Review

Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions

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Abstract

Chitin is the second most abundant natural biopolymer and the main structural component of invertebrate exoskeletons and cell walls of filamentous fungi. Fungal chitinases have multiple physiological functions including the degradation of exogenous chitin and cell wall remodelling during hyphal growth, but the regulation of the chitinolytic systems of filamentous fungi is not well understood. Fungi have on average between 10 and 25 different chitinases, but only the increasing number of fungal genome sequencing projects in the last few years has enabled us to assess the whole range and diversity of fungal chitinases. In this review the variety, domain architecture and subgroups of chitinases of filamentous fungi are shown, and how these data integrate with that from molecular biological studies on chitinases are discussed.

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1. Introduction

Chitin is a linear, insoluble homopolymer composed of β-1,4-linked subunits of the acetylated amino sugar N-acetylglucosamine. In nature two major types of chitin occur, which are characterized by an antiparallel (α-chitin) or a parallel (β-chitin) arrangement of the N-acetylglucosamine chains (Fig 1). After cellulose, chitin is the second most abundant polymer found in the biosphere (Tharanathan & Kittur 2003). It is the main compound of invertebrate exoskeletons and an essential structural component of the cell walls of filamentous fungi.

Previous reviews of fungal chitinases already emphasized the diversity of these enzymes as well as their multiple functions in fungi (Gooday 1990; Jollès & Muzzarelli 1999). However, only the recent advent of fungal genome sequencing projects has enabled us to assess the whole range and variety of fungal chitinases (http://genome.jgi-psf.org/mic_home.html, http://www.broad.mit.edu/seq/msc/). While in bacterial genome databases on average only between two and four chitinases can be found, the genomes of filamentous fungi typically contain between 10 and 25 different chitinases. The reasons why fungi have so many chitinases are not well understood. Potential physiological roles of fungal chitinases, as already discussed in previous reviews (Adams 2004; Cohen-Kupiec & Chet 1998; Duo-Chuan 2006; Gooday 1990; Jollès & Muzzarelli 1999; Yang et al. 2007), include: (i) degradation of exogenous chitin present in fungal cell walls of dead hyphal fragments or in the exoskeletons of dead arthropods, and the use of the degradation products as a nutrient source; (ii) cell wall remodelling during the fungal life cycle, which includes putative roles of chitinases during hyphal growth, branching, hyphal fusion and autolysis; and (iii) competition and defence against other fungi or arthropods in the fungal habitat. Some fungi have even developed lifestyles which
involve the use of chitinases to actively attack of other fungi (mycoparasitism), insects (entomopathogenic fungi) or nematodes (nematode-trapping fungi).

This review aims at highlighting the lessons that we have learned about chitinases in the past few years from the increasing number of available fungal genome sequences and how these data integrate with the already available knowledge derived from molecular biological studies on chitinases.

2. Mechanisms of chitin degrading enzymes

Chitinolytic enzymes can be divided into N-acetylglucosaminidases and chitinases, which substantially differ in their cleavage patterns (Fig 2). N-acetylglucosaminidases (EC 3.2.1.52) catalyze the release of terminal, non-reducing N-acetylglucosamine (GlcNAc) residues from chitin, but in general they have the highest affinity for the dimer N,N′-diacetylchitobiose (GlcNAc)₂ and convert it into two monomers (Horsch et al. 1997). According to the CAZy classification (Carbohydrate Active Enzymes database, Coutinho & Henrissat 1999; http:// www.cazy.org) N-acetylglucosaminidases belong to glycoside hydrolase (GH) family 20. It is important that those enzymes must not be referred to as exochitinases, which is unfortunately often confused in the literature. In contrast to that, chitinases (EC 3.2.1.14) are members of GH families 18 and 19 and catalyze the hydrolysis of the β-1, 4 linkages in chitin and chitooligomers, resulting in the release of short-chain chitooligomers. GH families 18 and 19 do not share sequence similarity, have different three-dimensional structures (Kezuka et al. 2006; Perrakis et al. 1994; Terwisscha van Scheltinga et al. 1996; van Aalten et al. 2000) and different catalytic mechanisms with β-anomeric products being formed by GH 18 chitinases (retaining mechanism, Brameld et al. 1998), whereas α-anomers are produced by GH 19 chitinases (inverting mechanism, Brameld & Goddard 1998). Furthermore, depending on their cleavage patterns, chitinases can be divided into endo- and exochitinases (Fig 2). Endochitinases degrade chitin from any point along the polymer chain forming random-size length products while exochitinases cleave from the non-reducing chain end and the released product is (GlcNAc)₂. However, the enzymatic properties of chitinases are more complex and versatile than reflected in the exo-/endo classification. Detailed studies of the chitinolytic system of the bacterium Serratia marcescens demonstrated another way to classify the enzymatic properties of chitinases by grouping them into processive and non-processive enzymes (e.g. Horn et al. 2006; Sorbotten et al. 2005; Uchiyama et al. 2001). Processive chitinases do not release the substrate after hydrolytic cleavage but slide it through the active site-tunnel for the next cleavage step to occur. The presence of a carbohydrate binding domain can enhance processivity, but is not essential for it. Non-processive chitinases dissociate completely from the substrate after hydrolysis. This leads for

