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Review

Extracellular peptidases of insect-associated fungi and their possible use in biological control programs and as pathogenicity markers

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ABSTRACT

This review deals with characteristics of peptidases of fungi whose life cycles are associated with insects to varying degrees. The review examines the characteristic features of the extracellular peptidases of entomopathogenic fungi, the dependence of the specificity of these peptidases on the ecological characteristics of the fungi, and the role of peptidases in the development of the pathogenesis. Data on the properties and physiological role of hydrolytic enzymes of symbiotic fungi in "fungal gardens" are also considered in detail. For the development of representations about mechanisms of control over populations of insect pests, special attention is given to analysis of possibilities of genetic engineering for the creation of entomopathogens with enhanced virulence. Clarification of the role of fungi and their secreted enzymes and careful environmental studies are still required to explain their significance in the composition of the biota and to ensure widespread adoption of these organisms as effective biological control agents. The systematization and comparative analysis of the existing data on extracellular peptidases of insect-associated fungi will help in the planning of further work and the search for markers of pathogenesis and symbiosis.

1. Introduction

The development of microbial communities is largely determined by the availability of organic carbon, nitrogen, and sulfur sources. These elements are contained in peptides and proteins the breakdown of which requires vigorous secretion of extracellular peptidases by fungal mycelium.

Fungi and insects are in close contact in many terrestrial and in some aquatic habitats. The interactions of fungi with insects are of great interest for environmental microbiology, determination of the balance of ecosystems, biodiversity and evolution of eukaryotic organisms and pathogens, and insect pest control. Important mediators in these relationships include fungal peptidases, in one case providing penetration of the fungus into the tissues of the insect, causing disease and death of the host (Joop and Vilcinskas, 2016), or in other cases creating conditions for their mutually beneficial coexistence (Kooij et al., 2014). These proteolytic enzymes are not only used by fungi to utilize nutrient resources, but they are also components of defense–attack systems (Naumann et al., 2014, 2015; Samuels et al., 2011) that control the stability of biotic relations and allow the study of ecological interactions and metabolic activities of fungi.

The diversity of proteolytic enzymes is related to the fact that they perform different functions and are secreted and act under various conditions. Analysis of the secreted peptidases in fungi from different ecological groups indicates the possibility of using these enzymes as markers indicating the type of nutrient preferred for a particular fungus (Dubovenko et al., 2010). Insect-related fungi characterized by parasitic or saprotrophic lifestyles are a convenient model for testing this idea (Semenova et al., 2017).

Data on the physicochemical properties, mechanisms of action, and participation in physiological processes of characterized peptidases of insect-associated fungi are collected and documented in a number of recent reviews (Shahid et al., 2012; de Carolina Sánchez-Pérez et al., 2014; Mondal et al., 2016; Dar et al., 2017).Genes and cDNA from different classes of peptidases belonging to fungi related to insects have been cloned and sequenced (Yu et al., 2012; Wang et al., 2013); this will help to more fully understand their

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functional role using the techniques of molecular biology (gene knockout, site-directed mutagenesis). Nevertheless, although our understanding of the role of fungi and their secreted enzymes is growing rapidly, systematization of results and careful environmental studies are still required to explain their role in the composition of biota and to allow widespread adoption of these organisms as effective biological control agents. Based on the above, the purpose of the review was to assess the regulatory factors of the environment and the distribution of different types of peptidases among insect-associated fungi, to analyze their role in the pathogenesis of entomopathogenic fungi and as trophic enzymes in symbiotic relationships, as well as the possibility of their use in biological control programs. Detection of the relationship between the specific production of certain types of peptidases and virulence of fungi against insects can help to establish marker enzymes of pathogenesis, the identification of new isolates with increased virulence, as well as the development of new biopesticides controlling insect pests without the use of environmentally hazardous chemicals.

1.1. Extracellular peptidases of entomopathogenic fungi

It should be noted that entomopathogens have more genes encoding secreted peptidases than other fungi with known genomes. Thus, *Metarhizium anisopliae* has 132 such genes encoding secreted peptidases (Gao et al., 2011). In the studied fungal entomopathogens, a complete set of proteases was identified including various subtilisins, trypsins, chymotrypsins, metallopeptidases, aspartyl peptidases, and exopeptidases (Table 1). The presence among them, on one hand of aspartyl peptidases and metallopeptidases active at low pH, and on the other hand trypsins and subtilisins active at high pH, reflects the ability of entomopathogens to grow in media of widely differing acid–base properties (Gao et al., 2011).

