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EXPERIMENTAL ARTICLES

Relation between Ligninolytic and Phospholipase Activities in the Fungus *Lentinus tigrinus*

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Abstract—Effect of hydrocortisone, NaF, and $FeSO_4$ on ligninolytic and phosphatase activity of the fungus *Lentinus* (*Panus*) *tigrinus* VKM F-3616D was investigated. Hydrocortisone and NaF were shown to inhibit the enzymes of the ligninolytic complex—laccase (EC 1.10.3.2), secretory peroxidase (EC 1.11.1.7), and Mn peroxidase (EC 1.11.1.13). $FeSO_4$ exhibited no significant effect on the activity of these enzymes. Decreased activity of the enzymes of the ligninolytic complex was associated with inhibition of the activity and changes in the substrate specificity of phospholipase A_2 (EC 3.1.1.4) in the presence of hydrocortisone of NaF. Cultivation of *L. tigrinus* in the presence of these compounds resulted in higher affinity of this enzyme to saturated fatty acids, while in the control and in the presence of $FeSO_4$ affinity to unsaturated fatty acids was higher.

Keywords: *Lentinus tigrinus*, ligninolytic enzymes, phospholipases, inhibitors, biodegradation

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At present, research into biodegradation of lignocellulose materials and xenobiotics by fungal macromycetes, including basidiomycetes, has gained wide acceptance.

Basidiomycetes are known to be capable of effectively degrading aromatic compounds resistant to decomposition in nature due to the synthesis of a unique complex of extracellular oxidative and hydrolytic enzymes [1, 2]. For this reason, fungi and their enzymes are used in the manufacture of biosensors and compound materials, as well as to decolorize paper and textiles [3, 4]. On the other hand, together with other microorganisms they are capable of destroying construction materials made of wood [5]. In the opinion of some authors, membranes and their components, phospholipids, play an important role in the processes of secretion of enzymes into the extracellular medium. It was shown that lipid peroxides also take part in the biodegradation of lignin and xenobiotics of phenolic nature, together with the extracellular ligninolytic enzyme complex [6]. A relationship between the physiological activity and the lipid composition, the intensity of the processes of lipid peroxidation (LP), and antioxidant activity was revealed in mycelial fungi [7, 8].

Earlier, we showed phospholipase A_2 (PLA_2) (EC 3.1.1.4) to be activated in the process of cultivation of the fungus *Lentinus tigrinus*, and it was suggested that a large amount of fatty acids was released into the perimycelial layer. Lipoxygenases and reactive oxygen species are involved in formation of lipid peroxide radicals, which oxidize the inhibitors of fungal growth present in the wood (aromatic compounds, including lignin). Simultaneously, the fungal mycelium secretes a ligninolytic enzyme complex into the extracellular medium. These events make aromatic compounds (resins) more accessible to the action of ligninolytic enzymes [9]. There seems to be a relationship between the activity of ligninolytic and lipolytic enzymes, which may regulate the process of biodegradation of lignocellulose substrates. The study of the relationship between the ligninolytic and phospholipase activities of the fungus *Lentinus tigrinus* will allow the development of new preparations for biological protection of construction materials. In order to support this suggestion, it is necessary to investigate the influence of specific inhibitors of ligninolytic and lipolytic enzymes on the physiological and biochemical characteristics of the fungus in the process of cultivation.

It was shown that impaired functioning of the phospholipase-encoding gene changes the fungal virulence [10]. Hydrocortisone mediates its action via specific intracellular receptors, prevents phospholipase A_2 from being activated by stimulating production of its inhibitor, lipomodulin, and acting directly on the cell membranes.

In almost all organisms, the biosynthesis of fatty acids results in the formation of saturated acids, which then undergo a series of desaturation and elongation reactions to form polyunsaturated fatty acids. Since

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desaturases are iron-containing enzymes [11], it may be suggested that the concentration of iron ions in the medium must affect the synthesis of polyunsaturated fatty acids in fungi. The role of iron ions in lipid metabolism is probably associated with their involvement in the regulation of the synthesis of citrate; the role of the latter in the formation of fatty acids has been studied in detail [12, 13].

The problems related to the role of fluorine for living organisms and the mechanisms of influence of fluorine and its derivatives on the metabolic activity are being discussed at length in the literature. Fluorine is considered to be the most deleterious and the most phytotoxic micropollutant among other contaminants such as CO, SO_2, and NO_2. One of the possible mechanisms of the inhibitory action of fluorides may be a lipid metabolic disorder mediated by a cascade of biochemical reactions. For example, it was experimentally shown for a number of organisms that fluorides inhibited the phospholipase A_2 activity at certain concentrations [14].

The goal of the present work was to investigate the changes in the ligninolytic and phospholipase activities of the fungus *Lentinus tigrinus* VKM F-3616D in the presence of hydrocortisone, iron ions, and fluoride ions.

