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Review of fungal chitinases

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Abstract

Chitin is the second most abundant organic and renewable source in nature, after cellulose. Chitinases are chitin-degrading enzymes. Chitinases have important biophysiological functions and immense potential applications. In recent years, researches on fungal chitinases have made fast progress, especially in molecular levels. Therefore, the present review will focus on recent advances of fungal chitinases, containing their nomenclature and assays, purification and characterization, molecular cloning and expression, family and structure, regulation, and function and application.

Key words: expression regulation, fungal chitinase, gene cloning, mycoparasitism, purification and characterization

Abbreviations: pNP – *p*-nitrophenyl; pNP-GlcNAc – *p*-nitrophenyl-*N*-acetyl–D-glucosaminide; pNP-$(GlcNAc)_2$ – *p*-nitrophenyl-β-D-*N*,*N*′-diacetylchitobiose; pNP-$(GlcNAc)_3$ – *p*-nitrophenyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose; 4-MU – 4-methylumbelliferyl; 4-MU-GlcNAc – 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide; 4-MU-$(GlcNAc)_2$ – 4-methylumbelliferyl-β-D-*N*,*N*′-diacetylchitobioside; 4-MU-$(GlcNAc)_3$ – 4-methylumbelliferyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose

Introduction

Chitin, an unbranched homopolymer of 1, 4-β-linked *N*-acetyl-D-glucosamine (GlcNAc), is widely distributed in nature. It is believed to be the second most abundant and renewable polymer on earth, next to cellulose. Chitinases are chitin-degrading enzymes, and hydrolyze the β-(1, 4) linkages of chitin. The enzymes occur in a wide range of organisms including viruses, bacteria, fungi, insects, plant, and animals. The roles of chitinases in these organisms are diverse. In fungi, chitinases are thought to have autolytic, nutritional, and morphogenetic roles [1]. Chitinases in mycoparasitic fungi are most commonly suggested to be involved in mycoparasitism [2]. Chitinases in bacteria are shown to play a role in the digestion of chitin for utilization as a carbon and energy source and recylcling chitin in nature [3–6]. In insects, chitinases are associated with postembryonic development and degradation of old cuticle [7]. Plant chitinases are involved in defence and development [8]. Chitinases encoded by viruses have roles in pathogenesis [9]. Human chitinases are suggested to play a role in defense against chtinous human pathogens [10–12]. On the other hand, chitinases have shown an immense potential application in agricultural, biological and environmental fields.

Due to important biophysiological functions and applications of chitinase, a considerable amount of research on fungal chitinases has been carried out in recent years. Therefore, the present review will focus on fungal chitinases, containing their nomenclature and assays, purification and characterization, molecular cloning and expression,

family and structure, regulation, and function and application.

Nomenclature and assays of chitinase

Nomenclature of chitinlytic enzymes is confused. At first, chitinlytic enzymes in Enzyme Nomenclature are listed into chitinase 3.2.1.14 and *N*-acetyl-glucosaminidases 3.2.1.30 [13]. In this terminology chitinase is referred only to endochitinase. Furthermore, this terminology does not also contain all known chitinlytic enzymes. Therefore, it is insufficient. Aferwards, chitinlytic enzymes are defined as any enzyme that catalyzes the cleavage of chitin, and divided endochitinase, exochitinases (also called chitin 1,4-β-chitobiosidase or chititobiosidases) and *N*-acetyl-glucosaminidases [14]. This classification covers almost any enzymes that catalyze the cleavage of chitin. Hence it is reasonable. At the same time, a similar nomenclature is suggested, but chitinases rather than chitinlytic enzymes are used in this nomenclature. In this nomenclature, chitinases is defined as any enzyme that catalyzes the cleavage of chitin [8]. In this article, we accept this nomenclature. According to this nomenclature, chitinases can be divided into two major categories: endochitinases and exochitinases [8]. Endochitinases (EC3.2.1.14) cleave chitin randomly at internal sites, generating soluble, low molecular mass multimers of GlcNAc, such as chitotetraose, chitotriose, and diacetylchitobiose. Exochitinase can be divided into two subcategories: chitobiosidases and β-(1,4)-*N*-acetyl-glucosaminidases. Chitobiosidases (EC3.2.1.29) or chitin-1,4-β-chitobiosidases catalyze the progressive release of diacetylchitobiose starting at the nonreducing end of chitin chains. Its products are sole diacetylchitobioses, and no monosaccharides or oligosaccharides are formed. β-(1,4)-*N*-acetyl-glucosaminidases (GlcNAcase, EC3.2.1.30) or chitobiases split diacetylchitobiose and higher chitin polymer, including chitotriose and chitotetraose, into GlcNAc monomers in an exo-type fashion. The enzyme also cleaves the chromogenic substrates pNP-GlcNAc and *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide to release pNP and 4-MU-GlcNAc to release 4-MU. Since the enzyme has broad substrate specificity, it also called β-(1, 4)-*N*-acetylhexosaminidases (HexNAcase, EC3.2.1.52) [16].