Compliance of the pH optima of the secreted peptidases to the pH values of the culture medium has been demonstrated in several studies (St. Leger et al., 1998; Maccheroni et al., 2004; Dunaevsky et al., 2006; Penalva et al., 2008). That is, to have nutrient flexibility in a wide range of growth conditions, a fungus produces a specific set of enzymes only in certain conditions when their operation is most effective. Thus, at pH 3.0 *M. anisopliae* expressed maximum level of aspartyl peptidases, at pH 7.0 aminopeptidases and a number of metallopeptidases, and at pH 8.0 it expressed subtilisin-like (Pr1) and trypsin-like (Pr2) peptidases. Alkaline pH itself exerted a derepressive effect on the synthesis of Pr1, but in the presence of the insect cuticle this derepression was three-fold more effective (St Leger et al., 1998). The entomopathogen could itself regulate the pH of the external environment through the production of ammonia, which resulted in pH increase and thus launched the production of subtilisin peptidases (St. Leger et al., 1999; Abdelaziz et al., 2018).

Especially the sharp increase in peptidases of entomopathogens with wide range of hosts, such as the *M. anisopliae* and *Beauveria bassiana*, compared to the narrowly specialized pathogens *Metarhizium acridum* and *Cordyceps militaris*, indicates that this trait may represent adaptation to life on a variety of hosts (Gao et al., 2011; Zheng et al., 2011; Xiao et al., 2012). The number and variety of transcripts of proteases from *M. anisopliae* depends in a specific way on the composition of the culture medium (cuticle and its origin, hemolymph, or root exudates) (Freimoser et al., 2005; Wang et al., 2005). The ability to produce large quantities of secreted peptidases is apparently connected with the necessity of rapid degradation of protective barriers formed by the insect host, and their diversity is due to different functions for survival in various ecological niches outside the host and the need to overcome the ability of insects to produce inhibitors of fungal peptidases (Li et al., 2012).

Fungi are able to use a wide variety of compounds as a source of nitrogen, and vice versa, the nitrogen sources in the environment largely determine the composition of the secreted enzymes. Many species of fungi secrete peptidases with growth on culture medium containing protein as the sole source of nitrogen, others in the presence of protein dramatically increase the synthesis and secretion of peptidases (Monod et al., 2002; Dias et al., 2008). Under derepression conditions, Pr2 from *M. anisopliae* can be induced by a number of different protein substrates, whereas Pr1 was specifically induced only when insect cuticle or protein components of the cuticle, a substrate specific for entomopathogenic fungi, was added to the culture medium (Paterson et al. 1993, 1994). Demonstration of differential expression of Pr1 and Pr2 in different cultural media indicates that entomopathogens can regulate their expression during saprotrophic growth, and such regulation is a factor that can flexibly respond to changing environmental conditions (Dhar and Kaur, 2010; Leao et al., 2015).

Most fully and convincingly, the combined degradation of insect cuticle by peptidases was studied and well-documented on a *M. anisopliae* model system, mainly by a series of works by St. Leger and his colleagues (St. Leger et al., 1986a, 1986b; 1994a, 1994b; 1996a). It was shown that proteins of the cuticle are not only a structural but also a protective component, binding chitin and making it resistant to chitinases. Chitinase from the culture liquid of *M. anisopliae* was able to cleave 3–4 % of the cuticle chitin to N-acetylglucosamine. Simultaneous treatment of the cuticle with proteinase and chitinase increased the release of N-acetylglucosamine to 6 %, and preliminary treatment with peptidase increased the efficiency of chitinase 3.5-fold.

Using molecular biology techniques and genomic analysis further, it was shown that entomopathogens differ among other fungi by a high content of genes of subtilisin-like peptidases. Thus, the entomopathogenic fungus *Metarhizium robertsii* has the highest number of these genes – 48, while the fungus *Saccharomyces kluyveri* has only 1 subtilase gene (Li et al., 2017). The expression of these genes depends on changes in the environment, the stage of the life cycle, and the host (Beys-da-Silva et al., 2014; Varshney et al., 2016). The main function of widely represented subtilisin Pr1 is apparently the hydrolysis of the host cuticle, providing the fungus with nutrients. Among serine peptidases, subtilisin-like peptidases are found in all studied fungi of the indicated type (St. Leger, 2013; de Carolina Sánchez-Pérez et al., 2014; Firouzbakht et al., 2015; Mondal et al., 2016). The entomopathogen generalist *M. anisopliae* can express up to 11 different subtilisins, which are responsible for the degradation of the cuticle of insects belonging to different orders. Their differences in biological functions are related to differences in secondary substrate specificity, adsorption properties towards the cuticle, and stability in alkaline conditions (Bagga et al., 2004). It is likely that the observed differences in the regulation and structural–functional characteristics enable the entomopathogen to react flexibly, producing peptidases corresponding to the composition of the environment that change depending on the stage of the life cycle.

