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REVIEWS

Fungal Proteolytic Enzymes: Features of the Extracellular Proteases of Xylotrophic Basidiomycetes

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Abstract—Fungal proteolytic enzymes attract the attention of researches due to such features as high diversity, broad substrate specificity, and stability under extreme conditions. Their functional role is also interesting; it includes a number of processes from the hydrolysis of macromolecular substrates under extremely low nitrogen content to initiation and maintenance of pathogenesis. In the present review, the features of the extracellular proteases of xylotrophic basidiomycetes are discussed. This group is important for the functioning of biological communities and participates in the biological destruction of plant debris; moreover, they are widely used as a source of nutrients and medicines. The review stresses the issues of classification of fungal proteases, their biochemical characteristics and physiological role, as well as the regulation of their activity in the course of fungal growth.

Key words: extracellular proteases, fungi, basidiomycetes, xylotrophs.

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INTRODUCTION

Proteolytic enzymes play an important part in the metabolism of almost all organisms (plants, animals, fungi, bacteria, and viruses). Investigation of proteases is a central issue in enzymology due to both their immense physiological importance and wide application in research and economical activities [1].

Analysis of the literature on microbial proteolytic enzymes suggests three main cases for investigating fungal proteases: if a possibility exists for their medical or economic applications; if they can act as pathogens causing diseases in humans, animals, or plants; and if microbial producers are model objects for the study of physicochemical characteristics and biological role of their enzymes. In all these cases, micromycetes are the major object of investigation. However, in recent works dealing with the first task, an increased interest can be seen in basidial macromycetes, a diverse and ecologically important group; systematization and analysis of available date became urgent.

Xylotrophic basidiomycetes are extremely important for the functioning of biological communities. Moreover, these fungi have long been used as a source of nutrients and medicines. They constitute a major part of industrially cultivated fungal species. Xylotrophic basidiomycetes, mainly wood-degrading fungi, produce a number of extracellular cellulolytic, xylanolytic (hemicellulose-degrading), and lignolytic enzymes which have been extensively studied [2]. However, much less attention has been paid to the role of proteases in xylotrophic fungi, although secretion of proteolytic enzymes is highly important for these organisms (proteins are the main nitrogen source in wood).

Various *Pleurotus* species are presently widely cultivated in many countries in order to obtain their edible fruit bodies. The fruit bodies of these fungi are big and have an agreeable taste. Apart from high edibility characteristics [3], they have useful medicinal features due to the presence of biologically active compounds with a positive therapeutic effect. Numerous studies revealed antioxidant [4], immunomodulating, and antitumor activity [5–8], as well as antiparasitic and antibacterial effects [9, 10] of this group of fungi.

It is worth mentioning that the highest ratio of species with pronounced antitumor activity was found among wood-decomposing fungi (*Lentinus (L.) edodes, Flammulina (F.) velutipes, Inonotus obliquus, Pleurotus (P.) ostreatus, Pholiota nameko, Ganoderma (G.) lucidum, Trametes (T.) versicolor, Schizophyllum commune*, etc.) A number of studies demonstrated the efficiency of fungal substances for the treatment of other diseases, apart from cancer. Moreover, the compounds isolated from edible mushrooms have low toxicity and better tolerance than synthetic preparations [7, 11].

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Fibrinolytic activity of this fungal group also attracted the attention of researchers. Fibrin accumulation in blood vessels often leads to thrombosis which may cause cardiac infarction and other cardiovascular diseases. Fibrinolytic enzymes of microbial origin are of interest because of their low cost and absence of undesirable side effects [12, 13].

The capacity for fibrin hydrolysis was revealed in the extracts from mycelium and fruit bodies of a number of basidial fungi belonging to various taxonomic groups [14]. From *P. ostreatus* fruit bodies, a Zn^{2+}-dependent metalloprotease was isolated. It hydrolyzes fibrin, as well as β- and γ-chains of human fibrinogen and does not hydrolyze azocasein, azoalbumin, and elastin [15].

Xylotrophic basidiomycetes have recently become an object of comprehensive study, due to their relative ease of cultivation, high consumer characteristics, and important medical features. Secreted proteolytic enzymes of the fungi belonging to different ecological groups are presently being studied; both their physicochemical characteristics and their role in fungal activity are investigated. However, the information on the proteases of xylotrophic fungi lacks systematization. Due to the high functional and practical importance of these enzymes, summarizing the published material becomes urgent.