![Fig. 1](a) Chemical structure of chitin. The grey box indicates one N-acetylglucosamine subunit of the chitin chain. (b) The two major types of chitin are characterized by an antiparallel (α-chitin) or parallel (β-chitin) arrangement of the chains.

![Fig. 2](Schematic drawing of the predominant cleavage patterns of chitinolytic enzymes. The subunits of the chitin chain are shown in light blue and the reducing end sugar in dark blue. Dotted lines indicate that the polymer substrates are longer than shown in the figure.)
non-processive enzymes to substrate degradation patterns with a homogenous distribution of medium chain (6–30mer) products and for processive enzymes to remnants of the polymeric substrate and only 2–8mer short chain degradation products (Horn et al. 2006).

Chitinases can contain various carbohydrate-binding modules (CBMs). These CBMs include different families classically defined as chitin- and cellulose-binding domains due to their preferred affinity for these carbohydrates (Henrissat 1999). Extensive data, classification and applications of these CBMs can be found in recent reviews (Boraston et al. 2004; Shoseyov et al. 2006) and in the Carbohydrate-Binding Module Family Server (http://www.cazy.org/fam/acc_CBM.html).

3. The chitinolytic enzyme machinery of fungi

Completing the picture by in silico analysis of genomic data

Analysis of more than 25 fungal genomes so far has shown that fungal chitinases exclusively belong to GH family 18. GH 18 enzymes are not only present in fungi, but also in bacteria, animals, viruses and plants. They were traditionally subdivided into classes III and V, which – because of their predominant occurrence in selected organisms – were also termed fungal/plant (class III) and fungal/bacterial (class V) chitinases. Class III and V chitinases differ in the architecture of their substrate binding pockets (Terwisscha van Scheltinga et al. 1994; van Aalten et al. 2001). Class V (bacterial-type) enzymes have deep, tunnel-shaped substrate binding grooves, and class III (plant-type) enzymes have shallow, open substrate binding grooves. Consequently class V enzymes show exo-acting activities (corresponding to processive enzymes), whereas class III enzymes are endo-chitinases (non-processive enzymes) (Hoell et al. 2005; Hurtado-Guerrero & van Aalten 2007; Jaques et al. 2003).

The first generation of a complete list of chitinolytic enzymes based on genomic sequence data was carried out in H. jecorina (T. reesei) (Seidl et al. 2005). It revealed the presence of 18 open reading frames (ORFs) encoding putative chitinases and two additional ORFs encoding low-similarity GH 18 proteins, demonstrating that previous research approaches had missed more than two-thirds of the Hypocrean/Trichoderma chitinases. In contrast to that, the ORFs encoding the two orthologues of the already described N-acetylglucosaminidases, and only one additional hypothetical protein distantly related to \(2-N\)-acetylhexosaminidases, were the only GH 20 proteins that could be detected in the H. jecorina genome database.

These findings reflect a similar situation in other fungi with annotated genomes such as Neurospora crassa, Magnaporthe grisea, Aspergillus spp., Fusarium spp., Botrytis cinerea, Sclerotinia sclerotiorum, etc., which have ORFs for a large number of chitinases (ca. 10–25, GH 18) but only between two and three ORFs encoding \(2-N\)-acetylhexosaminidases (GH 20). This suggests that chitinases rather than N-acetylglucosaminidases dictate the chitin depolymerization potential of a fungus and are the key elements for the regulation of different aspects of chitin degradation. However, the total number of chitinases in different fungi is highly variable, ranging from, for example, two chitinases in Ustilago maydis to 27 in Fusarium oxysporum.

