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Mini-review

Extracellular enzymes serving as virulence factors in nematophagous fungi involved in infection of the host

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Abstract

Extracellular enzymes, including serine protease, chitinase and collagenase, corresponding to the main chemical constituents of the nematode cuticle and eggshell, have been reported to be involved in the infectious process as virulence factors. This review will focus on the categories, characterization, purification, cloning and potential function of these virulence enzymes and will attempt to provide new insights into the mechanisms of fungal pathogenesis in nematodes.
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Keywords: Nematophagous fungi; Virulence enzymes; Infection of nematodes; Mechanisms of infection

1. Introduction

Plant-parasitic nematodes, important agricultural pests, have been reported to cause damage amounting to more than 100 billion US dollars per year throughout the world [20], placing them behind fungi but ahead of bacteria and viruses in terms of damage. In recent years, nematophagous fungi, one of the natural enemies of nematodes, have been employed in biological control because of their unique ability to capture and infect nematodes. This alternative strategy is attracting more and more attention, since traditional methods, i.e., nematicides and antithelminthic drugs, have evoked major environmental concerns and development of resistance.

Critical events during the process of nematophagous fungal infection are as follows: the cuticle is penetrated, the nematode is immobilized and the prey is finally invaded and digested by the fungus [1]. The molecular mechanisms of the sequence are not well explained, but based on research on entomopathogens, whose infectious mode was believed to be somewhat similar to that of nematophagous fungi, it is likely that hydrolytic enzymes participate in several steps of host infection. Moreover, ultrastructural and histochemical studies have suggested that penetration of the nematode cuticle involves the activity of hydrolytic enzymes [7]. In the last decade, extracellular enzymes as virulence factors in the infection process have been intensively studied, and the identification of numerous enzymes has confirmed their involvement in the molecular mechanism of infection. This review will deal with reported virulent enzymes of nematophagous fungi infecting nematodes, including their cloning, characters and their potential roles in biological control.

2. The nematode cuticle and varieties of associated enzymes in nematophagous fungi

Nematophagous fungi, like other fungal pathogens, must penetrate extracellular barriers presented by the host. Thus, the cuticle of adult nematodes or the shells of eggs play an important role in preventing nematophagous fungal infection. The cuticle of adult nematodes, as a non-cellular layer produced by the hypodermis, consists mainly of proteins including keratin, collagen and fibers running diagonally in

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opposite directions from each other. Collagen is the principal structural component and in *Cunninghamella elegans*, an important model species, the genome contains 5–150 collagen genes, most of which are believed to encode the cuticle. Thus, the efficient degradation of collagen in the cuticle was believed to be absolutely necessary for infection of adults. The shell of nematode eggs is formed by three layers including the chitinous layer, which is composed of protein matrix (50–60% of the composition) embedding chitin microfibrils. This chitinous layer is the thickest and probably the major barrier to infection.

At the early stage of infection of nematodes by nematophagous fungi, penetration of the nematode surface (cuticle) has been assumed to be due to the combination of mechanical activity and hydrolytic enzymes. The latter extracellular enzymes, corresponding to the main chemical constituents of nematode cuticle and eggshell, such as protein and chitin, were revealed to contribute to this early stage of infection. Several extracellular enzymes from nematophagous fungi, including protease (especially the subtilisin family of serine proteases), chitinase and collagenase involved in the infection of nematodes, have been identified, cloned, and homologously or heterologously expressed, respectively. Those isolated virulent enzymes are listed in Table 1 and it is assumed that they are at least partly responsible for fungal penetration of the nematode cuticle and/or digestion of the internal tissues of the host. Additionally, recent studies have been carried out on the potential functions of hydrolytic enzymes at other stages of infection, especially as they may also participate in the process of releasing toxins which antagonize the nematode. Some of these studies have touched upon the molecular level and point to the extracellular enzymes as virulence factors in the mechanism of the infection, which is also very helpful in improving the control potential of nematophagous fungi with genetic engineering.