COMPARATIVE BIOCHEMISTRY OF SECRETED PROTEASES FROM XYLOTROPHIC BASIDIAL FUNGI

Since proteolytic enzymes are indispensable in supplying nitrogen to xylotrophs under natural growth conditions (on living and dead wood), the absence of sufficient systematic information on secreted proteases of higher xylotrophic fungi is a noticeable flaw in our knowledge of the biology of this group. Apart from its mechanical and conductive functions, wood is a site of accumulation for storage compounds, including nitrogen-containing ones. Nitrogen-containing compounds in wood are known to be mostly proteins, either localized in the xylem living cells or associated with the cell wall material. The wood protein content depends on the geographic location and part of the tree [16]. Although mineral nitrogen is also present in wood species, its content is extremely low [17, 18]. The fact of proteins being the main nitrogen source in wood is probably the reason for the role of extracellular proteolytic enzymes in the metabolism of xylotrophic fungi.

Investigation of the structural and functional characteristics of basidiomycete proteases commenced ca. 30 years ago. Publications exist on the isolation and characterization of proteolytic enzymes from the cultured mycelium and fruit bodies of basidial macromycetes, as well as on attempts at their classification. Due to the extreme diversity of their structure and mechanisms of action, proteases do not fit in well with the usual rules of enzyme nomenclature. Classification of these enzymes is therefore often difficult. Substrate specificity is one of the major criteria for classification of proteolytic enzymes; all proteolytic enzymes are subdivided into two big groups, exopeptidases and endopeptidases.

Exopeptidases require the presence of free terminal groups close to the cleaved bond. Aminopeptidases were found in a number of microorganisms, including bacteria and fungi. Aminopeptidases are usually intracellular enzymes, although few reports exist on the detection of extracellular aminopeptidases in fungi. For example, in *T. trogii* culture liquid, apart from endopeptidase activity, aminopeptidase, carboxypeptidase, and dipeptidyl aminopeptidase activity was revealed [19].

Endopeptidases (proteinases, peptidyl peptide hydrolases) cleave internal peptide bonds in proteins. Their classification into subgroups is based primarily on the structure of their catalytic center; usually, the functional groups of the active center are identified by means of specific inhibitors. According to the structure of their active centers, proteinases are subdivided into four subgroups: **serine** [with a triad of amino acid residues containing serine, aspartic acid, and histidine in the active center; are inhibited by diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride (PMSF), and such substrate-like haloid methylketones as tosyllysine chlormethyl ketone and tosylphenylalanine chlormethylketone (TPCK)]; **cysteine** [contain cysteine and histidine residues in the active center; are inhibited by such sulfhydryl agents as *p*-chlomercury benzoate (PCMB) or monoiodineacetic acid; are activated by dithiothreitol and 2-mercaptoethanol]; **aspartic** [contain two carboxyl groups in the active center; are inhibited by pepstatin, acetyl pepstatin, epoxy compounds, e.g., 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP), methyl ether of diazoacyl-DL-norleucine (DAN), etc.]; and **metalloproteinases** [require ions of bivalent metals (Mg^{2+}, Mn^{2+}, Co^{2+}, or Zn^{2+}) and are suppressed by compounds binding these metals: ethylenediaminetetraacetic acid (EDTA), *o*-phenanthroline, etc.].

Available data are often insufficient for the strict placement of an enzyme within a specific group. Moreover, not all proteases can be classified. A number of proteases are presently known with no place for them in the standard scheme of classification. Discovery of new groups and families of proteolytic enzymes is quite probable.

The features of those proteolytic enzymes from xylotrophic basidiomycetes, which have been characterized in some detail, are presented in the table. The species listed in the table belong to three orders of basidial fungi, *Polyporales, Boletales*, and *Agaricales.* Secreted proteases mostly have a low molecular mass of 26 to 50 kDa. The values of their isoelectric points vary from 3.5 to 8.8. Optimal pH for these enzymes is usually acidic, from 2.0 to 5.0; proteinases from *P. ostreatus, Hypsizygis* (*H.*) *marmoreus*, and *F. veluti-*

Characteristics of the extracellular proteolytic enzymes from xylotrophic basidiomycetes