Nevertheless, in the majority of ascomycetes and basidiomycetes on average of 15 different chitinase-encoding genes can be detected per species.

Classification into subgroups

Screening of fungal genomes for the presence of ORFs encoding putative chitinases revealed an even larger diversity of these proteins than previously anticipated. A phylogenetic analysis of chitinases from sequenced fungal genomes showed that they can be divided into three different subgroups (Fig 3, Seidl et al. 2005). Subgroups A and B (corresponding to classes V and III, respectively) contained all of the previously identified fungal chitinases, whereas subgroup C comprised a novel group of, high molecular weight chitinases that had not yet been described in filamentous fungi.

Subgroup A chitinases contain a catalytic domain, but no CBMs, and have on average a molecular mass of 40–50 kDa. Chitinases from subgroup A are present in all fungal genomes. On average ascomycetes have ca. six and basidiomycetes ca. four subgroup A chitinases, but depending on the total number of chitinases of a fungus, numbers of subgroup A chitinases range from two in U. maydis to ca. 12 in F. oxysporum.

The most strongly conserved and abundantly expressed chitinases range from two in F. oxysporum 2006) and in the Carbohydrate-Binding Module Family Server (http://www.cazy.org/fam/acc_CBM.html).

Subgroup B chitinases are very variable in their size and domain structure and their molecular masses range from 30–90 kDa. They can be grouped in small subgroup B chitinases (30–45 kDa) that contain frequently CBMs – a feature that is completely absent from subgroup A chitinases – and in large proteins of ca. 90 kDa that have long, unstructured, serine/threonine rich domains and/or a GPI-anchoring signal indicating that the mature proteins are bound to the plasma membrane. CHIA of A. nidulans is an example for such a

Subgroup A

Subgroup B

Subgroup C

Fig. 3 – Domain organization of fungal chitinases. SP, signal peptide. GH 18, glycoside hydrolase family 18, BD, binding domain.
GPI-anchored chitinase and it has been reported to be involved in cell wall remodelling processes (Takaya et al. 1998a).

The number of subgroup B chitinases is strongly variable among different fungal species. In most fungal genomes of *Asphaerella fijiensis* involved in cell wall remodelling processes (Takaya 1998a). Chitinases of filamentous fungi are studied in more detail and both have a CBM 1 domain as well. So far only two subgroup B chitinases containing CBMs were found. In contrast to that, a number of fungi such as Hypocreaceae spp. (H. jecorina, *H. atroviridis, H. vires*, Aspergillus spp. (A. fumigatus, A. clavatus, Neosatoya fischeri) and Rhizopus oryzae have up to five subgroup B chitinases. This indicates that these additional chitinases could have specialized functions in the respective fungi. Some of these proteins even belong phylogenetically to a separate clade that includes in addition orthologues described in the entomopathogenic fungus *Metharizium anisopliae*, which suggests a specialized function in chitin degradation.

Analysis of fungal genome sequences confirmed that several of the small (30–45 kDa) subgroup B chitinases possess CBMs, but interestingly these domains vary depending on the fungal species. The ascomycetes *B. cinerea, Hypocrea/Trichoderma* spp. (H. jecorina, *H. atroviridis, H. vires*) and *Aspergillus* spp. (A. fumigatus, A. clavatus, *Neosatoya fischeri*) and *Rhizopus oryzae* have up to five subgroup B chitinases. This indicates that these additional chitinases could have specialized functions in the respective fungi. Some of these proteins even belong phylogenetically to a separate clade that includes in addition orthologues described in the entomopathogenic fungus *Metharizium anisopliae*, which suggests a specialized function in chitin degradation.

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4. Regulation and functions of fungal chitinases

Degradation of exogenous chitin

Although chitinolytic enzyme activities have been reported from several fungi (see review by Duo-Chuan 2006), molecular biological studies on the regulation and function of individual filamentous fungal chitinases are generally rather scarce. The published data can be divided into two main aspects: induction by exogenous components and regulation by developmental stimuli.

On the regulation of enzyme production, the major regulator is the concentrations of extracellular chitin. The regulation is achieved through the expression of genes encoding chitinases. The expression of genes encoding chitinases is upregulated by chitin, while the expression of genes encoding other enzymes is downregulated by chitin.