Techniques and methods for detecting chitinases have been developed. In order to measure chitinase activity in solution, a chromogenic assay procedure is developed with *p*-nitrophenyl-labeled substrates, such as *p*NP-GlcNAc, *p*NP-$(GlcNAc)_2$, and *p*NP-$(GlcNAc)_3$. Glucosaminidase, chitobiosidase and endochitinase activities are determined by measuring the release of *p*-nitrophenyl from *p*NP-GlcNAc, *p*NP-$(GlcNAc)_2$ and *p*NP-$(GlcNAc)_3$, respectively [14, 17]. Endochitinase activity is also measured by the reduction of turbidity of a suspension of colloidal chitin [18]. In addition, chitinase activity is often determined by measuring the amount of reducing sugars liberated from colloidal chitin by enzyme activity [19]. To detect chitinases after SDS-PAGE, proteins are prepared in Laemmli buffer. The proteins are separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Enzymes are reactivated in the gels by removing SDS by the casein–EDTA procedure [20]. Enzyme activity is detected on gels by using fluorescent substrates [18]. The chitinase enzymes appear as fluorescent bands under UV light because of enzymatic hydrolysis of fluorescent 4-methylumbelliferone from the GlcNAc mono- and oligosaccharides, such as 4-MU-GlcNAc, 4-MU-$(GlcNAc)_2$ and 4-MU-$(GlcNAc)_3$. These substrates allow for detection of three different chitinase types by acting as demeric, trimeric, and tetrameric substrates, respectively. The dimer is the preferred substrate for GlcNAcases. Chitobiosidases release fluorescent product from only the trimeric substrate and endochitinases are identified by digestion of the tetrameric substrate [21]. Other zymogram procedures that may be used for detection of chitinase activity on gels have also been described [22–25].

Purification and characterization of chitinases from fungi

Although there have been a lot of research reports on production of chitinases from fungi, data on the purification and characterization of these enzymes are limited to a few fungi. In mycoparasitic fungi, purification mainly focuses on the chitinolytic system of *T. harzianum*. Several chitinases of *T. harzianum* have been purified, including endochitinases, chitobiosidases and *N*-acetyl-glucosaminidases [14, 21, 26–31]. The purification of

chitinases from other mycoparasitic fungi has also been reported, such as *Aphanocladium album* [32], *Gliocladium virens* [33], *Fusarium chlamydosporum* [34], *Trichothecium roseum* [35], *Stachybotry elegans* [36], and *Talaromyces flavus* [37]. In entomopathogenic fungi, *N*-acetyl-D-glucosaminidase, endochitinase, and exochitinase have been purified and characterized from *Beauveria bassiana* [38–40] and *Metarhizium anisopliae* [41–43]. Compared with mycoparasitic fungi and nematophagous fungi, to date only two chitinases from the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium* have been purified and characterized [44]. Recently, chitinases from thermophilic fungi have received attention for their thermostability. A thermostable chitinase from the thermophilic fungus *Thermomyces lanuginosus* is purified [45]. Except from the above fungi, the purified chitinases from other fungi are also reported, such as *Candida albicans* [46], *Sacchromyces cerevisiae* [47], *Trichoderma asperellum* [48], *Trichoderma reesei* [49], *Colletotrichum gloeosporioides* [50], *Rhizopus oligosporus* [51, 52], *Mucor fragilis* [53], *Aspergillus fumigatus* [54, 55], *Aspergillus nidulans* [56, 57], *Penicillium janthinellum* [58], *Penicillium oxalicum* [59], *Piromyces communis* [60, 61], *Acremonium obclavatum* [62], and *Isaria japonica* [63].

Fungal chitinases are purified to homogeneity by different methods, such as fractional ammonium sulphate precipitation, ion exchange chromatography, hydrophobic interaction chromatography, gel filtration chromatography, chitin-affinity chromatography, and isoelectri focusing (IEF). From value of fold purification of different fungal chitinases, the value of most purified chitinases is low [14, 27, 28, 32, 33, 35, 37]. This low value may be due to a synergistic action of the different isoforms for chitin degradation present in the crude supernatant and the loss of chitinase activity during purification progress [28].

Most chitinolytic fungi have been found to produce more than one kind of chitinase. *Trichoderma harzianum* may produce seven individual chitinases: two *N*-acetyl-glucosaminidases (102 and 73 kDa) [21], four endochitinases (52, 42, 33 and 31 kDa) [14, 21, 28] and one chitobiosidase (40 kDa) [14]. *Talaromyces flavus* produces at least two kinds of chitinases [37]. The mycoparasite *Stachybotrys elegans* produces two exochitinases and one endochitinase [36]. The entomopathogenic fungus *Metarhizium anisopliae* produces at least six different chitinases [41–43, 64]. Research finds that these multiple chitinases have a mutually synergistic and complementary effect between them. For example, chitinases CHIT33, CHIT37, and CHIT42 of *T. harzianum* show the synergistic action of on cell wall degradation [28]. Combining the activities of the endochitinase and chitobiosidase from *T. harzianum* results in a synergistic increase in antifungal activity [65]. A similar phenomenon has been reported in plant chitinases [66] and bacterial chitinases [67].

