has a sequence that is well conserved, and exhibits flexibility that could be relevant in catalysis. (Loop III actually shows less variation in PaChi than in some of the other structures, as it is constrained by crystal packing contacts.) This loop adjoins Glu122, the putative general base.

A careful comparison of the GlcNAc-bound papaya structure with the others (which are all apo forms) uncovers an additional feature, which has not been noted previously. When the structures are aligned with a cutoff appropriate to the sequence similarity of the structures involved (1 Å), it can be seen that the papaya structure has a different conformation. This can be envisioned as one lobe of the apo structures (residues 48-192 in PaChi numbering) moving as a rigid body, coming closer to the other lobe (residues 193-251 of PaChi) when the GlcNAc residues are bound; the motion occurs as a result of flexibility near loops IV and V (Fig. 6c). The decreased distance between the two lobes is indicated by some representative distances reported in Table 3. Such a conformational change might be expected to give rise to tighter binding of the substrate, and more optimal placement of the catalytic residues. Further, we observe that PaChi crystals cracked when soaked with either chitotriose or chitopentose; presumably the necessary conformational changes could not be accommodated within the existing crystal lattice. Most of the predicted binding interactions involve the earlier region of the sequence (i.e. the larger lobe), so substrate almost certainly binds there first.

Differences in the remaining loops (i.e. I, II, IV and V) of the various chitinases are probably linked to substrate specificity rather than to catalytic action. It seems worth noting that, unlike family 18 chitinases and cellulases, there are no family 19 chitinases that have an enclosed tunnel, that is, all appear to be endo-acting enzymes directed at large insoluble substrates. The number and location of GlcNAc subsites seem to vary. In class I/II enzymes, subsites -2 to +2 have been described (Huet et al 2008). The bacterial enzyme, which lacks loops I, II and V, has also been demonstrated experimentally to have four subsites (Hoell et al 2006). By contrast, class IV enzymes lack loops II, IV and V, and are also shorter at the C-terminus. The result is a more open binding cleft, which may lack subsites -3 and +2. The enzymes thus seem most appropriate for the recognition of three GlcNAc units, although a bulkier substrate can extend out from the binding cleft at both ends without obstruction. The endogenous substrate for PaChi is presently unknown, and to our knowledge, class IV enzymes have not been tested with smaller substrates, but clearly the determination of substrate preferences should be the subject for future experiments with this class of enzyme.

The fungus *Heterobasidion annosum*, the causative agent of root and butt rot, is the most destructive conifer pathogen in the northern hemisphere. As both CtBMs and chitinases are among the most common pathogenesis related proteins, we wished to know if PaChi might be linked to antifungal action, and if so, which domains of the protein were responsible. The results reported here suggest that the CtBM accounts for most, if not all, of the antifungal activity of PaChi (Fig. 3). Similar inhibition has been observed for other chitinases on various fungal species (Arlorio et al 1992; Kawase et al 2006; Vierheilig et al 2001). However, the time scale of this type of experiment is sufficiently long that proteolysis can occur, and so it is not clear if the active species in this case is the CtBM itself, or if the antifungal activity of the CM depends on it being anchored to the fungus via the binding module. Modeling studies indicate that conserved surface of the CtBM, including three aromatic residues, will form a maximum of three subsites for GlcNAc units (Fig. 4b). The indication that the CM itself may recognize only three GlcNAc units is thus intriguing. It has been reported (Arlorio et al 1992) that chitinases inhibit fungal growth by attacking the apex of fungal hyphae. In the process, the hyphal walls become thinner and the tips swell until the plasma membrane ruptures. It has been demonstrated that the inhibitory activity of chitinase against pathogenic fungi is directly correlated with both the extent of exposure and proportion of chitin in the fungal cell wall (Yan et al 2008). Correspondingly, if class IV enzymes are able to act on relatively small segments of exposed substrate, this may well give rise to improved function in this biological context. Further, the observation that increased PaChi expression is associated with programmed cell death, a process that is linked to GlcNAc-containing glycoproteins that would be expected to bind more easily in a wider, less restricted active site cleft, is also thought-providing (Wiweger et al 2003). Clearly, more studies aimed at this class of proteins are needed.

