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EXPERIMENTAL ARTICLES

Degradation of Proteinaceous Substrates by Xylotrophic Basidiomycetes

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Abstract—The ability of various xylotrophs to produce extracellular proteolytic enzymes has been studied, with emphasis on medium-related factors regulating their secretion. Direct measurement of proteolytic activity in the culture liquid and postelectrophoresis determination of protease activity in polyacrylamide gel copolymerized with gelatin demonstrated that the secreted enzymes are quantitatively and qualitatively diverse. Activity levels of extracellular proteolytic enzymes strongly depend on pH and contents of protein and carbohydrate in the medium. All secreted proteases notably differed in molecular weight (of 51 kDa or higher and in excess of 95 kDa) and belonged mostly to two classes of proteolytic enzymes (serine proteases and metalloproteinases).

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Xylotrophic macromycetes occupy an important place in the structure of plant biocenoses. The fungi of this group are involved in degradation of complex biopolymers of plant cell walls (such as cellulose, lignin, pectic substances, hemicelluloses, and proteinaceous substances). The interest of researchers in these fungi is largely accounted for by the possibility of their use as degraders of plant waste and producers of active hydrolytic enzymes [1].

Biodegradation of natural substrates of xylotrophic fungi is a multiple-stage process involving both extracellular hydrolases (cellulases, xylanases, pectinases, proteases, etc.) and the complexes of oxidative enzymes. Successive interactions with substrates of enzymes of certain groups, secreted by the fungi into the environment, ensures degradation of the target molecules and the formation of readily assimilable low-molecular-weight oligomers and monomers, which are used for replenishing energy and as intermediates in metabolic processes [2–4].

In working with xylotrophs, attention has been given to studies of cellulases [5] and oxidative enzymes, which degrade lignin [6–9]. This is understandable, given the use of these fungi for processing plant material (waste of timber and agricultural industry). On the other hand, efficient extraction of the small amounts of nitrogen contained in wood (the natural substrate for xylotrophs) is vital for the fungi themselves, which usually grow on media deficient in nitrogenous compounds. Because protein accounts for the major part of wood nitrogen, the role of proteolytic enzymes participating in wood degradation and providing the fungi with nitrogenous compounds becomes all the more important.

Proteinases are currently identified in several basidiomycetes. Kalisz *et al.* [10] demonstrated that three species of basidiomycetes grow in media containing protein as the sole source of nitrogen, carbon, and sulfur, and that they secrete proteolytic enzymes. The enzymes themselves were not identified, however. Venables and Watkinson [11] detected protease activity in eight species of basidiomycetes. Major extracellular metalloproteinases were found in *Chondrostereum purpureum* [12] and the vegetative mycelium of *Lentinus edodes* [13]. Nevertheless, on the whole, proteolytic enzymes of xylotrophic basidiomycetes remain poorly studied, and little is known about the mechanisms that control their production.

For all the above reasons, our aim was to identify and characterize extracellular proteases in various species of xylotrophs, with a view to studying the conditions that affect the secretion of these enzymes.

MATERIALS AND METHODS

In this work, seven fungal species were used, which were isolated either in the territory of Moscow oblast, from trunks of live trees, deadwood, and apple stumps—*Aurantiporus fissilis* (Berck. & M.A. Curtis) H. Jahn ex Ryvarden, *Pleurotus ostreatus* (Jacq.) P. Kumm., *Bjerkandera adusta* (Willd.) P. Karst., *Phellinus pomaceus* (Pers.) Maire, *Inonotus cuticularis*

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Fig. 1. Postelectrophoresis determination of proteolytic activity in polyacrylamide gel copolymerized with gelatin: *1*, *A. fissilis*; *2*, *P. ostreatus*; *3*, *B. adusta*; *4*, *T. ochracea*; and *5*, *G. lucidum*.

(Bull.) P. Karst., and *Tranmetes ochracea* (Pers.) Gibb. & Ryvarden,—or in Sochi, from hornbeam trees—*Ganoderma lucidum* (Curtis) P. Karst.

The fungi were grown in 250-ml Erlenmeyer flasks, each filled with 100 ml of liquid modified Czapek medium containing the following components (g/l): sucrose, 30; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5, KCl, 0.5; $FeSO_4$, 0.01; and 0.1 M phosphate buffer (pH 7.3), in which mineral nitrogen was substituted by 1% casein solution (sterilized for 30 min at 0.5 atm). Following inoculation, the fungi were cultured under continuous shaking (100 rpm). The culture liquid was filtered after 2, 4, 6, 8, and 11 days and used for measuring proteolytic activity.