Most purified fungal chitinases have been characterized. The characteristics mainly contain molecular weight, pI, optimal pH, optimal temperature, thermostability, inhibitors, and antifungal activity. Usually, fungal endochitinases and chitobiosidases are single polypeptides, whereas *N*-acetyl-glucosaminidases are dimeric, such as *N*-acetyl-glucosaminidases of *T. harzianum*, *Stachybotrys elegans*, and *Beauveria bassiana* [26, 38, 36]. Molecular masses of fungal chitinases have a wide range from 27 to 190 kDa with an about pI 3–8. Optimal pH and temperature are similar for the majority of the purified chitinases from fungi. Fungal chitinases are active in the pH 4.0–7.0 range. The optimum temperature for most fungal chitinase activity is at 20–40° [14, 27, 28, 32, 33, 35, 37]. However, chitinases from two thermophilic fungi *Thermomyces lanuginosus* and *Talaromyces emersonii* have high optimum temperature and thermostability. A chitinase of *Talaromyces emersonii* has maximum activity at 65° and a half-life of 20 min at 70° [68]. A chitinase of *Thermomyces lanuginosus* exhibits optimum catalytic activity at 55°. The half-life time of the enzyme at 65° is 25 min [45]. Fungal chitinases have a known specific inhibitor, allosamidin or demethylallosamidin. The inhibitor is produced by *Streptomyces* sp. It can specifically inhibit family 18 chitinases from yeast, fungi, and insects, and has been used for functional research and identification of chitinases. Much evidence has been shown that fungal chitinases can degrade fungal cell walls and inhibit fungal growth *in vitro*. The purified endochitinase and chitobiosidase produced by *T. harzianum* inhibit spore germination and germ tube elongation of different fungal species [65]. Similar results have been also reported in other fungal chitinases [14, 34, 35, 37, 45, 69].

It should be emphasized that chitinases of *T. harzianum* are substantially more active and

effective against a wide range of fungi than chitinolytic enzymes from plants and other microorganisms [65]. Hence chitinases of *T. harzianum* are particularly attractive. *T. harzianum* has been thought to be rich source of chitinolytic enzymes for biocontrol. In addition, chitinases from thermophilic fungi are also promising for biocontrol and enzymatic conversion of chitin because of their thermostability [45].

Cloning and expression of fungal chitinase genes

From references and GenBank, a great number of genes encoding chitinases have been isolated and analyzed from a wide range of fungi containing yeast and filamentous fungi, such as *Saccharomyces cerevisiae* [47, 70], *Candida albicans* [16, 71–73, GenBank], *Kluyveromyces lactis* [74, 75], *Coccidioides immitis* [76, 77, GenBank], *Paracoccidioides brasiliensis* [78], *Trichoderma harzianum* [48, 79–87], *Trichoderma reesei* [49, 88, GenBank], *Trichoderma atroviride* [89, 90], *Trichoderma virens* [91, 92], *Trichoderma aureoviride* [93], *Rhizopus oligosporus* [51, 52], *Aspergillus nidulans* [94, 95, GenBank], *Aspergillus fumigatus* [96], *Metarhizium anisopilae* [97–101, GenBank], *Beauveria bassiana* [40], *Aphanocladium album* [102], *Nomuraea rileyi* [103], *Stachybotrys elegans* [104], *Puccinia triticina* [105], *Penicillium chrysogenum* [106], *Thermomyces lanuginosus* [107], *Amanita muscaria*, *Torrubiella confragosa*, *Magnaporthe grisea*, *Cryptococcus neoformans*, *Verticillium lecanii*, *Verticillium fungicola*, *Botrytis cinerea*, *Blumeria graminis*, *Ajellomyces dermatitidis*, *Ustilago maydis*, *Coniothyrium minitans*, *Metarhizium flavoviride*, *Rhizopus niveus*, *Paracoccidioides brasiliensis*, *Aspergillus oryzae*, and *Hypholoma fasciculare*[GenBank].

In recent years, genome sequencing of several model fungi has been completed, such as *Saccharomyces cerevisiae*, *Candida albicans*, *Coccidioides immitis*, *Neurospora crassa*, *Gibberella zeae*, *Magnaporthe grisea*, *Aspergillus nidulans*, *Aspergillus fumigatus* and *Trichoderma reesei*. By genome analysis, new and more chitinase genes have been found. Genome analysis reveals the presence of at least 5, 9, 17, 11, 21, 16 and 18 ORFs encoding putative chitinases in *C. albicans*, *N. crassa*, *G. zeae*, *M. grisea*, *A. fumigatus*, *A. nidulans* and *T. reesei*, respectively [88, 108, GenBank].

Chitinase genes of fungi are well expressed functionally active in hosts, such as *E. coli*, yeast, filamentous fungi, and plants. Expression of *ech42* encoding endochitinase of *T. harzianum* in *E. coli* results in high chitinase activity [86]. Transformation of *Schizosaccharomyces pombe* with cts1 from *Saccharomyces cerevisiae* results in the appearance of about a 5- to 13-fold increase in chitinase activity [70]. Introduction of *Aphanocladium album* chitinase gene into *Fusarium oxysporum* results in high chitinase levels in the host [109]. Expression of a gene encoding endochitinase from *T. harzianum* in *T. reesei* results in a transformant that produces 20 times more chitinase than *T. reesei* [110]. When *ech42* from *T. harzianum* is transferred to *T. harzianum*, level of chitinase activity increases up to 42-fold in transgenic *T. harzianum* strains carrying multiple copies of *ech42* as compared with *T. harzianum* wild strain [111]. The *ThEn42* gene of *T. harzianum* endochitinase is transferred to tobacco, potato and apple, and highly expressed in different plant tissues [112, 113]. Overxpression of a gene (*Bbchit1*) encoding endochitinase of the entomopathogenic fungus *Beauveria bassiana* results in high chitinase activity [40]. Expression of some fungal chitinase genes in the heterologous hosts is summarized in Table 1.