Acknowledgments

The authors would like to thank Dr. Fred Asiegbu (Swedish University of Agricultural Sciences) for providing *Heterobasidion annosum* (strain FP5), and Dr. Mark Harris (Uppsala University) for photographing the chitinase-inhibited fungal plate. The work was supported by the Swedish Research Council (VR) and the Swedish Foundation for Strategic Research via the Glycoconjugates in

Biological Systems network, GLIBS (SLM), as well as by the University of Hong Kong (ORA10208034) (MLC).

References

- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389-402
- Andersen OA, Dixon MJ, Eggleston IM, van Aalten DM (2005) Natural product family 18 chitinase inhibitors. *Nat Prod Rep* 22: 563-79
- Arlorio M, Ludwig A, Boller T, Bonfante P (1992) Inhibition of Fungal Growth by Plant Chitinases and Beta-1,3-Glucanases - a Morphological-Study. *Protoplasma* 171: 34-43
- Berger S, Menudier A, Julien R, Karamanos Y (1995) Do de-N-glycosylation enzymes have an important role in plant cells? *Biochimie* 77: 751-60
- Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE (2000) The Protein Data Bank. *Nucleic Acids Res.* 28: 235-42
- Boraston AB, Bolam DN, Gilbert HJ, Davies GJ (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. *Biochem. J.* 382: 769-81
- Brameld KA, Goddard WA, 3rd (1998) The role of enzyme distortion in the single displacement mechanism of family 19 chitinases. *Proc. Natl. Acad. Sci. U.S.A.* 95: 4276-81
- Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Broglie R (1991) Transgenic Plants with Enhanced Resistance to the Fungal Pathogen *Rhizoctonia solani*. *Science* 254: 1194-1197
- Brunner F, Stintzi A, Fritig B, Legrand M (1998) Substrate specificities of tobacco chitinases. *Plant J.* 14: 225-34
- Collaborative Computational Project N (1994) The CCP4 Suite: Programs for Protein Crystallography. *Acta Crystallogr. D Biol. Crystallogr.* 50: 760-763
- Engh RA, Huber R (1991) Accurate Bond and Angle Parameters for X-Ray Protein-Structure Refinement. *Acta Crystallogr. A* 47: 392-400
- Evans PR: Scaling of MAD data. In: Wilson KS, Davies G, Ashton AW, Bailey S (eds) *CCP4 Study Weekend*, pp. 97-102 (1997).
- Frettinger P, Herrmann S, Lapeyrie F, Oelmüller R, Buscot F (2006) Differential expression of two class III chitinases in two types of roots of *Quercus robur* during pre-mycorrhizal interactions with *Piloderma croceum*. *Mycorrhiza* 16: 219-23
- Fukamizo T (2000) Chitinolytic enzymes: catalysis, substrate binding, and their application. *Curr. Protein Pept. Sci.* 1: 105-24
- Hahn M, Hennig M, Schlesier B, Hohne W (2000) Structure of jack bean chitinase. *Acta Crystallogr. D Biol. Crystallogr.* 56 (Pt 9): 1096-9
- Harris M, Jones TA (2001) Molray-a web interface between O and the POV-Ray ray tracer. *Acta Crystallogr. D Biol. Crystallogr.* 57: 1201-3
- Hart PJ, Pflugger HD, Monzingo AF, Hollis T, Robertus JD (1995) The refined crystal structure of an endochitinase from *Hordeum vulgare* L. seeds at 1.8 Å resolution. *J. Mol. Biol.* 248: 402-13
- Henrissat B, Bairoch A (1993) New Families in the Classification of Glycosyl Hydrolases Based on Amino-Acid-Sequence Similarities. *Biochem. J.* 293: 781-788
- Hietala AM, Kvaalen H, Schmidt A, Johnk N, Solheim H, Fossdal CG (2004) Temporal and spatial profiles of chitinase expression by norway spruce in response to bark colonization by *Heterobasidion annosum*. *Appl. Environ. Microbiol.* 70: 3948-53
- Hoell IA, Dalhus B, Heggset EB, Aspö SI, Eijsink VG (2006) Crystal structure and enzymatic properties of a bacterial family 19 chitinase reveal differences from plant enzymes. *FEBS J.* 273: 4889-900
- Huet J, Rucktooa P, Clantin B, Azarkan M, Looze Y, Villeret V, Wintjens R (2008) X-ray structure of papaya chitinase reveals the substrate binding mode of glycosyl hydrolase family 19 chitinases. *Biochemistry* 47: 8283-91
- Jones TA, Zou JY, Cowan SW, Kjeldgaard (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr. A* 47 (Pt 2): 110-9