Activities of secreted proteolytic enzymes were determined by the method of Erlanger [4], using 5 mM solutions of synthetic *p*-nitroanilide substrates: Glp–Ala–Ala–Leu–pNa (GAALPA) for subtilisin-like proteases and Bz–Arg–pNa (BAPA) for trypsin-like proteases. The reactions were performed in 0.1 M phosphate buffer (pH 7.3) at 37°C; *p*-nitroaniline that formed as a result of the reaction was determined spectrophotometrically at 410 nm. In addition to synthetic substrates, a proteinaceous substrate was used (1% azocasein); the reaction was performed under the same conditions. Following addition of 10% TCA (stop reagent) and incubation at 4°C for 30 min, the precipitate formed (residual protein) was removed by centrifugation at 10 000 rpm for 10 min and the supernatant was collected. Freshly prepared 0.5 M NaOH (400 μl) was added to an equal volume of supernatant. The intensity of the color was measured at 440 nm. In order to detect acid proteases, the activity of the enzymes was measured using 1% azocasein as a substrate; in this case, 0.1 M phosphate buffer (pH 7.3) was replaced by citrate–phosphate buffer (pH 4.5). One unit of enzymatic activity was defined as the amount of the enzyme in the culture fluid, which causes the optical density of the reaction mixture to increase by 0.01 U as a result of substrate hydrolysis under the conditions described above.

Effects of the medium composition on the secretion and activity of proteases was determined as follows: *B. adusta* was cultured in 100-ml Erlenmeyer flasks, each filled with 30 ml modified liquid Czapek medium, two variants of which were used. Variant (a) contained casein (1.0, 1.5, 2.0, or 3.0%) as the sole source of nitrogen (samples were taken on days 2, 4, 5, 6, and 8 of culturing); variant (b), 0.2 M solutions of carbohydrates (glucose, galactose, mannose, mannitol, or fructose) as the sole source of carbon. When $NaNO_3$ was used as the sole source of nitrogen, samples were taken on days 4, 6, 8, and 11 of culturing. The activity of the enzymes was measured as described above. The mycelium was separated by filtration and dried at 105°C until the attainment of constant weight (for 30 min).

Postelectrophoresis determination of the enzymatic activity in gels involved the use of copolymers of 0.026% gelatin and polyacrylamide. The protein was applied onto the gel without warming, and electrophoresis was carried out at 4°C and a direct current of 25 mA. When the procedure was completed, the gel was washed twice with 2.5% Triton X-100 and twice with 20 mM Tris–HCl (pH 8.0), followed by incubation in the same buffer at 37°C for 2 h.

After the incubation, the gel was stained with 0.1% solution of Coomassie Brilliant Blue R-250 in a 4 : 1 : 5 mixture of methanol, acetic acid, and water and washed with the same solute (without the dye). The protease appeared as a light band against the dark background.

Effects of inhibitors capable of specific binding to proteases of various classes on extracellular proteases of the fungi under study were assessed as follows: 10 μl inhibitor solution was added to 100 μl enzyme solution, and the mixture was incubated 20 min at room temperature; following addition of the substrate, the residual activity was measured as described above. The solutions used in these experiments included inhibitors of cysteine proteases (40 mM iodine acetamide (IAA)), metalloproteinases (100 mM EDTA), and serine proteases (10 mM phenylmethylsulfonyl fluoride (PMSF), 30 mM *N*-α-*p*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), and 25 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK)).

RESULTS AND DISCUSSION

Data derived from analyses of extracellular proteolytic activities in several species of basidiomycetes are presented in Table 1 and Fig. 1. In assessing these activities, we used synthetic substrates for trypsin-like and subtilisin-like proteases (Bz–Arg–pNa and Glp–Ala–Ala–Leu–pNa), which are the most frequently encountered species among extracellular enzymes, and proteinaceous substrates, azocasein and gelatin. The

Table 1. Activity of extracellular proteases of the fungi studied

Fungus	Activity (measured using BAPA/GAALPA as substrates), U/ml				
	Duration of culturing, days				
	2	4	6	8	11
Ph. pomaceus	0.93/0.04	1.06/0.2	0.74/0.01	0.36/0.00	0.37/0.00
B. adusta	0.46/0.00	0.44/1.78	0.7/5.13	1.35/9.11	2.82/9.46
I. cuticularis	0.53/0.00	0.82/0.00	1.03/0.00	1.02/0.03	0.88/0.00
P. ostreatus	0.00/0.00	0.01/0.00	0.12/0.00	0.36/0.00	0.42/0.06
G. lucidum	0.28/0.00	0.58/1.58	0.3/1.36	0.27/3.52	0.77/10.7

Table 2. Effects of inhibitors on the activity of extracellular proteases of basidiomycetes

Inhibitor	Concentration, mM	Residual activity, %			
		B. adusta	*G. lucidum*	*T. ochracea*	*P. octreatus*
IAA	4	99	94	100	94
EDTA	10	13	46	71	83
PMSF	1	88	61	54	62
TPCK	3	91	100	100	100
TLCK	2.5	97	98	100	96

latter was used as a copolymer of polyacrylamide, which allowed the proteolytic activity to be measured directly in the gel, following electrophoretic separation of proteins. As shown in Table 1, *B. adusta* exhibited high levels of both activities assessed using the synthetic substrates; for this reason, this basidiomycete was used as a model organism in the majority of subsequent experiments.