Family and structure of fungal chitinase

Family of fungal chitinases

Chitinases are classified as glycosyl hydrolases. According to the glycosyl hydrolase classification system that is based on amino acid sequence similarity of the catalytic domains, chitinases have been placed in families 18, 19 and 20 [119]. Family 18 chitinases are found in bacteria, fungi, yeast, viruses, plant and animals, and hence the family is diverse in evolutionary terms. Family 19 members are almost exclusively present in plants. A single family 19 chitinase is identified in *Streptomyces griseus* [120, 121]. Chitinases of the two families (family 18 and family 19) do not share amino acid sequence similarity and have completely different 3-D structures and molecular mechanisms, suggesting that they have arisen from a different ancestor [122, 123]. Family 20 consists of the β-*N*-acetylhexosaminidases or β-*N*-acetylglucosaminidases from bacteria, fungi and humans.

Table 1. Expression of fungal chitinase genes in the heterologous hosts

Source of gene	Designation	Host	Vector	Reference
Trichoderma harzianum	*ThEn-42*	*Trichoderma reesei*	pCL-9, pCL-7	[110]
Trichoderma harzianum	*ech42*	*E. coli*	pSPORT	[86]
Saccharomyces cerevisiae	*cts1*	*Schizosaccharomyces pombe*	Yep24	[70]
Saccharomyces cerevisiae	cts1	*Saccharomyces cerevisiae*	pCT33	[47]
Aphanocladium album	*chit1*	*Fusarium oxysporum*	pBL1	[109]
Trichoderma harzianum	*chi1*	*Saccharomyces cerevisidae*	PC1CH1	[114]
Trichoderma harzianum	*ech42*	*Trichoderma harzianum*	pGEM-T	[115]
Trichoderma harzianum	*chit42*	Tobacco, potato	pBin19:p35S	[112]
Trichoderma harzianum	*ech42*	Apple	pBIN19ESR:p35S	[113]
Metarhizium anisopilae	*chit1*	*Metarhizium anisopilae*	p324-chit1	[99]
Beauveria bassiana	*Bbchit1*	*Beauveria bassiana*	pBANF-bar-pAN	[40]
Aspergillus nidulans	*nagA*	*Saccharomyces cerevisidae*	pYES2	[95]
Aspergillus nidulans	*nagA*	*Aspergillus oryzae*	pNGA142	[95]
Aspergillus fumigatus	*chiB1*	*Pichia pastoris*	pPICZαA	[96]
Thermomyces lanuginosus	*chit*	*Pichia pastoris*	pPIC9K	[107]
Metarhizium anisopilae	*chit42*	*E. coli*	PT7-7	[116]
Trichoderma harzianum	*ech42*	*Trichoderma harzianum*	pQuiA9	[111]
Trichoderma harzianum	*ech30*	*E. coli*	pBluescript	[117]
Trichoderma reesei	*chi46*	*E. coli*	pTrc46c	[49]
Trichoderma aureoviride	*ech42*	*Saccharomyces cerevisiae*	pYES2	[118]

Structure of fungal chitinases

Most fungal chitinases belong to family 18 of the glycohydrolase superfamily [124]. A characteristic of the family 18 chitinases is their multi-domain structure. Typically, fungal chitinase of family 18 basic structures are composed of five domains or regions: N-terminal signal peptide region, catalytic domain, serine/theronine-rich region, chitin-binding domain, and C-terminal extension region. However, most fungal chitinases lack the last three domains, which do not seem to be necessary for chitinase activity because naturally occurring chitinases that lack these regions are still enzymatically active. The derived amino-acid sequence of a *Saccharomyces cerevisiae* endochitinase, CTS1, shows four distinct domains: a signal sequence, a catalytic domain, a serine/theroninerich region and chitin-binding domain [47]. *Rhizopus oligosporus* two chitinases (chitinase I and chitinase II) have five distinct domain of chitinase: a signal sequence, a catalytic domain, a serine/theronine-rich region, chitin-binding domain, and an additional C-terminal domain [51]. *T. harzianum* 33 kDa chitinase includes a putative signal peptide region and a catalytic domain, but has the lack of three distinct domains of chitinase, a ser/thr-rich region, a chitin-binding region and a C-terminal region [82] (Figure 1).

Fungal chitinase structure domains have different biochemical functions. In fungal chitinases sequenced, except *Rhizopus oligosporus* chitinase III [52] and *Trichoderma harzianum* Chi18-2, Chi18-3 and Chi18-7 [88], a signal peptide is predicted to precede the N-terminal region of the mature protein. The signal peptide presumably mediates secretion of the enzyme and it is cleaved off by signal peptidases after the protein has been transported across the membrane. The chitinases lacking secretory signal sequence are shown to be intracellular chitinase, and they may function during morphogenesis [52, 88]. The fungal chitinase catalytic domain, responsible for the hydrolysis of the substrate, comprises the N-terminal half of the enzyme. Sequence alignments reveal two highly conserved regions within the catalytic domain. The

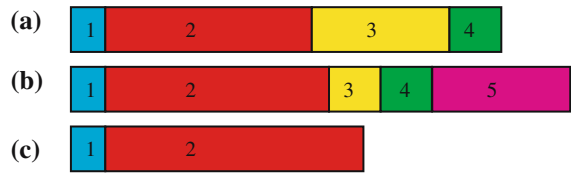

Figure 1. The structure of fungal chitinase of family 18. a: *Saccharomyces cerevisiae* endochitinase (CTS1); b: *Rhizopus oligosporus* chitinase (CHI1); c: *T. harzianum* chitinase (CHIT33). 1: signal peptide region; 2: catalytic domain; 3: serine/theronine-rich region; 4: chitin-binding domain, 5: C-terminal extension region.

two consensus regions or motifs, SxGG and DxxDxDxE, correspond to a substrate-binding site and a catalytic domain, respectively, in family 18 chitinases [125].