Trypsin-like peptidase Pr2 as well as a thermolysin-like metalloproteinase, metalloexopeptidases, serine dipeptidyl peptidase, and serine carboxypeptidase are formed in smaller amounts in the culture medium. Hydrophilic Arg-Xaa and Lys-Xaa bonds are apparently cleaved by Pr2, and the bonds formed by hydrophobic residues are cleaved by Pr1. A metallocarboxypeptidase functions as a supplement to Pr1, after which peptides terminated with Phe or other amino acids with hydrophobic side groups are released. They become the target for a carboxypeptidase that cleaves them to single amino acids (Joshi and St. Leger, 1999). In that case, if Pr1 and

Table 1
Best-studied extracellular peptidases of fungal entomopathogens.

Enzymes	Source, class/family	Mol. mass, kDa	IEP	pH optimum/T optimum/ stability	Substrates	Inhibitors/affect	References
Chymoelastase Pr1	*Metarhizium anisopliae*, serine, subtilisin-like similar to proteinase K	29	10	8.0–10.0	Insect cuticle, cuticle structural proteins, elastin	PMSF, not inhibited by EDTA and 1,10-phenantroline	St. Leger et al. (1994a)
Subtilisin-like peptidase VlPr1	*Verticillium lecanii*, serine	38		9.0/40 °C/stable at pH 6–11	Casein	PMSF, not inhibited by EDTA. Strongly activated by Ca^{2+}	Yu et al. (2012)
Subtilisin-like peptidase Pr1	*Beauveria bassiana*, serine	105		8.0/35 °C	N-Suc-(Ala) 2-Pro-Phe-*p*Na	AEBSF, to a lesser extent with TLCK and TPCK, not affected by EDTA and 1,10-phenantroline	Firouzbakht et al. (2015)
Elastase	*Conidiobolus coronatus*, serine	28.6	8.4	8.0/30 °C/stable at pH 10 (94 %)	N-Suc-(Ala) 2-Pro-Leu-*p*Na	PMSF	Wloka (2010)
Trypsin-like peptidase Pr2	*Beauveria bassiana*, serine	103		8.0/40 °C	Bz-Phe-Val-Arg-*p*Na	AEBSF and TLCK, to a lesser extent EDTA and 1,10-phenantroline, not affected by TPCK	Firouzbakht et al. (2015)
Trypsin-like peptidase Pr2a	*Metarhizium anisopliae*, serine	30	4.4	8.0	Casein, solubilized cuticle proteins, bonds in synthetic substrates formed by Arg and Lys	PMSF, DFP, leupeptin, antipain, not inhibited by cystatin, EDTA, and 1,10-phenantroline	St. Leger et al. (1996a)
Trypsin-like peptidase Pr2b	*Metarhizium anisopliae*, serine	27	4.9	8.0	Solubilized cuticle proteins, bonds in synthetic substrates formed by Arg carboxyl group	DFP, leupeptin, antipain, not inhibited by cystatin, EDTA, and 1,10-phenantroline	St. Leger et al. (1996a)
Trypsin-like peptidase	*Metarhizium anisopliae*	27	7.6		Insect cuticle, colloidal chitin		Pei et al. (2000)
Chymotrypsin-like peptidase CHY1	*Metarhizium anisopliae*	18.5	8.3	8.0	Hydrophobic amino acid residues		Screen and St. Leger (2000)
Thermolysin-like metallopeptidase	*Metarhizium anisopliae*, metallopeptidase		7.3	5.2–6.0	N-Suc-(Ala) 2-Pro-Phe-AFC	1,10-phenantroline, phosphoramidon	St. Leger et al. (1994a)
Metallopeptidase ZrMEP1	*Zoophthora radicans*, similar to both fungalysin and thermolysin	46			Gelatin	Zn-dependent, affects larvae of cabbage butterfly *Pieris brassicae*	Xu et al. (2006)
Cysteine peptidase P1	*Metarhizium anisopliae*, trypsin-like	71		9.0	Hemoglobin	Affects larvae of *G. mellonella*	Kucera (1980); Cole et al., (1993)
Carboxypeptidase	*Metarhizium anisopliae*	30	9.97	6.8	C-terminal bonds formed by aromatic amino acid residues and amino acids with long hydrophobic side chains	DFP and 1,10-phenantroline, activity not restored by Zn^{2+} or Co^{2+}	St. Leger et al. (1994b)
Metal-dependent aminopeptidase	*Metarhizium anisopliae*, metallopeptidase	45	4.51	7.0	Wide specificity with preference to Ala residues	Bestatin, amastatin, EDTA, 1,10-phenantroline	Samuels et al., 2011; St. Leger et al., (1993)
Prolyldipeptidyl peptidase	*Metarhizium anisopliae*, serine	74	4.01	8.0	N-terminal dipeptides containing Pro at the penultimate position	Diprotin, DFP, PMSF	St. Leger et al. (1993)
Aspartyl protease	*Metarhizium anisopliae*,		5.5–6.9	3.0	Hide protein azure	Pepstatin	St. Leger et al. (1998)