Producer. Type of tree rot	Systematic position (family)[1]	Enzyme name	Molecular mass, kDa	Isoelectric point	Decomposed substrates (pH optimum)	pH range of stability	*T* optimum (heat stability, °C)	Inhibitors	Source
Order *Polyporales*									
Phanerochaete chrysosporium Burds. White rot	*Phanerochaetaceae*	Endoprotease	40	5.6	Azocoll (4.0)	–	–	$HgCl_2$, PHMB, N-bromosuccinylimide	[20]
Sporotrichum pulverulentum Novobr. (Anamorphic *Phanerochaete chrysosporium* Burds.) White rot	*Incertae sedis* (of undetermined position)	Proteinase I EC 3.4.24.4p	28	4.7	Azocoll (5.0), human fibrinopeptide A (cleaves C-terminal Arg)	–	–	$AgNO_3$, $HgCl_2$, PCMB, EDTA (partially), α,α'-dipiridyl (partially)	[21, 22]
		Proteinase II EC 3.4.24.4q	26	4.2	Azocoll (5.2), human fibrinopeptide A (hydrolysis of Phe^8–Leu^9 and Leu^9–Ala^{10} bonds)	–	–	$AgNO_3$, $HgCl_2$, PCMB, EDTA (partially), α,α'-dipiridyl (partially)	[21, 22]
Fomitopsis pinicola (Sw.) P. Karst. Brown rot	*Fomitopsidaceae*	Protease	32	4.0	Milk-setting activity	–	–	Cu^{2+}, Hg^{2+}, EDTA	[23, 24]
Ganoderma lucidum (Curtis) P. Karst. White rot	*Ganodermataceae*	Aspartyl proteinase EC 3.4.23.32	36	–	Casein (2.0), hemoglobin (3.2), oxidized B chain of insulin (hydrolysis of the Phe^{24}–Phe^{25} bond)	3.2–5.2	52 (>58 complete loss of activity)	MDAH, DAN, S-PI, not inhibited by pepstatin A and EPNP	[25]
Irpex lacteus (Fr.) Fr. (=*Polyporus tulipiferae* (Schwein.) Overh.) White rot	*Steccherinaceae*	Proteinase	34	5.2	Milk-setting activity (2.5)	2.5–5.5	>60 complete loss of activity	Hg^{2+}, EDTA, PCMB, Fe^{3+}	[26, 27]

Table. (Contd.)

Producer. Type of tree rot	Systematic position (family)[1]	Enzyme name	Molecular mass, kDa	Isoelectric point	Decomposed substrates (pH optimum)	pH range of stability	*T* optimum (heat stability, °C)	Inhibitors	Source
		Aspartyl proteinase ILAP EC 3.4.23.29	36	5.3	Hemoglobin (3.0), oxidized B chain of insulin (hydrolysis of the Leu^{11}–Val^{12}, Ala^{14}–Leu^{15}, Phe^{24}–Phe^{25}, and Thr^{27}–Pro^{28} bonds), high milk-setting activity	3.0–7.0	45 (resistant to 60)	EPNP, DAN, pepstatin	[28, 29]
		Acid proteinasea EC 3.4.23.32	34	5.0	Hemoglobin (2.8), casein (3.0), Z-tetrapeptide ZFLAA (4.0 hydrolysis of the L–A bond)	3.0–5.0	45 (0–30)	Chymostatin, TPCK, is not inhibited by EPNP, DAN, and pepstatin	[30]
Laetiporus sulphureus (Bull.) Murrill Brown rot	*Polyporaceae*	Aspartyl proteinase	50	3.5	Casein (2.6), milk-setting activity, hemoglobin	–	(>35 unstable)	Pepstatin A	[31]
Pycnoporus sanguineus (L.) Murrill (=*Pycnoporus coccineus* (Fr.) Bond. & Singer =*Trametes sanguinea* (L.) Imazeki) White rot	*Polyporaceae*	Acid proteinasea Ia EC 3.4.23.6	34	3.5	Casein (2.8), oxidized B chain of insulin (hydrolysis of the Ala^{14}–Leu^{15}, Tyr^{16}–Ley^{17}, and Phe^{24}–Phe^{25} bonds), native insulin (hydrolysis of the same bonds), the RDVYIHPF-HLLVYS (hydrolysis of the Y^4–I^5 and L^{11}–V^{12} bonds)	2.3–6.5	55 (resistant to 60)	–	[32–35]

Table. (Contd.)