Subgroup C is a novel subgroup of fungal chitinases. The first chitinase described from this subgroup was *H. atroviridis* (*T. atrovirens*) CHI18-10 (Seidl et al. 2005). Subgroup C chitinases have typically a molecular mass of 140–170 kDa and despite their large size, which was confirmed by cDNA sequencing for *H. atroviridis* chI18-10 (Seidl et al. 2005), they have an N-terminal signal peptide targeting them to the secretory pathway. In addition, subgroup C chitinases contain several features which clearly distinguish them from other fungal chitinases. They have a chitin-binding domain (CBM 18) and multiple LysM-motifs, which are short peptide domains implicated in binding peptidoglycan and structurally related molecules such as chitin in bacterial and eukaryotic proteins, respectively (Ohnuma et al. 2007; Zhang et al. 2007). The LysM domains of the plant chitinase PrChi-A were shown to be essential for the antifungal activities of the protein (Onaga & Taira 2008).
concentrations as low as 1 mM (Mach et al. 1999; Peterbauer et al. 2002).

CH18-5 (ECH42) is among the most strongly conserved chitinases in the fungal kingdom. In Hypocrea/Trichoderma spp. it is the most abundant chitinase in culture extracts grown on chitinase inducing carbon sources and ch18-5 expression is triggered by chitin degradation products, irrespective of whether they originate from exogenous chitin or cell wall autolysis (Mach et al. 1999; Seidl et al. 2005). In addition H. atroviridis ch18-5 is induced during stages of the mycoparasitic attack (Carsolio et al. 1994; Zeilinger et al. 1999). Kullig et al. (2000) determined that the induction of ch18-5 by cell walls of R. solani requires the diffusion of a factor with a size between 12–90 kDa and concluded from their experiments that an as yet unidentified chitinase releases oligomers from the host cell-walls which then act as inducers of further chitinase (such as CH18-5) formation. On the other hand, ch18-15 (chi36), which is also expressed during similar growth conditions was shown to be induced by a soluble molecule <12 kDa, directly released from R. solani cell walls (Viterbo et al. 2002). The induction of chi18-5, chi18-12 (chi3) and chi18-15 is not only influenced by the presence of chitin or fungal cell walls, but also regulated by carbon catabolite repression, the nitrogen source and starvation (see also Cell wall remodelling; Carsolio et al. 1994; de las Mercedes Dana et al. 2001; Mach et al. 1999; Seidl et al. 2005).

Despite the high expression levels of CH18-5 during mycoparasitic growth conditions, ch18-5 gene-knockout studies did not show any alterations in the ability of H. atroviridis to overgrow other fungi in mycoparasitism plate confrontation assays (Carsolio et al. 1999; Woo et al. 1999), although culture filtrates of the knockout-strains had reduced antifungal activities against B. cinerea in vitro (Woo et al. 1999). This suggests that CH18-5 is expressed as a consequence of the chitin degradation events during mycoparasitic attack, but that it is not a major determinant for this process. In contrast to the broad inducibility of ch18-5, the H. atroviridis chitinase-encoding genes chi18-10 and chi18-13 (ech30), are not induced by colloidal chitin or starvation, but by the presence of complex chitinous carbon sources such as R. solani cell walls (Klemsdal et al. 2006; Seidl et al. 2005), demonstrating a more specific regulation of these H. atroviridis chitinases.

In a number of other fungi similar findings have also been reported with respect to the induction of individual chitinases by chitin, antagonization of fungi or insects, and by autolysis were reported. These studies include subgroup A chitinases that all belong phylogenetically to the CH18-5 clade: B. cinerea BcchiA (Choquer et al. 2007), Paecilomyces javanicus PfChi-1 (Chen et al. 2007), Stachybotrys elegans sechi44 (Morissette et al. 2003), Paracoccidioideas brasiliensis Pbcta1 (Bonfim et al. 2006), Clonostachys rosea Rcchi1 (Gan et al. 2007b), Lecanicillium psalliotaec Lpci1 (Gan et al. 2007a) and two subgroup B chitinases from Metarhizium anisopliae: chi2 (Baratto et al. 2006) and chi3 (da Silva et al. 2005).