PMSF, phenylmethylsulfonylfluoride; EDTA, ethylenediaminetetraacetic acid disodium salt; TLCK, tosyl-L-lysyl-chloromethane hydrochloride; TPCK, tosyl-L-phenylalanyl-chloromethane; DFP, diisopropylfluorophosphate; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; SBTI, trypsin inhibitor from soybean.

Pr2 are inactivated by inhibitors of serine peptidases contained in the cuticle and hemolymph of insects (Meekins et al., 2017; Yang et al., 2017), a metalloproteinase or cysteine peptidase P1 and trypsin-like cysteine peptidase Pr4 can be activated (Kucera, 1980; Cole et al., 1993). The specificity of the latter is similar to Pr2 trypsin-like peptidase, but it clearly prefers elongated peptide substrates with Arg in P1 position.

Aminopeptidases release individual amino acids that are used for nutrient purposes. Prolyldipeptidase breaks the bonds formed by Pro residues, since Pr1, Pr2, carboxypeptidase, and aminopeptidase cannot cleave Pro residues, which constitute 10 % of all amino acids of the insect cuticle (St. Leger et al., 1986a). The studied exopeptidases alone practically failed to hydrolyze the intact cuticle. However, in combination with Pr1 they effectively increased the release of amino acids from the cuticle.

There is practically no experimental data on the role of aspartyl peptidases of entomopathogens, but it is assumed that they are similar to aspartyl peptidases that assist the human pathogen *Candida albicans* to degrade protein molecules of the cell surface (Schaller et al., 2005).

The involvement of Pr1 peptidase in insect pathogenesis was demonstrated using *M. anisopliae* with mutations in the gene responsible for Pr1 synthesis. These mutants attacked *Manduca sexta* larvae significantly less, but the absence of Pr1A was partially offset by enhanced secretion of Pr1B and metalloproteinase. The introduction of additional genes for Pr1 in the *M. anisopliae* genome reduced the lifetime of infected larvae of *M. sexta* by 25 % (St. Leger et al., 1996b). The involvement of extracellular proteolytic activity in determination of virulence properties is indicated in the case of isolates of *Paecilomyces fumosoroseus* parasitizing whiteflies and other insect pests (Castellanos-Moguel et al., 2007), as well as isolates of *B. bassiana* that are pathogenic against Khapra beetle (Zare et al., 2014). According to these authors, the studies do not demonstrate that the enzymes play a major role in the determination of virulence, but on the basis of the data it is possible to develop rapid methods for the determination of virulence of a strain by its enzymatic activity.

Although the significance of Pr1 subtilisin-like peptidase in pathogenesis is clearly defined and its participation in the hydrolysis of the cuticle is quite clearly established, the role of Pr2 trypsin-like peptidase is less well understood. It requires a specific substrate sequence, and its occurrence is not always associated with the presence of cuticle in the culture medium (Tiago et al., 2002) and can significantly precede the appearance in the medium of the Pr1 peptidase responsible for the degradation of cuticle. Therefore, its role may be more specific and much more diverse – the induction or activation of Pr1 hydrolysis of antifungal protective proteins/antimicrobial peptides of the insect host and specific cleavage of a limited number of bonds formed by Arg or Lys residues in the cuticle.

Nevertheless, the production of a high level of subtilisin-like peptidase, which is one of the virulence factors of entomopathogenic fungi, can hardly be regarded as a marker of pathogenicity, since such subtilisin-like enzymes are widely represented in many saprotrophs, including saprotroph-mutualists (Kooij et al., 2014; Li et al., 2017). Rather, trypsins can claim this role because in the overwhelming majority of cases they are found mostly in fungal pathogens of plants, animals, and fungi (Dubovenko et al., 2010) and are widely represented among the peptidases of entomopathogens, with a clear tendency to an increase in pathogens with a wide range of hosts – 32 genes in *M. anisopliae*, a widespread pathogen of more than 200 insect species, which is almost two-fold more than in *M. acridum*, a highly specialized pathogen of insects of the grasshoppers and locusts families, and 6–10-fold higher than in other studied taxa (Gao et al., 2011). A characteristic feature of many entomopathogens is the presence of chymotrypsin, absent in most fungi from other ecological groups, and possibly derived from a bacterium as a result of horizontal gene transfer (Screen and St. Leger, 2000; Wichadakul et al., 2015).