- Karasuda S, Tanaka S, Kajihara H, Yamamoto Y, Koga D (2003) Plant chitinase as a possible biocontrol agent for use instead of chemical fungicides. *Biosci. Biotechnol. Biochem.* 67: 221-4
- Kasprzewska A (2003) Plant chitinases--regulation and function. *Cell. Mol. Biol. Lett.* 8: 809-24
- Kawase T, Yokokawa S, Saito A, Fujii T, Nikaidou N, Miyashita K, Watanabe T (2006) Comparison of enzymatic and antifungal properties between family 18 and 19 chitinases from *S. coelicolor* A3(2). *Biosci Biotechnol Biochem* 70: 988-98
- Kezuka Y, Ohishi M, Itoh Y, Watanabe J, Mitsutomi M, Watanabe T, Nonaka T (2006) Structural studies of a two-domain chitinase from *Streptomyces griseus* HUT6037. *J. Mol. Biol.* 358: 472-84
- Kim JS, Kim YO, Ryu HJ, Kwak YS, Lee JY, Kang H (2003) Isolation of stress-related genes of rubber particles and latex in fig tree (*Ficus carica*) and their expressions by abiotic stress or plant hormone treatments. *Plant Cell Physiol.* 44: 412-4
- Kleywegt GJ, Jones TA (1996) Phi/psi-chology: Ramachandran revisited. *Structure* 4: 1395-400
- Kleywegt GJ, Jones TA: Detecting folding motifs and similarities in protein structures. In: Carter J, C.W., Sweet RM (eds) *Methods in Enzymology, Macromolecular Crystallography, Pt B*, pp. 525-545. Academic Press (1997).
- Kleywegt GJ, Zou JY, Kjeldgaard M, Jones TA: Around O. In: Rossmann MG, Arnold E (eds) *International Tables for Crystallography, Vol. F. Crystallography of Biological Macromolecules*, pp. 353-356 366-367. Kluwer Academic, Dordrecht (2001).
- Kraulis PJ (1991) Molscript - a Program to Produce Both Detailed and Schematic Plots of Protein Structures. *J. Appl. Crystallogr.* 24: 946-950
- Leslie AGW (1992) Recent changes to the MOSFLM package for processing film and image plate data. *Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography*
- Matthews BW (1968) Solvent Content of Protein Crystals. *J. Mol. Biol.* 33: 491-&
- McCarter JD, Withers SG (1994) Mechanisms of enzymatic glycoside hydrolysis. *Curr. Opin. Struct. Biol.* 4: 885-92
- Meins F, Fritig B, Linthorst HJM, Mikkelsen JD, Neuhaus JM, Ryals J (1994) Plant chitinase genes. *Plant Mol. Biol. Rep.* 12: S22-S28
- Melchers LS, Apotheker-de Groot M, van der Knaap JA, Ponstein AS, Sela-Buurlage MB, Bol JF, Cornelissen BJ, van den Elzen PJ, Linthorst HJ (1994) A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity. *Plant J.* 5: 469-80
- Mizuno R, Fukamizo T, Sugiyama S, Nishizawa Y, Kezuka Y, Nonaka T, Suzuki K, Watanabe T (2008) Role of the loop structure of the catalytic domain in rice class I chitinase. *J. Biochem.* 143: 487-95
- Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr. D Biol. Crystallogr.* 53: 240-255
- Neuhaus JM, Fritig B, Linthorst HJM, Meins F, Mikkelsen JD, Ryals J (1996) A revised nomenclature for chitinase genes. *Plant Mol. Biol. Rep.* 14: 102-104
- Otwinowski Z, Minor W: Processing of X-ray diffraction data collected in oscillation mode. In: Carter J, C.W., Sweet RM (eds) *Methods in Enzymology, Macromolecular Crystallography, Pt A*, pp. 307-326. Academic Press (1997).
- Patil RS, Ghormade V, Deshpande MV (2000) Chitinolytic enzymes: an exploration. *Enzyme Microb. Technol.* 26: 473-483
- Potterton E, Briggs P, Turkenburg M, Dodson E (2003) A graphical user interface to the CCP4 program suite. *Acta Crystallogr. D Biol. Crystallogr.* 59: 1131-1137
- Robertus JD, Monzingo AF (1999) The structure and action of chitinases. *Exs* 87: 125-35
- Schultze M, Staehelin C, Brunner F, Genetet I, Legrand M, Fritig B, Kondorosi E, Kondorosi A (1998) Plant chitinase/lysozyme isoforms show distinct substrate specificity and cleavage site preference towards lipochitooligosaccharide Nod signals. *Plant J.* 16: 571-580
- Scorer CA, Clare JJ, McCombie WR, Romanos MA, Sreekrishna K (1994) Rapid selection using G418 of high copy number transformants of *Pichia pastoris* for high-level foreign gene expression. *Biotechnology (N Y)* 12: 181-4
- Song HK (1996) Refined Structure of the Chitinase from Barley Seeds at 2.0 Å Resolution. *Acta Crystallogr. D Biol. Crystallogr.* 52: 289-298
- Tang CM, Chye ML, Ramalingam S, Ouyang SW, Zhao KJ, Ubhayasekera W, Mowbray SL (2004) Functional analyses of the chitin-binding domains and the catalytic domain of *Brassica juncea* chitinase BjCHI1. *Plant Mol. Biol.* 56: 285-98