Producer. Type of tree rot	Systematic position (family)[1]	Enzyme name	Molecular mass, kDa	Isoelectric point	Decomposed substrates (pH optimum)	pH range of stability	*T* optimum (heat stability, °C)	Inhibitors	Source
Order *Boletales*									
Serpula lacrymans (Wulfen) J. Schrot. Brown rot	*Coniophoracee*	Aspartyl proteinas S4	30	–	Gelatin (4.0), azocasein	–	–	Pepstatin A	[36]
Order *Agaricales*									
Hypsizygus marmoreus (Peck) H.E. Bigelow. Brown rot	*Tricholomataceae*	Metalloproteinase	29.5	8.8	Casein (7.0), hemoglobin, serum albumin, Z-Glu-Leu·NH_2	6.2–7.6	50 (to 40)	EDTA, *o*-phenanthroline, talopeptin, phosforamidon	[37]
Lentinula edodes (Berk.) Pegler (=*Lentinus edodes* (Berk.) Singer) White rot	*Marasmiaceae*	Aspartyl proteinas EC 3.4.23.32	40	–	Casein (2.7), hemoglobin (2.9)	3.2–5.2	50 (>55 complete loss of activity)	PCMB, PMSF, DAN, MDAH, S-PI, is not inhibited by pepstatin A and EPNP	[38]
Flammulina velutipes (Fr.) Karst. White rot	*Marasmiaceae*	Aminopeptidase	27	–	L-leucine *p*-nitroanilyde (7.0)	–	–	EDTA, *o*-phenanthroline	[39]
Pleurotus ostreatus (Fr.) Kumm. White rot	*Pleurotaceae*	Subtilisin-like serine proteinase (PoS1)	75	4.5	SucAAPF*p*NA (8.0), SucAAPL*p*NA (7.0), azocoll, BTEE, oxidized B chain of insulin	–	–	Antipain, chymostatin, DCI, PMSF, APMSF	[40]
Disporotrichum dimorphosporum (Arx) Stalpers (=*Sporotrichum dimorphosporum* Arx, Anamorphic *Agaricales*)	*Incertae sedis* (of undetermined position)	Protease	34	7.4	Azocoll(5.0)	–	–	–	[41]

[1] The systematic position according to the presently accepted system as presented in the "Index Fungorum" database (http://www.speciesfungorum.org/Names/Names.asp).

pes are exceptional, since their pH optima lie in the neutral range.

According to the results of inhibitor analysis, most of the studied proteases belong to aspartate proteinases. Their low pH optimums are an indirect confirmation of such classification. Metalloproteinases are the second widespread group among xylotrophic basidiomycetes; they are secreted by *Hypsizygus* [37] and *Flammulina* [39]. They were revealed in the vegetative mycelium of *G. lucidum* [42] and *L. edodes* [43] and in *P. ostreatus* fruit bodies [15, 44].

The *P. ostreatus* extracellular proteinase PoS1 belongs to serine proteinases [40, 45]. For many of the enzymes listed in the table, the nature of the catalytic center has not been determined. However, some of them exhibit a pronounced tendency to lose activity in the presence of such specific SH group inhibitors as PCMB, PHMB, $HgCl_2$, etc. These are proteases from *Phanerochaeta (Ph.) chrysosporium, Sporotrichum (S.) pulverulentum, L. edodes*, and *Irpex (I.) lacteus* (=*Polyporus tulipiferae*). Existence of fungal intracellular cysteine proteinases is reliably known. For example, in 1998, Shin and Choi isolated a cysteine proteinase from *P. ostreatus* fruit bodies [46]. The issue of secretion of cysteine proteinases by fungi is still open. Analysis of a number of published sources suggests that at least most of micromycetes secrete only serine, aspartate, and metalloproteinases.

All the enzymes listed in the table exhibit endopeptidase activity with various proteins and synthetic substrates; only *F. velutipes* protease is an aminopeptidase (it hydrolyses L-leucine *p*-nitroanilide). Proteinase I from *S. pulverulentum*, apart from endopeptidase activity (azocoll hydrolysis), also exhibits an exopeptidase one (removes the C terminal arginine from human fibrinopeptide A).

The possibility of the glycosylation of some of the enzymes was investigated. Proteinases from *P. ostreatus* and *Ph. chrysosporium* are glycosylated [20, 40] and *L. edodes* proteinase is not [38].