Apart from N-terminal signal peptide region and catalytic domain, the structure of some fungal chitinases reported has serine/theronine-rich region and chitin-binding domain (ChBD), such as *Saccharomyces cerevisiae* endochitinase, CTS1 [47], *Kluyveromyces lactis* endochitinase, KlCts1p [75], *Trichoderma reesei* Chi18-13 and Chi18-16 and *Rhizopus ologosporus* chitinase I and Chitinase II [51]. The serine/theronine-rich region of fungal chitinases is ususlly glycosylated with sugar chains post-translationally to yield the mature protein. The glycosylation sites may be necessary for the secretion of the protein and maintenance of its stability. Fungal chitinases are thought to be anchored to wall cell or their substrate through the chitin-binding domain [47]. A conserved six-cysteine motif, which likely mediates the tertiary structure or protein–protein interaction through the formation of disulfide bridges, is found in the chitin-binding domain of CTS1 and KlCts1p [47, 75]. The chitin-binding domain of *Trichoderma reesei* Chi18-13 and Chi18-16 has high similarity with a fungal cellulose-binding domain (CBD), consisting of four strictly conserved aromatic amino acid residues that are implicated in the interaction with cellulose [88, 126]. It should be noted that the chitin-binding domain differs from the substrate-binding site in the catalytic domain [124]. The chitin-binding domain of *Kluyveromyces lactis* KlCts1p functions independent of the catalytic domain [75]. It has been demonstrated that the presence of the chitin-binding domain in *Saccharomyces cerevisiae* CTS1 does not increase the rate of chitin hydrolysis [47], but addition of ChBD from *Nicotiana tabacum* ChiA chitinase to *Trichoderma harzianum* chitinase Chit42, which lacks a ChBD, increases chitin-binding capacity of the native Chit42 [127].

It is not clear how the C-terminal region of fungal chitinase functions. Interestingly, it has been found using the DGPI facility at the SIB website that at the C-terminus of a *Candida albicans* chitinase, CHT2, and two *Aspergillus fumigatus* chitinases, ChiA1 and ChiA, there are putative glycosyl-phosphatidylinositol (GPI)-anchor and cleavage sites. By a GPI anchor, CHT2 and ChiA1 are likely to be anchored to the cell wall or cell membrane, and to perform roles during growth and morphogenesis [94, 96, 128].

So far crystal structure of only two fungal chitinases is available. The three-dimensional model of a *Coccidioides immitis* chitinase, CiX1, and a *Aspergillus fumigatus* chitinase, ChiB1, has been reported [129, 130]. The active site of CiX1 is composed of conserved amino acid residues (W47,131,315,378, Y239,293, R52,295). The amino acid residue E171, by analogy to other class 18 glycohydrolases, is thought to be the catalytic amino acid, as site-directed mutagenesis of E171 eliminates any detectable enzyme activity. The structure model reveals that the chitinase is an eight-stranded beta/alpha-barrel. The properties of the chitinase structure are eight parallel beta-strands, forming the barrel's core, which is surrounded by eight alpha-helices connected to the barrel. It is suggested that the barrel structure of chitinases forms a groove on the enzyme's surface. This groove is considered as the active center, which binds sugar units of chitin, possibly $(GlcNAc)_6$ moieties, which are subsequently cleaved [131].

It should be noted that studies on fungal β-*N*-acetyl-glucosaminidases, belonging to family 20 of the glycohydrolase superfamily, have been done recently. Genes encoding β-*N*-acetyl-glucosaminidases have been cloned from several fungi, such as *T. harzianum*, *C. albicans*, and *A. nidulans* [16, 81, 95]. Molecular cloning and sequencing of fungal β-*N*-acetyl-glucosaminidases reveals that fungal β-*N*-acetyl-glucosaminidase structure is composed of signal peptide, propeptide, zincin-like domain, catalytic domain, and C-terminal segment [132].

Subfamily of fungal chitinases

Based on chitinase structure and amino acid sequence, chitinases are separated into different subfamilies, classes, or groups. Bacterial chitinases are clearly separated into three major subfamilies, A, B, and C [133]. Plant chitinases are divided in five different classes. Class I and Class II chitinases are similar in their catalytic domains. Class I chitinases have a chitin-binding domain. This domain is separated from the catalytic domain by a hing region. Class II chitinases lack both the chitin-binding domain and the hinge region. Class III chitinases have higher homology to fungal

chitinases than to other plant chitinase classes. Class IV chitinases are similar to Class I chitinases but they are smaller in size due to certain deletions. Class V chitinases show some homology with bacterial exochitinases [134].