- Ubhayasekera W, Tang CM, Ho SW, Berglund G, Bergfors T, Chye ML, Mowbray SL (2007) Crystal structures of a family 19 chitinase from *Brassica juncea* show flexibility of binding cleft loops. *FEBS J.* 274: 3695-3703
- Vagin A, Teplyakov A (1997) MOLREP: an automated program for molecular replacement. *J. Appl. Crystallogr.* 30: 1022-1025
- van Aalten DM, Komander D, Synstad B, Gaseidnes S, Peter MG, Eijssink VG (2001) Structural insights into the catalytic mechanism of a family 18 exo-chitinase. *Proc. Natl. Acad. Sci. U.S.A.* 98: 8979-84
- van Hengel AJ, Tadesse Z, Immerzeel P, Schols H, van Kammen A, de Vries SC (2001) N-acetylglucosamine and glucosamine-containing arabinogalactan proteins control somatic embryogenesis. *Plant Physiol.* 125: 1880-90
- Vierheilig H, Alt-Hug M, Wiemken A, Boller T (2001) Hyphal in vitro growth of the arbuscular mycorrhizal fungus *Glomus mosseae* is affected by chitinase but not by beta-1,3-glucanase. *Mycorrhiza* 11: 279-282
- Wirth SJ, Wolf GA (1990) Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. *J. Microbiol. Methods* 12: 197-205
- Wiweger M, Farbos I, Ingouff M, Lagercrantz U, Von Arnold S (2003) Expression of Chia4-Pa chitinase genes during somatic and zygotic embryo development in Norway spruce (*Picea abies*): similarities and differences between gymnosperm and angiosperm class IV chitinases. *J. Exp. Bot.* 54: 2691-9
- Wright CS (1990) 2.2 A resolution structure analysis of two refined N-acetylneuraminyl- lactose--wheat germ agglutinin isolectin complexes. *J. Mol. Biol.* 215: 635-51
- Yan R, Hou J, Ding D, Guan W, Wang C, Wu Z, Li M (2008) In vitro antifungal activity and mechanism of action of chitinase against four plant pathogenic fungi. *J. Basic Microbiol.* 48: 293-301

Table 1. Activity of recombinant chitinases.