The quantitative and qualitative diversity of proteolytic enzymes secreted by the basidiomycetes under study was also confirmed in experiments where the activity was measured directly in polyacrylamide gels (Fig. 1). The basidiomycetes studied can be separated into several types secreting one to three groups of proteases (exhibiting appreciable proteolytic activity) that differed in molecular weights. Only one group of proteases was identified in *A. fissilis* (93 kDa) and *P. ostreatus* (63 kDa). *B. adusta* and *T. ochracea* were found to secrete two groups of proteases each, one comprising enzymes with molecular weights in the range 85–93 kDa and the other, with the weights exceeding 95 kDa. *G. lucidum* produces three groups of enzymes, with molecular weights of 51, 73, and more than 95 kDa. Unlike specific synthetic substrates, which allow determining protease types with sufficient probability, direct determination in gels characterizes the total proteolytic activity and reveals all proteases capable of hydrolyzing the proteinaceous substrate. Due to this, the proteinaceous substrate was used for determining (by inhibitor analysis) the classes to which hydrolytically active proteases belonged. In xylotrophic fungi, these enzymes belong largely to two classes—serine proteases and metalloproteinases—as Table 2 demonstrates. The ratio of these proteases is species-dependent and varies from predominance of metalloproteinases in *B. adusta* to the reverse picture in *T. ochracea*.

Although the genotype of a producer strain determines the ability to synthesize particular enzymes, its realization depends to considerable extent on the conditions of culturing and the composition of the medium. In stark contrast with mycelial fungi described previously [15, 16], phytopathogens, and saprotrophs—the basidiomycetes under study did not require the presence of a proteinaceous substrate in the culture medium for secreting extracellular proteases. The base level of all activities determined was detected in the presence of sodium nitrate as the sole source of nitrogen. The result of measurements of extracellular activities depended on both the duration of culturing and the concentration of protein in the medium (Fig. 2). Of note is the fact that the increase in total proteolytic activity (judged by the hydrolysis of the proteinaceous substrate azocasein; Fig. 2b) occurred 2–4 days prior to the elevation of specific proteolytic activities determined using selected synthetic substrates. It is possible that early effects of nonspecific proteases are prerequisites to subsequent involvement of more specialized enzymes. Maximum augmentation of all activities studied was observed when 1.5–2% azocasein served as the sole source of nitrogen. Thus, the presence of protein in the culture medium was not a prerequisite to secretion of extracel-

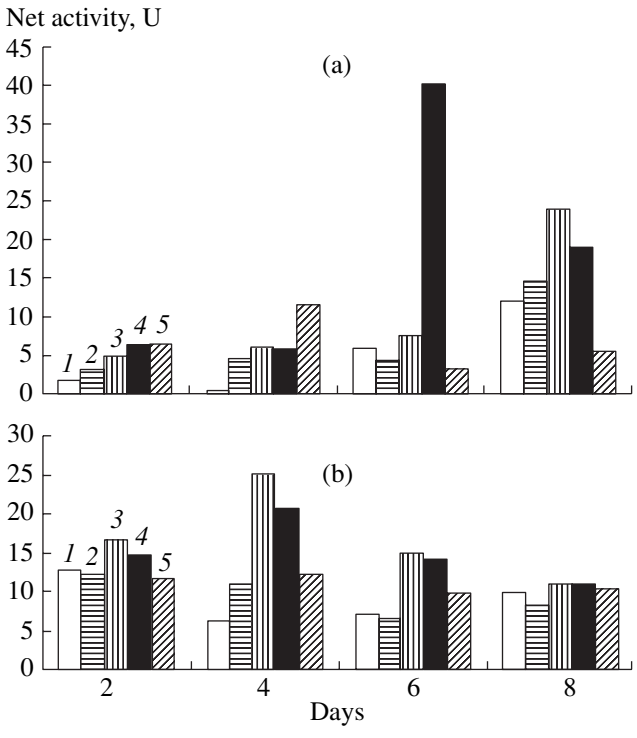

Fig. 2. Effect of casein concentrations on the activity of extracellular proteases hydrolyzing BAPA (a) and azocasein (b): *1*, $NaNO_3$; *2*, 1.0% casein; *3*, 1.5% casein; *4*, 2.0% casein; and *5*, 3.0% casein.