One of the extracellular proteinases, which features and functional roles were most completely characterized, has been isolated in 2001 by Palmieri et al. from *P. ostreatus* [40] and is termed PoS1. The purified enzyme hydrolyzed *N*-benzoyl-DL-tyrosine methyl ether (BTEE), *N*-succinyl-alanyl-alanyl-prolyle-phenylalanyle-*p*-nitroanilide (SucAAPF*p*NA), *N*-succinyl-alanyl-alanyl-prolyle-leucyle-*p*-nitroanilide (SucAAPL*p*NA), azocollagen (azocoll), and the oxidized B chain of bovine insulin anddid not hydrolyze *p*-tosyl-L-arginine methyl ether. Proteinase PoS1 activity increased in the presence of $CaCl_2$ and Mn^{2+} ions. Physicochemical analysis of the enzyme revealed that it is a monomeric glycoprotein with a molecular mass of 75 kDa. Its isoelectric point is 4.5; pH optimum was in the alkaline range and was 8.0 and 7.0 for SucAAPF*p*NA and SucAAPL*p*NA, respectively. The carbohydrate part of the glycoprotein contains various monosaccharide residues; mannose is the most important one.

Proteinase PoS1 is almost completely inhibited by antipain, chymostatin, PMSF, 3,4-dichloroisocoumarin (DCI), and 4-aminophenyl methanosulfonyl fluoride, (APMSF) and thus belongs to the group of serine proteases. For more precise determination of its specificity, the bonds hydrolyzed in the bovine insulin oxidized B chain were determined and its action was compared with other serine proteases (trypsin, chymotrypsin, elastase, and subtilisin). The results confirmed that PoS1 is a subtilisin-like proteinase. The amino acid sequence was determined for the N terminal part and for three internal sites of the proteinase. A homological sequence was found for one of the internal regions; it belonged to an enzyme of the subtilisin proteinase family.

It should be noted that the structural and kinetic characteristics of PoS1 are different from those of the known proteases from other xylotrophic fungi.

The sequencing data enabled construction of oligonucleotide primers; several fragments of the relevant *P. ostreatus* gene (*posl*) were amplified, cloned, and sequenced. Since two of these fragments corresponded to the 3' and 5' regions of the *posl* gene, the complete sequence of this gene was recovered from the *P. ostreatus* genome library. Subsequent analysis of the nucleotide sequence of the *posl* gene and of the amino acid sequence of its product, the PoS1 protein, revealed that this proteinase is synthesized as a pre-pro-protein, i.e., a more high-molecular precursor, which forms the active PoS1 enzyme after removal of peptides of a specified length [45]. This feature is characteristic of most of the known subtilisin proteases [47]. The amino acid sequences to be removed are usually attached to the N-terminal part of the enzyme. The prepeptide acts as a signal sequence in the enzyme transfer through the cell membrane; the propeptide participates in the proper folding of the enzyme and deactivates it [48]. Such prosequences are usually removed by autoproteolysis. The possibility of self-activation is not excluded for PoS1.

The first goal-oriented research in the proteolytic activity of higher basidial fungi commenced as an applied task and dealt mainly with milk-setting enzymes; in the 1970s, these enzymes were urgently required in many countries due to an animal material shortage. An active search for rennin (chymosin) substitutes in various microbial groups was carried out. Basidial fungi were among the objects of such research. Casein preparations were therefore traditional substrates for determination of proteolytic activity in basidiomycetes. Hemoglobin and gelatin were used to a lesser degree. Most of the proteinases from basidial fungi was obtained in pure form and characterized due to their high milk-setting activity [14]. However, the enzymes with such characteristics should not only be close to rennin in their substrate specificity, but also in activity under low pH and in heat resistance [31]. Such

proteinases were found in *G. lucidum, I. lacteus, Laetiporus sulphureus, L. edodes, Pycnoporus* (*Pyc.*) *sanguineus* (= *Pyc. coccineus* = *T. sanguinea*), etc. These features were found in aspartate basidiomycete proteinases. The seeming predominance of this type of proteinases in xylotrophic fungi may reflect the results of dedicated research, rather than a real situation.

All milk-setting enzymes used in industry belong to aspartate proteinases. Proteinase ILAP from *I. lacteus* has even been used to prepare some varieties of high-quality cheese. Its ratio of milk-setting and proteolytic activity is not lower than that of the widely used enzymes from *Mucor* (*M.*) *pusillus* and *M. miehei* [29].