Recombinant Proteins	Specific activity
	Umin ⁻¹ μg ⁻¹
PaChi(l)	0.015±0.003
PaChi(s)	0.015±0.001
PaChi(l)-Y158C	0.002±0.004
PaChi(s)-Y158C	0.001±0.002
KM71with pPIC9K vector only (control)	0.002±0.002

One enzyme unit of chitinase was defined as the amount of enzyme liberating 1 mg of soluble GlcNAc from chitin per hour at infinite dilution.

Table 2. Data collection and refinement statistics for PaChi(s) structures. Information in parentheses refers to the highest resolution shell.

Data collection	PaChi(s)-1	PaChi(s)-2	PaChi(s)-3
Environment	ESRF ID23:1	ESRF ID 14:4	ESRF ID23:2
Wavelength (Å)	0.9998	0.9395	0.873
Space group	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2
Cell dimensions (Å)	67.0 65.6 36.5	67.2 65.8 36.5	67.6 66.4 36.6
Resolution (Å)	50-1.8 (1.9-1.8)	50-1.55 (1.63-1.55)	50-2.25 (2.29-2.25)
Unique reflections	14815	23744	8202
Average multiplicity	4.2 (3.6)	6.8 (6.4)	5.9 (2.6)
Completeness (%)	97.4 (88.5)	97.4 (92.6)	99.1 (92.9)
R _{merge}	7.8 (48.1)	8.8 (21.9)	10.5 (22.5)
Mn (I/sd)	13 (3.1)	16.7 (6.3)	
Refinement			
Number of reflections (completeness, %)	14262 (96.7)	22318 (97.1)	7796 (98.8)
Resolution range (Å)	50-1.8	50 – 1.55	36.61-2.25
R-factor / R-free (%)	17.6 (21.0)	16.3 (19.4)	15.1 (22.4)
Number of protein atoms (Average B, Å ²)	1511 (21.3)	1567 (12.5)	1511 (13.9)
Number of water molecules (Average B, Å ²)	91 (31.7)	226 (27.4)	127 (21.4)
r.m.s. bond length (Å) ^a	0.014	0.009	0.021
r.m.s. bond angle (°) ^a	1.3	1.0	1.6
No. Ramachandran plot outliers (%) ^b	2 (1.2)	2 (1.4)	3 (1.7)

^a Using the parameters of Engh & Huber (Engh and Huber 1991).

^b Calculated using a strict-boundary Ramachandran plot (Kleywegt and Jones 1996).

Table 3. Comparisons of PaChi(s)-2 with other available chitinase structures. The sequences are listed in descending order, according to similarities reported by a BLAST search (Altschul et al 1997) with the PaChi sequence. Where multiple molecules were present in the asymmetric unit, values refer to the A molecule. For each structure, the C α -C α distance is reported for the pair of residues that is equivalent to 152-214 and 129-215 (the equivalent distance is given for PaChi in the column heading, for comparison).

Protein source	PDB entry	Number Ca atoms matched	R.m.s. difference (Å)	Sequence identity to PaChi	Equivalent to 152-214 distance (11.0 Å)	Equivalent to 129-215 distance (16.4 Å)	Plant chitinase class	Ref.
Papaya	3CQL	194	1.3	51	9.2	13.6	I or II*	(Huet et al 2008)
<i>Streptomyces coelicolor</i>	2CJL	166	1.1	48	10.5	14.8	-	(Hoell et al 2006)
Jack bean	1DXJ	171	1.1	49	10.9	16.7	II	(Hahn et al 2000)
Barley	1CNS	197	1.0	53	10.5	16.1	II	(Song 1996)
<i>Streptomyces griseus</i>	1WVU	182	1.1	47	10.3	15.2	-	(Kezuka et al 2006)
Leaf mustard	2Z38	193	1.1	48	10.4	15.9	I-like	(Ubhayasekera et al 2007)
Barley	2BAA	198	1.2	51	11.4	18.0	II	(Hart et al 1995)
Rice	2DKV	197	1.1	50	10.5	16.3	I	(Mizuno et al 2008)

*No gene sequence information is available to assign the exact class.