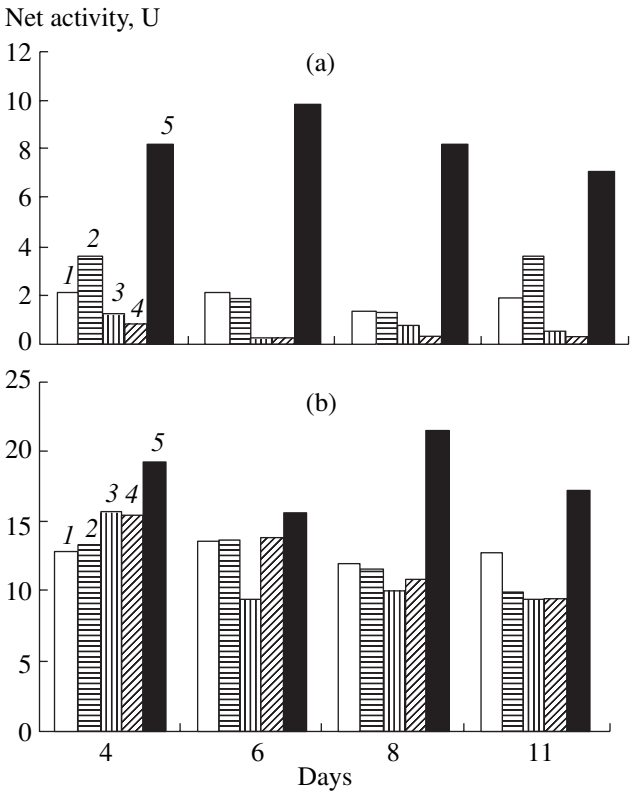

Fig. 3. Effect of various carbohydrates added to the culture medium on the activity of extracellular proteases hydrolyzing BAPA (a) and azocasein (b): *1*, glucose; *2*, galactose; *3*, mannose; *4*, mannitol; and *5*, fructose.

lular proteases, even though it is required for them to attain maximum activity levels.

Carbohydrates play an important role in determining the values of extracellular proteolytic activity. Among the monosaccharides tested, fructose turned out to be the optimum component of the medium: in the presence of fructose, extracellular activities (measured using Bz–Arg–pNa and azocasein) increased considerably (Fig. 3). This result, which was reproduced with total proteolytic activity and specific activity (calculated per unit dry weight of the mycelium), may be underlain by more efficient transportation or metabolism of fructose (compared to other carbohydrates).

In addition to the factors mentioned above, the regulation of extracellular protease production was appreciably affected by the pH of the medium. This was clearly demonstrated in experiments where total extracellular proteolytic activity was measured at diverse pH values using azocasein as the substrate. Table 3 demonstrates that *B. adusta*—growing at pH 5.9 and 7.3—produced considerable amounts of extracellular proteases, the activity of which is measured (with azo-

Table 3. Effect of pH of the medium on the production of extracellular proteases by *B. adusta*

pH of the medium	Activity, U/ml									
	with azocasein as the substrate*					with azocasein as the substrate**				
	Time, days									
	2	4	6	8	12	2	4	6	8	12
4.5	0.28	0.22	0.24	0.24	0.36	1.02	0.94	0.9	0.92	0.82
5.9	0.54	0.56	0.62	0.6	0.56	0.56	0.42	0.4	0.44	0.58
7.3	0.84	0.62	0.6	0.54	0.66	0.64	0.62	0.6	0.66	0.64

* Measured in 0.1 M phosphate buffer (pH 7.3).
** Measured in 0.1 M citrate–phosphate buffer (pH 4.5).

casein as the substrate) at pH 7.0; in the case of the fungus growing at pH 4.5, conversely, we observed a three- to fourfold increase in the activity of the proteases, measurable at acidic pH (with the same substrate). Similar pH-dependences were obtained for *Botrytis cinerea* [17] and *Aspergillus niger* [18]; in the latter case, the effect of pH was more pronounced than that of any other factor regulating the production and secretion of extracellular proteases. The fungus *Metarrhizum anisopliae* grows within a wide pH range (2.5–10.5) [19, 20]. In this case, various components of the proteolytic complex were produced at those pH values that corresponded to their pH optima.

Thus, our study demonstrates that the production of extracellular proteolytic enzymes in xylotrophs is not induced by the presence of protein (as is the case of mycelial fungi studied previously [15, 16]), in spite of the quantitative dependence on its content, as well as on the type of carbohydrate used and the pH of the medium. Such regulation may ensure maintenance of (1) the level of protease activity, which is needed for efficient degradation of cell walls of plant cells, and (2) the flow of nitrogenous compounds from the plant to the fungus.

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