Peptidases produced by fungi can be used for biological control of insect pests and various pathogens including bacteria, fungi, and nematodes. It was shown that extracellular alkaline peptidases from *Aspergillus fumigatus* cultured in the presence of exoskeleton of the weevil *Callosobruchus maculatus* can facilitate colonization of the insect host (Pereira et al., 2006). Peptidases of entomopathogenic fungi might become the main participants of insecticide preparation or supervector with the use of peptidases, lipases, and chitinases of these fungi for solving problems of insect pest control.

1.2. Hydrolytic enzymes secreted by symbiotic fungi in "fungal gardens"

The set of peptides produced by saprotrophic fungi that are in mutualistic relations with insects (Table 2) have certain differences from the set of peptidases of entomopathogenic fungi (Table 1).

Studies of hydrolytic enzymes produced by fungal symbionts have been carried out mainly on colonies of leaf-cutting ants that form relatively large gardens that are convenient for experiments. Carboxymethylcellulases, laccase, pectinases, xylanases, and peptidases belonging to the fungal symbiont were found in the fungal gardens (Ronhede et al., 2004; Schiott et al., 2008, 2010; De Fine Licht et al., 2013).

Before leaf fragments are placed in a fungal garden, the ants crush the leaf mass to a homogeneous state and moisten it with fecal liquid, forming pellets that are inoculated with mycelium and placed on the top layer of the fungal garden. Hydrolytic enzymes identical to enzymes of the fungal symbionts grown on artificial nutrient media were found in the fecal liquid of the ants (Boyd and Martin, 1975a; 1975b). Subsequently, the identity of the enzymes in the secretions of ants with hydrolases of the fungal symbiont was suggested by isoelectric focusing (Ronhede et al., 2004), and for some enzymes this was confirmed by protein sequences (Schiott et al., 2008) as well as comparative genomic analysis (Kooij et al., 2014). Their overexpression was observed at the ends of the thickened hyphae (gongylidia), which is the food of the ants. The peptidase disappeared from the fecal fluid when the ants were kept in 10 % sucrose and blackberry leaves in the absence of a fungal symbiont (Kooij et al., 2014). Mechanisms by which the fungal enzymes pass through the digestive tract of the ant without loss of activity have not been studied. However, the treatment of the inserted plant material by fungal enzymes probably plays an important role in the colonization of leaf mass by the fungal mycelium. They can speed the digestibility of proteins of plant cells in the leaf pulp that the ants add to the fungus garden, although the participation of these proteases in the activation of certain proenzymes is not excluded. In this case, we are faced with an example of a symbiotic adaptation allowing the use of ants for targeted transport of enzymes from the lower layers of the fungal garden, where the fungus has already entered into a rapid growth phase, and where gongylidia are formed and peptidases actively expressed, to the upper layers where fresh vegetable material is supplied and where high enzymatic activity is necessary for the rapid processing of leaves, but the initiation of rapid growth of the fungal symbiont has not yet been reached. It is noteworthy that the secretion of enzymes by a fungal symbiont in synthetic medium is very weak, and this mutualistic association with ants effectively replaced extracellular secretion in the fungus (Boyd and Martin, 1975b). Peptidases of the fungal symbiont, although synthesized and localized in gongylidia, act as secreted enzymes. It is assumed that hydrolytic enzymes of fungi are concentrated in the digestive

Table 2
Peptidases of fungal symbiont from fungal gardens of ants.