The second reason for the interest in aspartate proteinases lies within the scope of theoretical enzymology. Among basidiomycetes, enzymes were revealed, which had all the characteristics of aspartate proteinases, but were not sensitive to pepstatin, a widely used aspartate proteinase inhibitor [49]. *L. edodes, G. lucidum, I. lacteus, F. velutipes*, and *P. ostreatus* are known to secrete such proteinases. However, only the proteinases from *L. edodes, G. lucidum, and I. lacteus* have been purified and characterized. The latter species secretes two acid proteinases; one of these is inhibited by pepstatin and the other is not [30]. Apart from the two major families of aspartate proteinases (those of pepsin and rennin), a new family should probably be formed to comprise the fungal proteinases of nonpepsin type.

Decomposition of food proteins is not the only function of the proteases from xylotrophic fungi. In a number of species, proteases were shown to participate in the regulation of activity of other secretory enzymes. Proteinase PoS1 from *P. ostreatus* plays the key role in the posttranslational regulation of *P. ostreatus* laccases, POXA1b (phenol oxidase A1b) and POXA3, which belong to the enzymes of the ligninolytic complex [50]. Incubation of proteinase PoS1 together with laccase POXA3 resulted in 30% increased activity of the latter. Limited proteolysis of POXA3 possibly transfers it to a more active form. Another isoform, POXA1b, lost activity completely in the presence of proteinase [40]. The same authors report specific cleavage of POXA1b laccase during the early stages of fungal growth due to the proteases of the culture liquid. The disappearance of POXA1b correlated with appearance of the extracellular proteolytic activity [51]. Additional experiments suggested that PoS1 did not degrade POXA1b directly, but possible acted via induction of a cascade mechanism involving other extracellular proteases of the fungus [40].

A similar relation was observed between *Ph. chrysosporium* lignin peroxidases and extracellular proteinases. In this case, emergence of proteinase activity resulted in an almost complete degradation of all lignin peroxidases and thus, a loss of lignin peroxidase activity [52, 53]; in the case of *S. pulverulentum* proteinases I and II, activation of endo-1,4-β-glucanase was observed [21, 22].

In this respect, *P. pulmonarius* differs from both *Ph. chrysosporium* and from *P. ostreatus*, its closest relative. In *P. pulmonarius*, secreted proteases probably do not participate in the regulation of peroxidase activity. Peroxidase activity in the culture liquid disappeared completely several hours after its separation from the mycelium. However, the suggestion that peroxidases are cleaved by the proteolytic enzymes present in the culture liquid was refuted by addition of various protease inhibitors, including PMSF, the inhibitor which efficiently protected *Ph. chrysosporium* peroxidases. In this case, loss of peroxidase activity is caused by their high sensitivity to hydrogen peroxide H_2O_2; its concentration increased significantly several hours after collection of the culture liquid. Acidification in the culture liquid during storage is the second possible factor leading to peroxidase inactivation [54].

The differences in enzyme content may be explained by the differences in the way metabolic processes proceed in different organisms due to the differences in their way of life. The interspecies differences developed in the course of evolution result from the differences in genotypes and therefore in proteins which are mostly enzymes. The type of induced tree rot is the most important ecologo-physiological characteristics of xylotrophic fungi. Brown (destructive) rot is caused by the fungi mainly decomposing and utilizing cellulose; lignin remaining after cellulose decomposition gives a brown stain to the wood; the wood itself begins to crumble. The fungi utilizing mainly lignin, cause white (corrosive) rot. The wood is delaminated into individual fibers. Since practically all xylotrophs can decompose cellulose to a certain degree, while only white rot fungi decompose lignin, this classification is relatively arbitrary [11].

Denisova, having analyzed the data on the extracellular proteolytic activity of xylotrophic basidiomycetes, concluded that a correlation exists between the characteristics of secreted proteinases and the trophic type of the fungi. Most of the extracellular proteinases from xylotrophic basidiomycetes have acidic pH optimum. In nature, the wood decomposed by fungi also has low pH values. This is probably an evolutionary developed feature; attack on the proteins of a host tree required active acid proteases. In cultures, brown rot fungi acidify the medium to pH 2–4. Numerous researches demonstrated that such low pH values resulted from accumulation of free oxalic acid [14, 55]. The cultures of white rot fungi generally have higher pH values of approx. 4–6, due to decarboxylase biosynthesis preventing accumulation of high amounts of oxalic acid. The more advanced enzymatic apparatus of white rot fungi indicates greater evolutionary progress of this group [14].