Compared with bacterial chitinases and plant chitinases, fungal chitinases are not well classified. Within family 18 two distinct classes of fungal chitinase may be identified based on the similarity of enzymes to family 18 chitinases from plants or bacteria [76, 94]. Therefore, fungal chitinases are divided into fungal/bacterial (corresponding to Class V) chitinases, similar to chitinases found in bacteria, and fungal/plant (corresponding to Class III) chitinases, which are similar to chitinases from plants [1, 94, 96]. Recently, according to phylogenetic analysis, it has been suggested that fungal chitinases of family 18 can be divided into three groups: groups A and group B (corresponding to class V and III of plant chitinases, respectively), whereas a novel group C comprises high molecular weight chitinases that have a domain structure similar to *Kluyveromyces lactis* killer toxins [88]. Unfortunately, none of these proteins in group C has as yet been characterized from any filamentous fungus.

Regulation of fungal chitinase genes

Regulation under the condition of culture

Chitinases produced from fungi are inducible in nature. When fungi are grown in chitin-containing medium, chitinase activity is detected [14, 21, 28, 33, 35-37, 45]. Chitinase gene expression in fungi has been reported to be controlled by a repressor/inducer system, in which chitin or other products of degradation (such as *N*-acetyl-β-D-glucosamine and glucasamine) act as inducers whereas glucose or easily metabolizable carbon sources act as repressors [15, 109].

Recently, it has been studied in detail that regulation of chitinase genes of *ech42* encoding 42 kDa endochitinase, *chit36* encoding 36 kDa endochitinase, *chit33* encoding 33 kDa endochitinase, and *nag1* encoding *N*-acetyl-β-glucosaminidase from *T. harzianum*. The four genes are induced by fungal cell walls or colloidal chitin [81, 83, 85, 86] or by carbon starvation [82, 85, 110, 135], whereas the presence of high concentrations of glucose or glycerol inhibits the expression of *ech42*, *chit36*, *chit33*, and *nag1* [85, 86, 135–138]. In addition, *chit33* and *nag1* are also induced by GlcNAc, but GlcNAc fails to induce *ech42* and *chit36* expression [48, 81, 135].

On the basis of northern analysis of CHIT42 mRNA of *T. harzianum*, it is found that the CHIT42 mRNA is specifically induced by chitin as a carbon source or chitin-containing cell walls [83]. Similar results are also reported [86, 115]. Further research demonstrated that *ech42* expression appears to be not directly induced by chitin but by carbon starvation (nutrient depletion). Furthermore, *ech42* gene transcription is also induced by other physiological stress, such as low temperature and high osmotic pressure. Four copies of a putative stress response element (CCCCT) are found in the *ech42* promoter [137]. Similar the stress response element is also reported in *Saccharomyces cerevisiae*. It is shown that the stress response elements in *S. cerevisiae* mediate various stress responses, including nutrient depletion, low temperature and high osmotic pressure [139, 140]. Potentially, the stress response element of *T. harzianum* is likely to have similar functions. Recently, a new BrlA-like cis-acting element, different from the CCCCT element, has been identified in the *ech42* promoter under nutrient depletion. The element is possibly also involved in *ech42* regulation, as expression of the *ech42* under carbon starvation is antagonized via the BrlA-like cis-acting element [141]. The BrlA binding boxes have proposed to modulate the expression of several light-induced genes involved in sporulation in *Aspergillus nidulans* [142].

It has been demonstrated that the mechanism of regulation of *nag1* and *ech42* of *T. harzianum* expression is different. Expression of *nag1* is induced by low-molecular-weight chitooligosaccharides and by its own catabolic products (GlcNAc), whereas *ech42* expression is not induced by GlcNAc and chitooligosaccharides [137]. Induction of a hydrolase gene by its own products has also been reported for *T. reesei* genes such as α-galactosidase, β-xylosidase and α-arabinosidase [143]. On the other hand, it is shown that an *N*-acetyl-glucosaminidase of *T. harzianum* is produced at a low constitutive level when the fungus is grown on glucose as sole carbon source [21, 86, 144]. Therefore, it is possible that such low constitutive *N*-acetyl-glucosaminidase, which may be

secreted extracellularly, may be sufficient to initiate chitin degradation and to release GlcNAc, which, in turn, further induce *nag1* expression. Recently two regulating elements, a CCCCT motif and a $CCAGN_{13}CTGG$ motif, have been identified in *nag1* promoter. Similar CCAGNCTGG motifs are also identified in the promoters of *T. asperellum* two GlcNAcase genes, *exc1y* and *exc2y* [48]. Further research shows that mutation of the two regulating elements in *nag1* promoter results in loss of inducibility of *nag1* expression by GlcNAc [145].

As regard to *chit33* from *T. harzianum*, it is reported that the gene is strongly repressed by glucose, and de-repressed under starvation conditions [82]. Northern blot analysis shows that the *chit33* mRNA is strongly expressed under starvation conditions, but very little chitinase activity is observed, suggesting post-transcriptional regulation of *chit33* or a high rate of proteolysis of CHIT33. On the contrary, the mRNA and protein levels of *chit42* are similar, implying that no post-transcriptional regulation is involved in the expression of the *chit42* gene [115]. In addition, *chit33* expression is also induced by heating [135], whereas *ech42* expression is induced by low temperature [137].