Enzyme	Class/family	Mol. mass, kDa	IEP	pH optimum/ stability	Substrates	Inhibitors	Reference
Proteinase 1	Serine peptidase	70		8.0/stable at pH 5.5–7.5	Azocoll, Bz-Met-*o*Me, N-Ac-Tyr-*o*Me, N-CBZ-Ser-*o*Me	DPF, but not TLCK and SBTI	Boyd and Martin (1975a), 1975b
Proteinase II	Metallopeptidase	41		7.0/stable at 5.5–7.5	Azocoll, oxidized insulin A	Hg^{+2}, EDTA, 1,10-phenantroline; Zn^{+2} restores activity	Boyd and Martin (1975a), 1975b
Proteinase III	Metallopeptidase	15		7.0/stable at pH 5.5–7.5	Azocoll, oxidized insulin A	Hg^{+2}, EDTA, 1,10-phenantroline; Zn^{+2} restores activity	Boyd and Martin (1975a), 1975b
Protease A			6.9–7.0		Skim milk		Ronhede et al. (2004)
Protease B			9.4–9.6		Skim milk		Ronhede et al. (2004)
Serine peptidase	*Leucocoprinus gongylophorus* Serine, subtilisin-like			7.0–7.5	Azocasein, Glp-Ala-Ala-Leu-*p*Na, Suc-Ala-Ala-Pro-Phe-*p*Na, but not Bz-Arg-*p*Na	PMSF, but not TLCK and TPCK	Semenova et al. (2011)
Metallopeptidase				5.2–6.0	Azocasein	EDTA	Semenova et al. (2011)
Serine carboxypeptidase SerI	*Leucoagaricus gongylophorus* Serine, S8A, subtilisin-like	50.1		7.0	Azoalbumin	PMSF; increased 3-fold in gongylidia	Kooij et al. (2014)
Serine carboxypeptidase SerII	Serine, S53, *Laccaria bicolor* grifolisin-like	63.4		7.0	Azoalbumin	PMSF; increased 8-fold in gongylidia	Kooij et al. (2014)
Peptidyl-Lys-metallopeptidase Met I	Metallopeptidase M35, similar to metallopeptidase of *Pleurotus ostreatus*	37.2		6.0/stable to pH 10.0	Azoalbumin	1,10-phenantroline; increased 5-fold in gongylidia	Kooij et al. (2014)
Peptidyl-Lys-metallopeptidase Met II	Metallopeptidase M35, similar to metallopeptidase of *P. ostreatus*	37.5		6.0/stable to pH 10.0	Azoalbumin	1,10-phenantroline; increased 20-fold in gongylidia	Kooij et al. (2014)

PMSF, phenylmethylsulfonylfluoride; EDTA, ethylenediaminetetraacetic acid disodium salt; TLCK, tosyl-L-lysyl-chloromethane hydrochloride; TPCK, tosyl-L-phenylalanyl-chloromethane; DFP, diisopropylfluorophosphate; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; SBTI, trypsin inhibitor from soybean.

tract of the ant due to absorption of water by the intestinal wall (Ronhede et al., 2004). Such concentration of enzymes of the fungal symbiont becomes of great importance in view of the low activity of secreted peptidases characteristic of most saprotrophic fungi.

Among the peptidases produced by a fungal symbiont and used for hydrolysis of plant material, representatives of mainly two classes of proteolytic enzymes have been found – serine and metallopeptidases (Boyd and Martin, 1975a; Semenova et al., 2011; Kooij et al., 2014). Although in the works of Boyd and Martin (1975a) and Semenova et al. (2011) only the presence of activities of serine peptidases or metallopeptidases was demonstrated, Kooij et al. (2014) identified both of them. All of these enzymes possessed broad substrate specificity and were stable in the pH range of 5–9. In all cases a serine peptidase was represented by a subtilisin-like enzyme that plays an important role in the penetration and capture of nutrient sources of saprotrophs, phytopathogens, and entomopathogens (Dunaevsky et al., 2008; Bryant et al., 2009; Li et al., 2017). Two serine peptidases, SerI and SerII, are similar to subtilisin of *Laccaria bicolor* (SerI) and grifolisin (SerII) (Kooij et al., 2014). Apparently, in mutualistic association the functions of subtilisin and metalloendopeptidases from gongylidia are primarily the destruction and digestion of freshly harvested plant material.

It should be noted that trypsin-like peptidases are lacking among the transported or secreted peptidases of the fungal symbiont. If we consider that the presence of trypsin-like enzymes is characteristic of phytopathogenic (Dubovenko et al., 2010) and entomopathogenic (St. Leger et al., 1996a; Pei et al., 2000) fungi, the lack of this type of peptidases suggests the saprotrophic nature of a fungal symbiont.

Comparison of enzymatic activity in colonies of ancestral and derived Attini ant tribes showed that the enzyme systems of the symbionts of “higher ants” are characterized by a significantly higher proteolytic activity. In colonies of leaf-cutting ants, high activity of the enzymes responsible for the breakdown of carbohydrates was also revealed. Analysis of enzyme systems in colonies of higher ants has demonstrated high specificity to the cleavage of the content of protoplasts of plant cells. While fungal symbionts of lower ants secreted enzymes able to only partially breakdown the cell walls of plant cells, this is also characteristic of enzymes of free-living agaricoid fungi (De Fine Licht and Boomsma, 2010).