Table 1
Extracellular enzymes in nematophagous fungus infecting nematodes

Types of extracellular enzymes	Identified and purified enzymes	Origins of enzymes
Subtilisin-like serine protease	P32	*V. suchlasporium*
	PII, Aoz1	*A. oligospora*
	pSP-3	*P. lilacinus*
	VCP1	*P. chlamydosporia*
Chitinase	CHI43	*V. chlamydosporium*, *V. suchlasporium*
Collagenase	–	

3. Virulence enzymes involved in infection of nematophagous fungi

3.1. Subtilisin-like serine protease

As is generally known, subtilisin-like serine protease is a virulence enzyme characterized by its catalytic domain of aspartic acid–histidine–serine, and its substrate with a serine residue susceptible to organic phosphate fluorine was most intensively studied in nematophagous fungi. In 1988, a Spanish research team detected extracellular protease in fungal endoparasites of the cyst nematodes *Verticillium suchlasporium* isolated from infected eggs [11]. Two years later, the first serine protease of P32 was purified from a strain of those *V. suchlasporium* isolates and the purified protease was systematically characterized, especially its activity of degrading certain cyst nematode proteins [12]. Furthermore, localization of P32 in the process of infection was completed with immunocytochemistry and supported the conclusion that serine protease was involved in egg penetration by *V. suchlasporium* [13]. Up to now, four additional kinds of serine protease in nematophagous fungi have been purified and cloned, including PII and Aoz1 from *Arthrobotrys oligospora* [1,2,8,9,30], pSP-3 from *Paecilomyces lilacinus* [17] and VCP1 from *Paecilomyces chlamydosporia* [16,22,23], among which PII was the best studied (the purified serine proteins and their corresponding properties are shown in Table 2).

A. oligospora is a major species of nematode-trapping fungi that captures nematodes with special hype of three-dimensional networks. It can penetrate and immobilize nematodes within a few hours, which is assumed to be partly

Table 2
Properties of extracellular serine proteases isolated from nematophagous fungi

Nematophagous fungi	Serine protease	Molecular mass (kDa)	p*I*	Inhibitor of protease	Optimum pH	Substrate	Gene cloning
V. suchlasporium	P32	32	–	PMSF, *p*CMB	8.5	Proteins from mature eggs	–
A. oligospora	PII	35	4.6	PMSF	7–9	Casein, BSA, gelatin, denatured collagen, the cuticle of nematode	Genome sequence of PII (X94121)
A. oligospora	Aoz1	38	4.9	PMSF, SSI	6–8	Casein, gelatin, azocoll the cuticle of nematode	Full cDNA sequence (AF516146)
P. lilacinus	pSP-3	33.5	>10.2	PMSF	10.3	Vitellin, the eggshells of immature eggs	Partial cDNA (L29262)
P. chlamydosporia	VCP1	33	10.2	PMSF	–	Casein, BSA, azocoll, eggshells of *M. incoymta*	Full cDNA sequence (AJ427454)

PMSF, phenylmethylsulfonylfluoride; *P*CMB, *p*-chloromercuric benzoic acid; SSI, an inhibitor of the subtilase family of serine proteases, Novozyme; BSA, bovine serine albumin.

due to the extracellular enzymes. The activity of extracellular protease was measured in the liquid culture and significantly decreased by the serine protease inhibitor of PMSF, so it seemed that the protease should belong to the serine protease family [1]. Then, with protein chromatography and screening of the genome library, protease PII was purified and cloned. The obtained nucleic acid sequence as well as the peptide sequence confirmed previous speculation that PII was a serine protease with a molecular mass of approximately 35 kDa, which had optimum activity between pH 7 and 9. As for the utilization of substrates, PII showed high hydrolytic activity against broad protein substrates, such as BSA, gelatin, denatured casein and collagen, and even the preparation of nematode cuticle, but low activity toward native collagen [2,8]. In 2002, Ahman et al. studied in detail the potential roles of protease II in host infection by generating several *PII* mutants, including disrupted *PII* gene mutants and *PII* overexpression mutants. Deletion of the *PII* gene caused a limited effect on pathogenicity, including decreased percentages of adhesion and immobilization of nematodes, while the overexpression of mutants demonstrated a higher capacity to kill nematodes [9]. The previous experiments all suggested that the serine protein of PII is an important pathogenic factor in the nematophagous fungus *A. oligospora* killing of its hosts.