However, the table also illustrates some instances counter to this tendency. For example, proteinase of a

white rot fungus *G. lucidum* most efficiently hydrolyzes hemoglobin and casein at pH 3.2 and 2.0, respectively. The *Pyc. sanguineus* proteinase also has a pH optimum of 2.8, On the other hand, *H. marmoreus*, a brown rot fungus, synthesizes a neutral proteinase. Apart from environmental conditions, the genetic characteristics of a producer determining its systematic position, also possibly affect the features of proteolytic enzymes. This is confirmed by the fact that among the species discussed, only the members of the order *Agaricales* synthesize neutral proteinases.

In a number of papers, the effect of cultivation conditions on activity of the extracellular proteases from xylotrophs was investigated [52, 53, 56–58]. For example, Dosoretz et al. revealed that two types of proteolytic activity developing in *Ph. chrysosporium* grown in a nitrogen-limited medium react differently to the changes in medium composition. The first activity (appearing during the phase of extensive growth and peaking on the second day) was not sensitive to the carbon content of the medium, while the second one (appearing on the eighth day, after the peak of ligninase activity) increased under carbon limitation. Varying glucose concentrations in the medium resulted in the earlier or later appearance of the second activity. Introduction of additional glucose on the sixth day of growth caused complete repression of the synthesis of the later proteolytic activity. Oxygen supply to *Ph. chrysosporium* culture affected both the degree and the time of appearance of the proteolytic activity; this effect was more pronounced for the second activity [52]. Addition of cycloheximide on the sixth day of cultivation had no sufficient effect on lignin peroxidase activity and on the content of extracellular protein; however, it resulted in an almost complete disappearance of proteinase activity. This is an indication of *de novo* synthesis of the relevant protease (or proteases) [53]. Further studies revealed that, apart from carbon limitation, the expression of the second activity was induced by nitrogen limitation [58]. Unfortunately, the authors did not isolate the individual enzymes responsible for *Ph. chrysosporium* proteolytic activity.

A comparison of the conditions influencing the secretion of proteolytic enzymes by some xylotrophic fungi was carried out. Seven species of basidiomycetes were studied: *P. ostreatus, G. lucidum, Aurantiporus fissilis, Bjerkandera (B.) adusta, Phellinus pomaceus, Inonotus cuticulares*, and *T. ochraceae.* Unlike the previously studied phytopathogenic and saprotrophic mycelial fungi, the basidiomycetes used in this work did not require the presence of a proteinaceous substrate in the medium for secretion of extracellular proteases. The presence of protein in the growth medium was not necessary for protease secretion, although it was required to obtain their maximal content [57].

The study of the effect of growth conditions, including the composition of the growth media, on the extracellular proteolytic activity of *P. ostreatus* revealed a significant increase in proteinase PoS1 activity on addition of vanillic acid or copper sulfate to the basic medium [45].

The regulation of production of extracellular proteolytic enzymes is affected by pH of the medium. This phenomenon was monitored by measuring the extracellular proteolytic activity (using azocasein) at different pH values. *B. adusta* grown at pH 5.9 and pH 7.3 produced significant extracellular activity (measured using azocasein and determined at pH 7.0). However, cultivation at pH 4.5 resulted in a three- to fourfold increase of activity (measured using azocasein and determined at pH 4.5). Thus, *B. adusta* demonstrates excellent adaptability to environmental changes [57].

Since investigation of the enzymatic activity of xylotrophic basidiomycetes and of its regulation enables better understanding of the peculiarities of the metabolic processes and the adaptation capacity of these organisms, it is of significant scientific interest. Unfortunately, the research of this fungal group is usually restricted to the species cultivated under artificial conditions; the main mass of wild fungi remains unexplored in this respect.

PHYSIOLOGICAL ROLE OF PROTEOLYTIC ENZYMES IN XYLOTROPHS AND PRACTICAL APPLICATION OF THESE ENZYMES

Proteases perform a number of complex physiological functions, including protein catabolism, blood clotting, cell growth and migration, morphogenesis and development [1]. Novel investigation techniques revealed highly specific and selective protein modifications performed by proteases, including the activation of the zymogenic enzyme forms by limited proteolysis, formation of hormones and other physiologically active peptides from precursor proteins, thrombus lysis, or the processing and transport of secreted proteins through the membrane. The important role of proteolytic enzymes in metabolic and regulatory processes explains their occurrence in all living organisms. The functions which have been unequivocally demonstrated, and are the most important for xylotrophs, will be discussed further in some detail.