How fungi respond to different chitin-conformations and calcification grades of chitin, and how these differences influence chitinase regulation has not been studied yet. Pure chitin is in general not a good carbon source for fungi and in many studies researchers use colloidal chitin which is more accessible to the fungus because it has undergone acidic hydrolysis pretreatment. Another question that has not been addressed is how fungi that secrete high levels of chitinolytic enzymes protect their own cell walls. The avirulence protein AVR4 from the plant pathogenic fungus Cladosporium fulvum has been shown to bind to the chitin of fungal cell walls and protect them against plant chitinases (van den Burg et al. 2006). Although no further AVR4 orthologues can be detected in fungal genome databases, it is possible that other fungal cell wall proteins such as hydrophobins fulfil similar functions (Wessels 1997). The cell wall protein QD74 from T. harzianum has recently been reported that to be involved in resistance to lytic enzymes (Rosado et al. 2007).

**Cell wall remodelling**

Cell wall remodelling during the fungal life cycle is thought to be regulated by a delicate balance between controlled lysis and synthesis of chitin (Barthicki-Garcia 2006; Gooday 1990; Merz et al. 1999). One would be expect that chitinases that are involved in cell wall remodelling, are not induced by exogenous chitin but are developmentally regulated (e.g. during vegetative growth, by the onset of sporulation) and/or show basal expression levels under all growth conditions. These kinds of transcript patterns have indeed been obtained for several chitinases, e.g. from H. atroviridis (Seidl et al. 2005, chi18-2, chi18-3, chi18-4, all subgroup A), B. cinerea (Choquer et al. 2007, BcchiB; subgroup A), Rhizopus oligosporus (Takaya et al. 1998b, chi3, subgroup A). However, morphological changes in knockout-strains have only been reported for one chitinase encoding gene, A. nidulans chiA (subgroup B). The respective strains displayed a decreased hyphal growth rate and lower germination frequency, whereas hyphal and conidio- phore morphology were normal (Takaya et al. 1998a).

Transcriptional evidence for the induction of chitinases by starvation and subsequent autolysis has been obtained in H. atroviridis (chi18-5, subgroup A; Mach et al. 1999; Seidl et al. 2005), chi18-12 (chi33, subgroup B; de las Mercedes Dana et al. 2001; Limón et al. 1995), in Aspergillus nidulans (chiB, subgroup A; Pusztahelyi et al. 2006; Yamazaki et al. 2007) and in A. fumigatus (chiB1, subgroup A; Jaques et al. 2003). Again, only in one case, A. fumigatus chiB1, the autolytic function of a chitinase was demonstrated with gene-knockout strains, which showed much lower levels of chitinase activity during the autolytic phase of batch cultures (Jaques et al. 2003).

It has been repeatedly suggested (Adams 2004; Hurtado-Guerrero & van Aalten 2007; Jaques et al. 2003) that subgroup B chitinases could rather be the ones responsible for cell wall remodelling in fungi. This suggestion is based on the finding that knockout strains of A. nidulans chiA, which encodes a subgroup B chitinase, but not of A. fumigatus chiB, which encodes a subgroup A chitinase, displayed morphological defects. However, as detailed above (Section 3), subgroup B consists of two types of proteins. While some chitinases, such as A. nidulans chiA are large proteins with unstructured serine/threonine rich domains and/or GPI anchors, the majority of subgroup B chitinases are small proteins that can contain a CBM. At least some subgroup B chitinase-encoding genes such as H. atroviridis ch18-13 have a carbon source-inducible regulation, whereas, a number of subgroup A chitinase-encoding genes such as chi18-2, chi18-3, chi18-4 and chi18-5, showed transcript levels under all tested growth conditions, which is rather
a transcription pattern that would be expected from chitinases involved in cell wall remodelling. In view of these data, it seems likely that not a certain subgroup of chitinases, but rather selected chitinases from all subgroups have functions in hyphal growth-related processes.

5. Conclusions

Filamentous fungi contain a large number of chitinases and genomic data mining has enabled us to form a detailed picture of the chitinolytic enzyme machinery of fungi, and has revealed an even higher variety of chitinases than previously anticipated. Fungal chitinases belong to GH family 18 and based on a phylogenetic analysis they can be divided into three subgroups (A, B and C), which have distinctive domain architectures (Fig 3). The potential roles of fungal chitinases are exogenous chitin degradation, defence and attack mechanisms against other fungi and arthropods and cell wall remodelling. Major questions to be addressed in the future are: (1) do these enzymes have specialized roles in one or more of the biological processes that chitinases have been implicated as being involved in, (2) do their functions partially overlap, (3) how do fungi regulate their large battery of chitinases, and (4) which signals trigger their expression. The rapid advances in omics techniques and in different interdisciplinary approaches will hopefully enable us in the next few years to further extend our knowledge about this group of enzymes and their versatile functions.

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