In fact, cloning and sequencing of all serine proteases from nematophagous fungi have illustrated that they have a high degree of similarity to protease K of the subtilisin-like serine protease family, with the conservation of the aspartic acid–histidine–serine catalytic triad and substrate binding domains, which is also the most important characteristic in this family (the alignment between all subtilisin-like serine proteases is shown in Fig. 1). In analysis of these serine proteases, they share biochemical traits: their molecular masses are mostly between 32–38 kDa; maximum activities can be obtained in an alkaline situation; serine protease inhibitors such as PMSF can significantly decrease their activities; the purified proteases can degrade the proteins from nematode cuticle and damage the adult nematode cuticle and nematode eggshells. Light microscope and scanning electron microscopy (SEM) showed that the outer layers of eggshells and cuticles of nematodes exfoliated; indeed, large flaws even appeared with treatment of the purified protease, which could serve as proof that the extracellular serine protease can destroy the integrality of the cuticle or eggshells to help in penetration by pathogenetic fungi. On the other hand, some experiments also supported the hypothesis that activities of serine protease initiate or trap nematophagous fungi, apart from degrading the cuticle or eggshell of the host. This hypothesis was based on the fact that deletion mutants of PII had a lower number and the multicopy mutants a larger number of traps in *A. oligospora*. Analysis of these results revealed two possible reasons: (1) peptides produced by extracellular protease hydrolyzing the cuticle of nematodes could simulate the formation of traps; (2) the extracellular protease could rapidly digest the tissues of the infected nematodes to ensure enough nutrients for the development of an infection structure [9]. Additionally, these serine proteases all have a relatively broad substrate specificity, based on which it is believed that the proteases have two independent functions: one involves saprophytic growth and the other involves infection of nematodes. Such a dual role has also been assigned to the homologous serine protease Pr1 from the entomophagous fungus *Metarhizium anisopliae* [25] and the serological and functional relationship between Pr1 and VCP1 seems to support the above hypothesis [19]. Another possible similarity is the intraspecific polymorphism that is a common phenomenon in subtilisins from pathothogenic fungi [3], although among the five kinds of subtilisin-like serine protease from nematophagous fungi, it has only been proven in VCP1 [16]. The observed intraspecific variation in fungal subtilisins is not usually in the structurally conserved regions such as the binding domain or active center of the catalytic domain. The small differences in amino acid sequences may have effects upon substrate utilization, kinetics and surface properties rather than on the essential activity of the enzyme [24], which is also confirmed by the difference in substrates between VCP1 from root knot and cyst nematode isolates [16].

3.2. Chitinases

Chitinases, which have been assumed to be required for hyphal growth [26] and participate in infection of mycoparasites and entomopathogenic or nematopathogenic fungi, are a kind of inducible enzyme catalyzing chitin that is one of the important components of invertebrate cuticles. Fungal chitinase and its potential role in infecting nematode eggs were first put forward by Wharton [28]. Then, chitinase activity was detected by enzymatic assay in *Verticillium* spp. isolated from infected nematode eggs. It was also revealed that the proportion of infected nematode eggs increased concurrently with enhancement of chitinase activity [4]. However, in succeeding years, no chitinases from nematophagous fungi have been purified and characterized, while a corresponding chitinase related to infection of mycoparasites and entomopathogenic fungi was monitored and cloned, which represents a good illustration of the chitinase role in antagonizing the host [10,18,29]. It was in 2002 that purification and identification of CHI43, the first chitinase serving as a nematicidal factor in infecting nematode eggs, was reported [27].

As it is a kind of inducible enzyme, the activity of chitinase could be detected in the filtrate and increased with time for more than 2 weeks when nematophagous fungi of *V. chlamydosporium* and *V. suchlasporium* were grown in semiliquid medium containing colloidal chitin as the main source of C and N. The same culture filtrates were synchronously analyzed by SDS–PAGE electrophoresis. An obvious protein band of 43 kDa, plus 32 kDa (it seemed consistent with the serine protein in *V. suchlasporium* identified by Lopez-Llorca), was observed and its concentration increased

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PII        MLTNGLISLLAIAGLATNAFAGPIRKVSNAGAAGAIADKYIVVLKKGLSDSAVQTYH.RISSFHSNVARDLTGARAHGVGRKFRFSSTG.  88
Azol       MLTNGLISLLAIAGLATNAFAGPIRKVSNAGAAGAIADKYIVVLKKGLSDSAVSKHTNRISSFHSNVARDLTGARAHGVGRKFRFSSTG.  89
VCP1       ..................MQLSVLLTLLPAVLAAPAIVEQRAEPAPLFTPKSSIIAGKYIVKFKDGVARIAADEAT.SALSAKADHVYSHL  72
pSP-3      ........................................ARAPLLTPRGASSSSTASTLSSSRTACPSPLSTRLSALCPRRPTASTTT  49
PK         ..................MRLSVLLSLLPLALGAPA.VEQRSEAAPLIEARGEMVANKYIVKFKEGSALSALDAAMEKISGKPDHVYKNVF  72
Consensus