MATERIALS AND METHODS

The fungus *Lentinus* (*Panus*) *tigrinus* was isolated at the Department of Biotechnology, Ogarev Mordovia State University, from the dry fungal fruit bodies growing on fallen birch deadwood in the vicinity of Saransk and was deposited in the All-Russian Collection of Microorganisms as the strain VKM F-3616D.

L. tigrinus was grown on Czapek medium containing 15 g/L of lignosulfonate. The fungus from slant wort agar (the inoculum size 1 × 1 cm) was introduced into conical 500-mL Erlenmeyer flasks with 100 mL of nutrient medium and grown for four days on Environmental Shaker-Incubator ES-20/60 orbital shaker (Biosan, Latvia) at 200 rpm and 26°C.

Further producer growth proceeded on the same medium supplemented with the inhibitors (5% of the inoculum) for 3, 6, 9, and 12 days under the same conditions as the inoculum.

The following compounds were used as effectors (g/L): hydrocortisone, 0.03 and 0.02; $FeSO_4$, 0.05 and 0.1; NaF, 0.05 and 0.1.

The biomass was determined by the gravimetric method. Protein in the culture liquid (CL) was determined according to Bradford method using bovine serum albumin as the standard [15]. The lignolytic activity was determined in cell-free culture liquid.

Laccase activity was recorded by pyrocatechol oxidation [16]. The amount of the enzyme that forms 1 μmol of the product over 1 min at 30°C was taken to be a unit activity. Peroxidase activity was determined by *o*-dianisidine oxidation [17]. The amount of the enzyme that cleaves 1 μmol of the substrate over 1 min at 20°C was taken to be a unit activity. Mn^{2+} peroxidase activity was determined by phenol red oxidation [18].

Determination of the activity of PLA_2 (EC 3.1.4.11). The *L. tigrinus* biomass (50 mg) was homogenized with 6 mL of 0.05 M acetate buffer, pH 5.2, containing 0.15 M NaCl. After sonication with ultrasound (22 kHz) for 1–2 min, the homogenate was allowed to stand for 18 h at 40°C. After acidification with concentrated HCl to pH 4.0, the homogenate solution was heated at 70°C for 5 min. After that, the homogenate was rapidly cooled down to 40°C, neutralized with 5 N NaOH, and centrifuged at 10000 *g* for 20 min. The pellet was discarded, and the supernatant fluid was filtered. The protein content was determined in the filtered clear yellow supernatant fluid.

The PLA_2 activity was determined by the accumulation of free fatty acids using gas chromatography. The reaction medium contained 10 mM Tris, 0.05 M NaCl, 5–20 mM $CaCl_2$, 0.5 mM EDTA, 0.5% detergent Triton X-100, pH 8.0. Egg yolk phosphatidylcholine (PC) purified chromatographically using the chloroform : methanol : acetone : icy acetic acid : water (40 : 13 : 15 : 12 : 8 vol/vol) system was used as substrate. For the micelles to be formed, the mixture was sonicated with ultrasound for 1–2 min. The fraction with phospholipase activity (protein content of 1–100 μg) was added to the incubation mixture. Hydrolysis was carried out for 1 h at 37°C. The reaction was stopped with the chloroform : methanol : water (1 : 2 : 0.8 vol/vol) mixture. The lower phase was evaporated, PL were precipitated with icy acetone; the free fatty acid content was determined in the supernatant layer.

The free fatty acid content was analyzed by gas-liquid chromatography. Methylation of fatty acids was carried out according to Morrison and Smith. Methanol (3 mL), boron trifluoride (50 μL) in methanol, and margaric acid (10 μg) were poured to the dry residue. The test tubes were tightly closed and placed into a thermostat with a temperature of 64°C for 1 h. The samples were then cooled; 1.5 mL of water, 2 mL of hexane, and 1.5 mL of hydrochloric acid were added to each test tube. The test tubes were sealed, shaken vigorously for 3 min, and centrifuged for 5 min. The upper phase containing methyl esters was collected and evaporated in the cannula under nitrogen flow. The methyl esters were dissolved in 10 μL of hexane.