Figure legends

Fig. 1. Schematic representation of the plant chitinase classes showing signal sequence, chitin binding module, linker region in black, blue and green respectively. Catalytic modules are shown in red and cyan. Classes I, II and IV belong to GH family 19, whereas class III enzymes are included in GH family 18. Loop differences are indicated, as defined in the text.

Fig. 2. Constructs used. (a) Domain organization of long and short constructs of secreted *Pichia*-expressed recombinant chitinases. (b) The sequence expressed in pPIC9K vector derivatives. Recombinant PaChi(l) and PaChi(l)-Y158C contain residues Ala1-Cys251, while PaChi(s) and PaChi(s)-Y158C include only the CM that starts at Ser48. Tyr158 is underlined and marked in italics. Cloning of the PCR-derived spruce chitinase into the vector generates a fusion including vector pPIC9K-derived residues, *YVEF* and *AAAN* at the N- and C-termini, respectively. The color-coding represents CtBM in blue, linker (L) in green, and CM in red.

Fig. 3. Antifungal activity against the plant pathogen *Heterobasidion annosum*. Fungus was inoculated in the center of the plate. Wells contained repeated additions of 30 μ l of PaChi2(l) (0.25 mg/ml = 9.7 μ M), PaChi(s) (0.25 mg/ml = 11.6 μ M) and 10 mM HEPES, pH 7 (control). The test was repeated several times with the same results.

Fig. 4. Homology model of the CtBM of PaChi(l). (a) Ribbon cartoon showing the binding module color-coded from green to blue, with disulfide-forming residues in gold. (b) CtBM shown as a coil representation in stereo; conserved residues that are likely to be important in chitin binding are colored slate blue whereas conserved glycine residues are colored in red. Modeled GlcNAc residues in the three potential subsites, based on the PDB entry 1ULM complex structure, are orange-red.

Fig. 5. Structure of PaChi(s). (a) Ribbon diagram showing the general structure of the CM. Residues that may be catalytically important are shown in orange-red. (b) Three modeled GlcNAc residues (black) are docked into the wide catalytic cleft, using the aligned papaya structure to place a chitin chain; labels indicate the proposed subsites. Conserved residues believed to be important for catalysis and substrate binding are shown in orange-red and blue, respectively.

Fig. 6. Comparison of chitinase structures. (a) Superposition of PaChi(s)-1, -2, and -3. (b) PaChi(s)-2 (red) is overlaid with *Streptomyces coelicolor* A3(2) chitinase G (PDB entry 2CJL, green) and *Brassica juncea* chitinase (PDB entry 2Z38, slate blue). Loops I-V of *Brassica* chitinase are comprised of residues 164-170, 217-222, 235-257, 308-311 and 325-332, respectively. Loops I and III of PaChi(s) include residues 65-71 and 123-144. (c) Comparison of the PaChi (orange-red) and papaya (steel blue; PDB entry 3CQL) enzymes, aligned using a tight cutoff (1 Å); 113 atoms match with an r.m.s. difference of 0.5 Å. Three GlcNAc units are modeled into the PaChi structure.

Fig. 7. Details of the active site. (a) Residues believed to be important in catalysis are shown, including alternate conformations observed for Glu113. Distances between Glu113 OE1 and Glu122 OE1 in the two conformations are shown in black dashed (8.7 Å) and dotted (9.7 Å) lines. The salt links between Glu113 and Arg230 in the two conformations are marked as red bubbled lines. Three water molecules that are consistently observed, named according to the PaChi(s)-2 structure. (b) Stereo representation of the electron density of some of the catalytically important residues in the PaChi(s)-2 structure.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7