PII        FNGYVGGFDKATLQEILNSPEVDYVEQDTVVTTYA..EQTDSTWGLDRISHEDYSAPYTYEYDETAAGAGTTVYYVIDTGIRISHDEFQTV 176
Azol       FNGYVGGFDKATLQEILNSPEVDYVEQDTVVTTYA..EQTDSTWGLDRISHEDYSAPYTYEYDETAAGAGTTVYYVIDTGIRISHDEFQTV 177
VCP1       FNGFAGSLTKEELQTLRNHPDVDFIEKDAVMTANAIVEQQGAPWGLGRISNRQKGST.TYRYDDS.AGNGACVYVLDTGIETTHPEFEG. 159
pSP-3      FSEASRNLNANDLKTLRDHPDVEYIEQDAIITINAYTQQPGAPWGLGRISHRSKGST.TYEYDTS.GGSGTCAYVIDTGVEASHPEFEG. 136
PK         SGFAATLDENMVRVLRAHPDVEYIEQDAVVTINAAQTN...APWGLARISSTSPGTS.TYYYDES.AGQGSCVYVIDTGIEASHPEFEG. 156
Consensus                                        a        wgl ris        ty yd    g g   yv dtg    h ef

PII        NGSSRATWGFNSVDKTDSDGNGHGTHCAGTIAGKTYGVSKKAKVVAVKVLSAGGSGSTAGVVSGMNWVAENAT....PNFSVASMSLGGS  262
Azol       NGSSRATWGFNSVDKTDSDGNGHGTHCAGTIAGKTYGVSKKAKVVAVKVLSAGGSGSTAGVVSGMNWVAENAT....PNFSVASMSLGGS  263
VCP1       ....RATWLKSFIDGENNDGHGHGTHCAGTVGSKTYGVAKKAKLLAVKVLDNGGSGSYAGVIAGMEFVSQDYKTRGCPNGAIASMSLGGP  245
pSP-3      ....RASQIKSFISGQNTDGNGHGTHCAGTIGSKTYGVAKKTKIYGVKVLDNSGSGSYSGIISGMDFAVQDSKSRSCPKGVVANMSLGGG  222
PK         ....RAQMVKTYYYSSR.DGNGHGTHCAGTVGSRTYGVAKKTQLFGVKVLDDNGSGQYSTIIAGMDFVASDKNNRNCPKGVVASLSLGGG  241
Consensus       ra            dg ghgthcagt    tygv kk     vkvl   gsg        gm            p    a  slgg

PII        KSAALNTAVDAIFNAGITIVVAAGNENQDAKNVSPASAPNAITVGAIDSSNKIASFSNWGTLIDVFAPGVGVLSSWATSDKETKTISGTS  352
Azol       KStALNaAVDcIFNAGITIVVAAGNENQDAKNVSPASAPNAITVGAIDSSNKIASlSNWGTLIDVFAPGVGVLSSWATSDKETKTISGTS  353
VCP1       FSASVNQAAAAMVSSGVFLSVAAGNDGADAARYSPASEPSACTVGATTSTDARSSFSNFGKLVDIFAPGSAILSTWINGGTRS..ISGTS  333
pSP-3      KAQSVNDGAAAMIRAGVFLAVAAGNDNANAANYSPASEPTVCTVGATTSSDARSSFSNYGNLVDIFAPGSNILSTWIGGTTNT..ISGTS  310
PK         YSSSVNSAAARLQSSGVMVAVAAGNNNADARNYSPASEPSVCTVGASDRYDRRSSFSNYGSVLDIFGPGTSILSTWIGGSTRS..ISGTS  329
Consensus       n         g    vaagn   a   spas p   tvga       s sn g   d f pg   ls w         isgts