It should be noted that there are other regulation mechanisms in fungal chitinases. Research demonstrates the presence of two mRNA species (spliced and unspliced mRNAs) of *chi18-3* and *chi18-13* from *T. harzianum*. The ratio of spliced and unspliced transcripts and their abundance seems to depend on growth conditions [88]. The presence of different levels of spliced and unspliced mRNAs has also been reported in the rice blast fungus *Magnaporthe grisea* [146]. Because of the difference in mRNA level, this regulation belongs to post-transcriptional regulation. Fungal chitinase activity may also be regulated by secretion. It is shown that *N*-acetyl-glucosaminidase activity in *T. harzianum* is divided into secreted activity and activity that is bound to the mycelium and that the ratio between those activities varies for different carbon sources [81]. Similar results are also reported in *N*-acetyl-glucosaminidase activity in *T. asperellum* [48]. In addition, there is the regulation between individual chitinases. Research finds that the *N*-acetyl-glucosaminidase of *Trichoderma atroviride* is essential for its *ech42* induction by chitin, as *chit42* is not induced by chitin in a mutant that does not express the gene *nag1* of *T. atroviride* [80]. However, there are contrary results. It is reported that the absence of expression of *exc2y* (a homologue of *nag1*) in *T. asperellum* does not affect the transcription of *chit42* [48]. This differential regulation of *chit42* may reflect differences in *nag1* and *exc2y* enzymatic activities [48].

Glucose repression is a common phenomenon in the regulation of fungal chitinase genes. At present, the molecular basis of glucose repression to fungal chitinase gene expression has been more understood. It is demonstrated that the URS (upstream regulatory sequence) in the fungal chitinase promoters plays a key role in the regulation of glucose repression. The protein product of the regulatory gene *creA*/*cre1* is a negatively acting transcription factor that binds to DNA sequence motif with the consensus sequence SYGGRG in the URS [147–150]. In the presence of glucose, the activated CreA/Cre1 protein binds to the consensus motif, and represses transcription of chitinase genes.

Regulation under the condition of mycoparasitism

Regulation of chitinase genes under the condition of mycoparasitism is also studied. Research shows the induction of chitinases (CHIT102, CHIT73) in *T. harzianum* during parasitism on *Sclerotium rolfsii* [144]. *T. harzianum* CHIT42 chitinase expression is strong enhanced during interactions of *T. harzianum* with *Rhizoctonia solani* [86] and *T. harzianum* with *Botrytis cinerea* [136]. Analysis of confronting cultures of *T. harzianum* and its hosts (such as *Botrytis cinerea* and *Rhizoctonia solani*) demonstrates that the transcript of *ech42* and *chit36* is observed during the pre-contact stage of the confrontation [85, 89, 151]. Recently, *ech42* expression has been shown to be triggered by diffusible factors whose formation does not require contact between *T. harzianum* and *R. solani* [152–153]. The diffusible factors are shown to be a macromolecule produced by *T. harzianum*. Further research shows that the macromolecule is an enzyme, most likely a constitutive chitinase as its action can be inhibited by allosamidin. The action of the enzyme on *R. solani* is crucial for *ech42* expression [153]. According to the above evidence, it is suggested a model in which an attack on *R. solani* by constitutive chitinase of *T. harzianum*

releases a low-molecular-weight inducer of *ech42* expression [89, 153]. Interestingly, it is also reported that mycoparasitic interaction relieves binding of the Cre1 carbon catabolite repressor protein to promoter sequences (SYGGRG) of the *ech42* gene [136]. However, mechanism of the relief is unknown.

GlcNAcase regulation in mycoparasitism is simpler than *ech42*, similar to the regulation of *nag1* under the condition of culture. Two GlcNAcases (EXC1Y and EXC2Y) of *T. asperellum* are up-regulated just before the physical interaction between the parasite and its host. GlcNAc is a diffusible element that up regulates the two GlcNAcases, and it may be generated by the action of the constitutive activity of GlcNAcases, starting positive feedback regulation [48].

Unlike *ech42*, *chit33* is expressed after direct contact of *T. harzianum* and *R. solani* [89, 135, 137, 153], indicating triggering of *chit33* is likely to be different from that of *ech42*. It is suggested the induction of *T. harzianum* chitinases is an early event, which is elicited by the recognition signal (i.e. lectin–carbohydrate interactions in mycoparasitism) [144]. A lectin from the cell walls of the host *Sclerotium rolfsii* is shown to serve as a recognition signal [144, 154, 155]. This suggestion implies that *T. harzianum* chitinases should be elicited after direct contact of *T. harzianum* and *R. solani*, namely after interaction and recognition between the lectin in cell surface of the host and carbohydrate in cell surface of *T. harzianum*. Whether *chit33* expression is involved in the recognition signal is not clear, and this requires further studies in the future.

Functions and applications of fungal chitinases

In fungi, chitinases play important biological and physiological roles, containing autolytic, nutritional, morphogenetic, and parasitic roles. In the yeast *Saccharomyces cerevisiae*, disruption of its chitinase gene (*CTS1*) results in cell clumping and failure of the cells to separate after division [47], complementing evidence that cell sepatation of *S. cerevisiae* is inhibited by the potent chitinase inhibitor demethylallosamidin [156]. Disruption of *CTS1* can also promote pseudohyphal growth [157]. Recently, the second chitinase gene, *CTS2*, has been identified in *S. cerevisiae*. The gene product is involved in sporulation, as disruption of *CTS2* results in abnormal spore wall biosynthesis and failure to form mature asci [158]. In the dimorphic yeast *Candida albicans*, results from disruption of its chitinase gene, *CHT2*, suggest a role for the gene product in cell separation [71]. Deletion of *CHT3* generates chains of unseparated cells in the yeast growth phase [159]. It has been shown that the membrane-bound endochitinase and *N*-acetyl-β-glucosaminidase of the dimorphic fungus *Benjaminiella poitrasii* significantly contribute to its morphological changes during the yeast-mycelium transition [160]. *Kluyveromyces lactis*, an industrially important yeast, secretes a killer toxin, which has an essential chitinase activity [161]. The toxin is a heterotrimeric glycoprotein composed of α, β and γ subunits encoded by the killer plasmid k1. Its toxicity to competitive yeast is thought to be involved in the exochitinase activity of α subunit [162]. Recently, the second chitinase (KlCts1p) secreted from *K. lactis* has been identified and characterized [75]. KlCts1p is shown to be a nucleus-encoded endochitinase. Disruption of *KlCts1* results in cell separation defect, suggesting that KlCts1p is required for normal cytokinesis.