1. Degradation of nutrient proteins to amino acids is the main and most evident function of proteolytic enzymes. Fungi are heterotrophic organisms; their nitrogen nutrition is of a mixed type, i.e., they can utilize both organic and inorganic nitrogen sources. Under natural conditions fungi usually have to secrete a variety of extracellular enzymes to decompose natural organic materials. In the course of protein decomposition, endo- and exopeptidases act in concord: the first ones produce a number of free C- and N-terminal ends; the second ones act on the peptide fragments thus formed. Hydrolytic enzymes in general have relatively broad specificity, i.e., they may attack more than one

substrate. This broad specificity is especially important for the fungal secreted proteases and other proteolytic enzymes decomposing food proteins. The fungus *T. rogii* uses these enzymes for efficient decomposition of a number of proteins and peptides contained in the substrate [19]. Any enzyme penetrating through the plasma membrane is usually termed an extracellular enzyme.

2. Proteases participate in the regulation of activity of other enzymes synthesized by a given organism. Zymogenic activation in fungi is an important step in the regulation of a number of physiological processes. In such xylotrophic fungi as *P. ostreatus* and *Ph. chrysosporium*, activity of ligninolytic enzymes is regulated via their specific activation or inactivation by the extracellular proteases secreted by these fungi [51, 53]. The proteases of xylotrophs, similar to the proteolytic enzymes of other microorganisms, are possibly able to degrade the regulatory proteins controlling heat shock response, SOS pathway of DNA reparation, and programmed cell death [1].

3. The PoMTP metalloprotease was found to play an important part in the fruit body formation in *P. ostreatus.* The data are confirmed by the fact that at the stages of primordia and fruit body formation, the content of appropriate mPNA is considerably higher than at the stage of vegetative mycelium [59].

4. Since the presence of the extracellular fungal proteolytic enzymes may indicate the possibility of a fungus to grow and develop on the host plant's living tissue, causing its gradual destruction, they may act as pathogenic factors. The data obtained for xylotrophic fungi correlate with the pattern observed for mycelial fungi: while subtilisin-like proteinases are most common among saprotrophs, phytopathogenic species also possess highly active trypsinlike proteinases [60]. Under natural conditions, *P. pulmonaris* develops only on dead wood. It exhibits a pronounced subtilisin-like proteolytic activity; trypsinlike activity was almost absent in the fungi grown on various media. In *P. ostreatus*, however, an extracellular trypsinlike activity was observed in the course of growth [61]; this organism is able to grow on living trees. Thus, the presence of a host plant' living tissue is probably required for the functioning of extracellular trypsinlike proteinases in xylotrophs. Importantly, proteolytic enzymes participating in attacking living plants may become efficient targets for antifungal preparations.

Commercial interest in fungal proteases supports the interest in their functional role. Successful cultivation of fungi on nutrient media and their ability to synthesize numerous biologically active compounds support this interest [62]. Widespread application of novel proteolytic enzymes stimulates their search and investigation. The estimated world sale of enzymes exceeds US$ 1 billion. Proteases are the greatest group of industrially required enzymes and constitute approx. 60% of the total world enzyme sales; 2/3 of this amount are microbial proteases [63].

Variety and specificity of fungal proteases favor their therapeutic applications. We have already stated above that the search for fibrinolytic enzymes, especially of microbial origin, became urgent in biology and medicine. Significant work has been carried out by Denisova on the fibrinolytic activity of cultured basidiomycetes from various ecological and taxonomic groups. As a result, she has proposed laboratory regulations for the production of a fibrinolytic preparation from the culture of a *Coprinus* fungus. High activity of the preparation has been confirmed in experiments on animals in the processes of fibrinolysis and thrombolysis [14].

Summarizing the review of the extracellular proteolytic enzymes from xylotrophic basidial fungi, it should be noticed that this review is among the first attempts to generalize and analyze available data concerning the biochemistry and role of these proteases. Isolation and analysis of the genes, encoding xylotrophic proteases will promote further investigation of their genetics, regulation, and evolution, as well as of the various types of their interactions with other enzymes of this important microbial group.

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