PII        MACPHVAGLAAYYISASEGGADPATITDKITSSAVSGQVTGNIRGSPNKIAYNGYA                    408
Azol       MACPHVAGLAAYYISASEGGADPATITDKITSSRRQWSGHREHPWLPKQDRLQRICLSTHSPKTNHQVTIVAS   426
VCP1       MATPHVAGLAAYLNALQGVVS.PAALCKKIQDTAIKNALTGVPASTVNFLAYNGA                     387
pSP-3      MATPHIVGLGAYLAGLEGFPG.AQALCKRIQTLSTKNVLTGIPSGTVNYLAFNGNPSG                  367
PK         MATPHVAGLAAYLMTLGKTTA.ASACRYIA.DTANKGDLSNIPFGTVNLLAYNNYQA                    384
Consensus  ma ph  gl ay
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Fig. 1. Alignment of serine protease amino acid sequences from *A. oligospora* (PII and Aoz1), *P. chlamydosporia* (VCP1), *P. lilacinus* (pSP-3), and *Tritir. Album limber* (PK). The amino acids underlined indicate the conserved catalytic triad of the aspartic acid–histindine–serine. The GenBank accession numbers of PII, Aoz1, VCP1, pSP-3 and PK are, respectively, X94121, AF516146, AJ427454, L29262 and CAA32820.

along with the rise in chitinase activity. Then the 43 kDa protein was purified with chromatography on a macroporous cross-linked chitin affinity matrix and zymography also confirmed the endochitinolytic activity. Although CHI43 from *V. chlamydosporium* or *V. suchlasporium* exhibited the same optimum activity toward colloidal chitin at pH 5.2–5.7, the p*I*s of CHI43 were different between the two fungal origins. Analytical IEF of CHI43 from *V. chlamydosporium* showed the presence of different isoforms, which had a main p*I* of 7.9 and weaker bands of p*I* 7.3 and 7.6, while the p*I*s of three isoforms in *V. suchlasporium* were separately 7.6, 7.1, 7.4. Since the nucleic acid and deduced protein sequences cannot be obtained, the minor differences in amino acids that result in isoforms with the same molecular mass and different p*I*s, and different resultant enzymatic properties, remain to be further studied. To reveal the effect of CHI43 on degradation of nematode eggshells, the eggs of *G. pallida* were treated with chitinase of CHI43 or/and serine protease of P32. Scars and slight peelings could be observed on the surface of eggshells when only CHI43 or P32 was recruited; a combination of CHI43 and P32 seemed to result in more serious damage, while the untreated eggs remained on a smooth and undisturbed surface as negative control. Thus, these results demonstrated that chitinase might be involved in the breakdown of nematode eggshells by fungal parasites and the collaboration of different virulent enzymes, for example chitinase and serine protease, seemed to be more efficient in infection.

3.3. Collagenase

Collagenase was defined as an enzyme that catalyzes the hydrolysis of collagen and gelatin rather than other proteinic substrates [14], and up to now many kinds of collagenases of different microorganic origin, including serine protein protease, have been identified. In 1980, Schenck et al. examined the capabilities of producing collagenase or keratinase in the eight species of nematophagous fungi during the process of infecting nematodes, and it was shown that all of the tested species could secrete extracellular collagenase with good activities of hydrolyzing collagen [21]. In that experiment, the fungi were grown in liquid cultures for 6 days at 28 °C followed by filtration through filter paper, and protease activity was determined with cultural supernatant. Then the collagenases were partially purified and detected for remaining activities in the range of pH 2.5–10. In a series of experiments with bacterial collagenase and culture of *C. elegans*, many characters of collagenase associated with the infection have been confirmed [5,6,15]. These characters include the capacity of purified collagenase to degrade proteins from the nematode cuticle; this could help in penetration of the host the cuticle; it also influences the plant-parasitic nematode in terms of life history, mobility and the hatching of eggs, which suggests that collagenase may have a nematotoxic composition in the process of nematophagous fungal infection. But it is not sure whether the other kinds of collagenases apart from serine protease could serve as infectious factors, since low activity against native collagen was obtained with serine protease alone, which does not seem efficient enough in infection.

4. Concluding remarks

As suggested by all of the studies above, enzymes undoubtedly serve as important virulence factors in the infectious process of nematodes. The complex componential nature of the cuticle and eggshells has demonstrated that penetration requires the synergistic action of several different enzymes. But limitations in enzyme categories and gaps in our knowledge of the action mode leave certain mechanisms of infection of nematodes unexplained. Detailed exploration of the function of virulence enzymes is needed to gain more information on such mechanisms involved in nematophagous fungal infection of hosts, which could serve as useful tools for improving nematicidal activity in biological control.

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