In filamentous fungi, chitinases may have roles during sporulation, spore germination, hyphal growth and hyphal autolysis. It is reported that the *chi3* chitinase gene of *Rhizopus oligosporus* is transcribed during hyphal growth and the deduced amino acid sequence of *chi3* has no potential secretary signal sequence in its amino terminus, suggesting that the *chi3* chitinase may possibly function in loosening the cell wall at the hyphal tip to enable turgor pressure to extend the hypha at the apex [52]. Research shows that disruption of a gene encoding *Aspergillus nidulans* chitinase, *ChiA*, leads to a decrease in the frequency of spore germination and a lower hyphal growth rate [94]. The specific chitinase inhibitors allosamidin or demethylallosamidin inhibit fragmentation of hyphae into arthroconidia of *Acremonium chrysogenum* [163]. Disruption of the *nagA* gene of *Aspergillus nidulans* results in poor growth on a medium containing chitiobiose as carbon source, but no phenotypic differences in growth with wild-type strains on a medium containing glucose as carbon source. This suggests that NagA is not essential for the growth of *Aspergillus nidulans* on the medium containing easily metabolizable carbon sources,

and its main role is assimilation of chitin degradation products [95]. A *nag1*-disruption strain of *Trichoderma atroviride* exhibits delayed autolysis [80]. However, it is demonstrated that disruption of the gene encoding the Cts1p chitinase of *Coccidioides immitis* and the gene encoding the ChiB1p chitinase of *Aspergillus fumigatus* has no effect on their growth or morphogenesis [96, 164]. One possible reason is that related enzymes in *A. fumigatus* and *C. immitis* may compensate for the loss of ChiB1p or Cts1p, as the two species contain large numbers of chitinase-encoding genes. On the other hand, it is also suggested that these secreted chitinases may not have morphogenetic roles, and they play more principal roles in the digestion and utilization of exogenous chitin for energy and biosynthesis and recycling of fungal chitin during autolysis [1, 96].

Chitinases have been suggested to be involved in mycoparasitism. Upon contact with the host, the mycoparasitic fungus *T. harzianum* mycelium coils around the host hyphae, forms hook-like structures that aid in penetration the host's cell wall, and finally absorb nutrients from host cells. It is suggested that chitinases play an important role in the above mycoparasitic progress, especially in the cell wall penetration and nutrient utilization by degrading the cell walls [144, 165–167]. Recent research shows that chitinase genes (such as *ech42*, *chi33*, *nag1*, *chi18-13*) from *T. harzianum* play a important role in mycoparasitism [88, 89, 153]. It has been reported that disruption of *ech42* gene affects mycoparasitism in *T. harzianum* [168].

Except important biophysiological functions, fungal chitinases have shown an immense potential application in various fields. Recently, the possibility for improving plant resistance using genetic manipulation techniques is promising. The *chit42* gene of *T. harzianum*, encoding a powerful endochitinase with a much stronger antifungal activity to a wide range of phytopathogenic fungi, is expressed constitutively in tobacco, apple and potato, and these transgenic plants show a high level resistance against phytopathogenic fungi tested [112, 113]. Transgenic tobacco plants expressing the *S. cerevisiae* Cts1 chitinase can inhibit spore germination and hyphal growth of *Botrytis cinerea* [169]. Fungal chitinases are also used for insect control. Overexpression of the *Bbchit1* gene from the entomopathogenic fungus *Beauveria bassiana* in transgenic *B. bassiana* can significantly enhance the virulence of *B. bassiana* for aphids. Insect bioassay results show significantly lower 50% lethal concentrations and 50% lethal times of the transformants overexpressing *Bbchit1* gene compared to the values for the wild-type strain, suggesting that overproduction of endochitinase Bbchit1 can promote the infection efficiency of *B. bassiana* in aphids and accelerate infection [140]. In biological research, chitinases from *T. harzianum* are used for the generation of fungal protoplasts [170]. Chitinases are also used in bioconversion of shellfish. The chitinases of *Myrothecium verrucaria* and *Saccharomyces cerevisiae* are utilized for the production of single-cell protein (SCP) from chitinous waste [171]. The enzymatic conversion of chitin to ethanol has been suggested [172].

Prospects

Fungal chitinase researches have made fast progress in molecular levels. The future researches should aim to discovery of novel chitinases, especially from extreme environmental fungi, determining biological functions of chitinase in growth and development and mycoparasitism, signaling in chitinase expression, regulation mechanism between different chitinases, gene evolution and generating new chitinases with protein engineering.

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