The pH optimum of the total proteolytic activity of the fungal symbiont from the lower ant colonies was 6.0, but it decreased to 5.0 in the colonies of higher ants, thus approaching the pH that the ants maintain in the fungal garden. Proteolytic activity of the lower ants was determined mainly by metallopeptidases, of the higher ants serine peptidases, and of leaf-cutting ants again metallopeptidases, but the pH optimum was lower than that of metallopeptidases of the fungal symbiont from the colonies of lower ants. The appearance of peptidases with pH optimum shifted to the acidic region was also observed among the serine peptidases of fungi from the colonies of higher ants. Thus, adaptive changes may be the cause for the change of the pH optimum of fungal peptidases in the course of the irreversible domestication of derived fungal symbionts (Semenova et al., 2011). Enzymes of the fungal symbiont of higher and lower ant colonies belonging to two different classes of peptidases markedly differ in substrate specificity, which can meet the requirements of the ecological niches occupied by the mutualist fungus (St Leger et al., 1997). Therefore, one explanation for the shift of proteolytic activity almost exclusively to the activity of serine peptidases could be that the ants cultivate these symbionts on collected leaf and flower material that can be more

efficiently degraded just by the serine peptidases (De Fine Licht and Boomsma, 2010).

Thus, analysis of the accumulated data indicates that the proteolytic enzymes of entomopathogenic fungi overcome all protection layers and allow penetration of the fungus into the body of the living insect, not only participation in the nutrition of the mycelium as in the case saprotrophic and mutualistic fungi. Therefore, the peptidases they secrete need to be more active, diverse, and plentiful. The task of the peptidases of fungal symbionts is to provide accessible nutrient to the growing mycelium and insect host. A very special way of moving fungal hydrolytic enzymes to the site of active processing of the nutrient substrate, developed in the process of evolution, helps the fungi to perform this task.

It is noteworthy that there are few studies on the relations between secretion of certain groups of enzymes, on one hand, and taxonomic position or belonging to different trophic groups of fungi, on the other hand, so comparative analysis of the results should help the planning of further work and the search for markers of pathogenesis and symbiosis.

1.3. Genetic engineering for creation of entomopathogens with enhanced virulence

Interest in chemical substances formed during the vital activity of fungi is due to the practical application of entomopathogens for regulating insect pests in agriculture and for studying the functional role of the enzymes in pathogenesis. Entomopathogenic fungi can keep the number of insects at manageable levels using natural processes within the ecosystem. They are unique because they do not require ingestion or other specialized ways to penetrate into the host (as in the case of viruses, bacteria, and most nematodes), and they grow directly through the cuticle of the insect. Fungi work by a contact method and have a fairly wide range of hosts. All this indicates their great potential for use to control even sucking insects (Mustafa and Kaur, 2009). The use of genetic engineering techniques has allowed in some cases improvement of the virulence of entomopathogenic fungi (Lovett et al., 2019).

Analysis of works using peptidases of entomopathogens to improve the virulence of fungi through genetic engineering allows us to distribute them in three directions. The first is the use of entomopathogens as a source of genes for improving virulence. Constitutive overexpression of the subtilisin-like Pr1A peptidase enhanced the pathogenic effect of *M. anisopliae*, reducing the time to death of insects by 25 % and nutrient consumption by 40 % (St. Leger et al., 1996b). The investigations showed the possibility of using the transformed secreted peptidase of entomopathogen of *Verticillium lecanii* to increase control over plant pathogenesis. The *VlPr1* gene of the subtilisin-like peptidase was used for transformation. The peptidase, expressed in *Escherichia coli*, showed a marked inhibitory activity against several plant pathogens, especially *Fusarium moniliforme*, a major parasite of rice, sugar cane, beans, soybeans, and maize (Yu et al., 2012). The *Pr1* gene from *M. anisopliae* has been used to increase the virulence of *B. bassiana* to the coffee berry borer *Hypothenemus hampei*, a dangerous pest of fruits and beans of the coffee tree. The transformed strain of *B. bassiana* increased the mortality of the insects by 21.7 % and reduced their lifetime by 14.3 % (Gongora, 2004). For its part, the gene of peptidase cdep1 from *B. bassiana* transformed into the nematophagous fungus *Lecanicillium attenuatum* significantly improved its nematicidal activity (Xie et al., 2016). Thus, the use of peptidase associated with pathogenesis from one entomopathogenic fungus can improve the virulence of other pathogenesis-related fungi.

Other strategies for changing the virulence of entomopathogenic fungi are increasing the effectiveness of Pr1 with double transformants or with chimeric proteinase recombinants. An attempt for genetic modification of *B. bassiana* through co-transformation with peptidase genes of Pr1A from another entomopathogen *M. anisopliae* and neurotoxin AaIT of the scorpion *Androctonus australis* did not produce the expected additive or synergistic effect on the insects, and it led rather to antagonistic interactions between the products of the transformed genes (Lu et al., 2008). These authors attributed this result to the degradation of AaIT protein by peptidase Pr1 and point to the need to consider the stage of the cell cycle in which products of transformed genes are expressed and to assess protein interactions in work with multiple genes, especially if one of them is for a peptidase.

A more successful attempt was the construction of a chimeric gene comprising a peptidase and chitinase. The *cdep1* gene encoding a homolog of Pr1A peptidase and the *chit1* gene that encodes chitinase were co-transformed in *B. bassiana*. Transformants of *B. bassiana* carrying the chimeric gene penetrated into the cuticle significantly faster than the wild-type entomopathogen or transformants overproducing only one of the two genes. In this case, the simultaneous use of two genes caused a synergistic effect on virulence. The time required for killing 50 % of insects decreased by 25 %, and the lethal concentration for conidia required for killing 50 % of insects (LC_{50}) decreased by 60.5 % (Fang et al., 2009).

Another case of noticeable effect on insecticidal activity is the acquisition of a chimeric gene using the cDNA of the subtilisin-like CDEP peptidase of *B. bassiana* and toxic crystal protein product of the *cry1Ac* gene of *Bacillus thuringiensis*. The transformants showed increased toxicity to *Helicoverpa armigera* larvae, manifesting a noticeable decrease in LC_{50}. It is assumed that in this case the role of the CDEP peptidase, besides the destruction of proteins of the cuticle, is the hydrolysis of the peritrophic membrane, which ensures the penetration of the spores and crystal toxins of *B. thuringiensis* into the hemolymph of the larvae (Xia et al., 2009).

Finally, reasonable design of a peptidase as a virulence factor might be a strategy for improving a fungal strain by genetic engineering. For example, only the addition of a chitin-binding domain from *Bombyx mori* chitinase to CDEP1 peptidase from *B. bassiana* allowed during expression of the modified enzyme increase in the virulence of the entomopathogen by 25 % compared to the virulence in the constitutive expression of the unmodified peptidase (Fan et al., 2010).

2. Conclusion

Recent advances in genomic biology have revealed features of genomes associated with adaptation of fungi to host insects and determination of the range of these hosts, as well as the evolutionary relationship between insect pathogens and non-pathogens. Molecular biological studies have identified genes for enzyme that function in the interactions of fungi with insects and provide a contribution to the virulence of the fungi. They include secreted subtilisin-like Pr1 peptidase of entomopathogenic fungi that take an active part in the hydrolysis of cuticular proteins and are considered as one of the factors of virulence of the entomopathogens. However, the presence of Pr1 not only in entomopathogens, but also in a wide range of saprotrophs, including saprotroph-mutualists, prevents their consideration as a marker of pathogenesis. For this role trypsin-like Pr2 peptidase is more suitable, which in contrast to Pr1 is present predominantly in pathogens and plays an important role in pathogenesis, but not through the destruction of the integument, but possibly through participation in protection against antifungal protein products. Thus, serine peptidases of entomopathogenic fungi can be biocontrol agents that have commercial potential for development into effective biopesticides.

Rapid development of new methods and interdisciplinary advances in coming y should facilitate understanding of the structural and functional features of these peptidases, which will greatly help with the design of effective biologically active agents. Careful experiments with the enzymes will lead to improved selection of entomopathogenic lines that can be effectively integrated into pest control programs. Analysis of the use of peptidases of entomopathogens alone or as a super-vector in combination with other enzymes and toxins is necessary to achieve maximum efficacy and compatibility, and this may lead to a revolution in the substitution of biopesticides for synthetic insecticides. More field testing is then required to assess the impact of biotic and abiotic factors on the efficacy and sustainability of the new biopesticides. It should be noted that while slowed progress in this direction can be due to the ban of a number of countries (including many EU countries and Russia) on the cultivation of genetically modified crops (GMC), laboratory studies are allowed and continue by various research teams. Although many European countries do not grow GMC, Europe remains one of the world's main consumers of GMC products, importing more than 30 million tons of genetically modified maize and soybean for livestock feed annually.

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