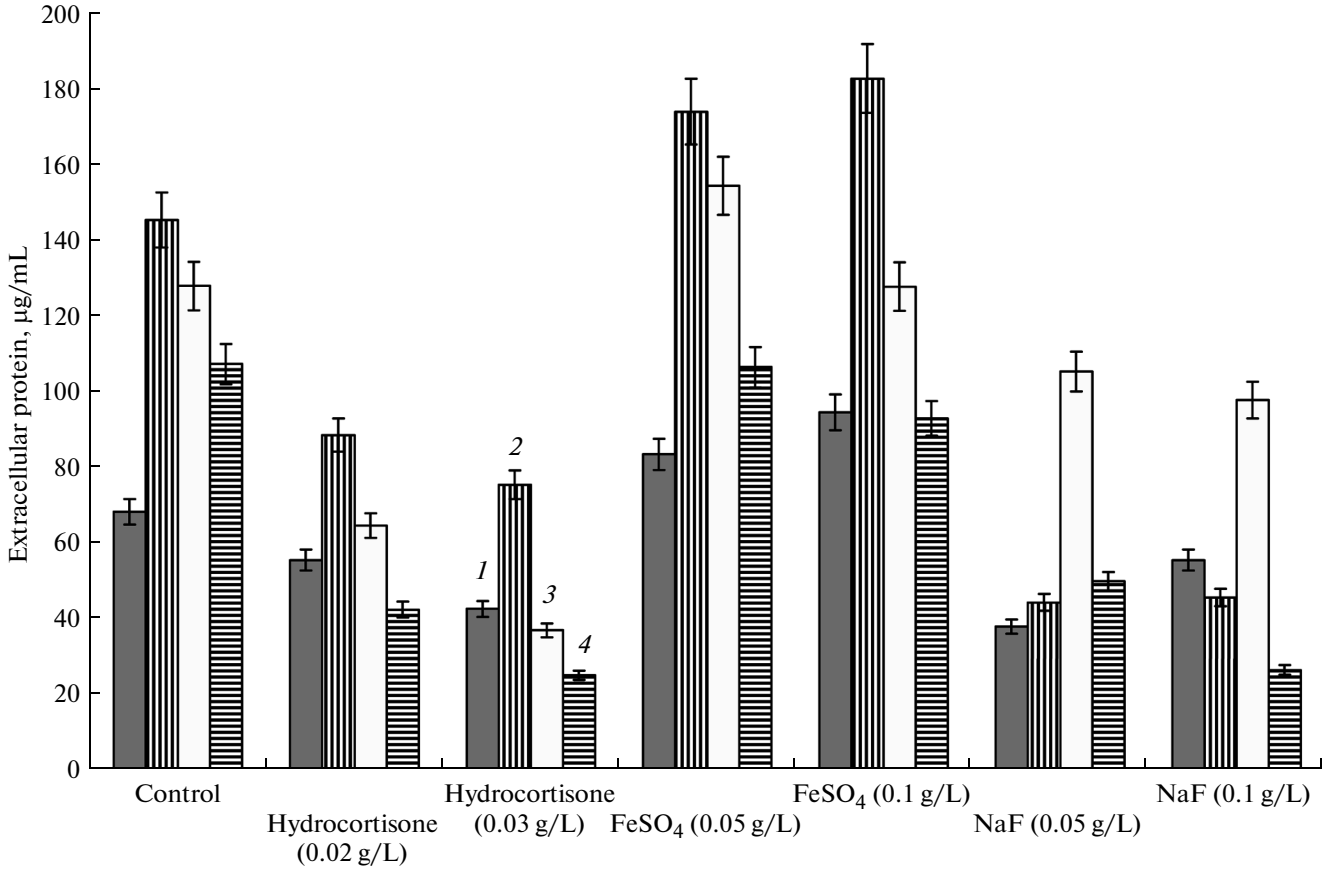

Fig. 1. Influence of the effectors on the accumulation of extracellular protein by *L. tigrinus*: 3 days (*1*); 6 days (*2*); 9 days (*3*); and 12 days (*4*).

The fatty acid methyl esters were separated on a Shimadzu GS-2010 Plus gas chromatograph (Shimadzu, Japan) with an Omegawax 250 30-cm capillary column with an inner diameter of 0.25 mm and a phase layer thickness of 0.25 μm (Supelco, United States). The initial temperature was 50°C; after 1 min, the temperature rate increment was 20 degrees/min up to 120°C followed by 4 degrees/min up to 170°C. The final temperature was 250°C. The amount of fatty acids was assessed by the peak areas of the corresponding fatty acid methyl esters compared to the internal standard—margaric acid (1 mg/mL). The enzyme activity was expressed in μg of the fatty acids formed over 1 h per 1 mg of protein [19].

The following reagents were used in the work: the standard phospholipids and fatty acids (Sigma, United States). The remaining reagents were of Russian make with the chemically pure quality. Freshly distilled solvents were also used.

All the results obtained in at least five parallel experiments were statistically processed using the Microsoft Excel 2000 software package.

RESULTS AND DISCUSSION

It was established that the extracellular protein content in the control changed in the course of cultivation: at days 3, 6, and 12 of growth, it was 68.2, 145.4 and 107.3 μg/mL, respectively. In the previous works with this strain, the highest values of this parameter were obtained at days 4–7 [20, 21]. Addition of hydrocortisone and sodium fluoride to the medium decreased the protein content in the culture liquid in relation to the control (Fig. 1) by 40–60%, depending on the cultivation time. On the contrary, addition of iron sulfate increased, although insignificantly, the level of synthesis of extracellular protein by the fungus. The most noticeable increment was observed at the initial stages of cultivation.

Earlier, we discovered the main role of laccase, secretory peroxidase of the plant type, and Mn peroxidase in the processes of biodegradation of lignocellulose substrates by the fungus *L. tigrinus* [22, 23]. In this series of experiments we studied how the effectors influenced the change in the activity of these enzymes during submerged cultivation.

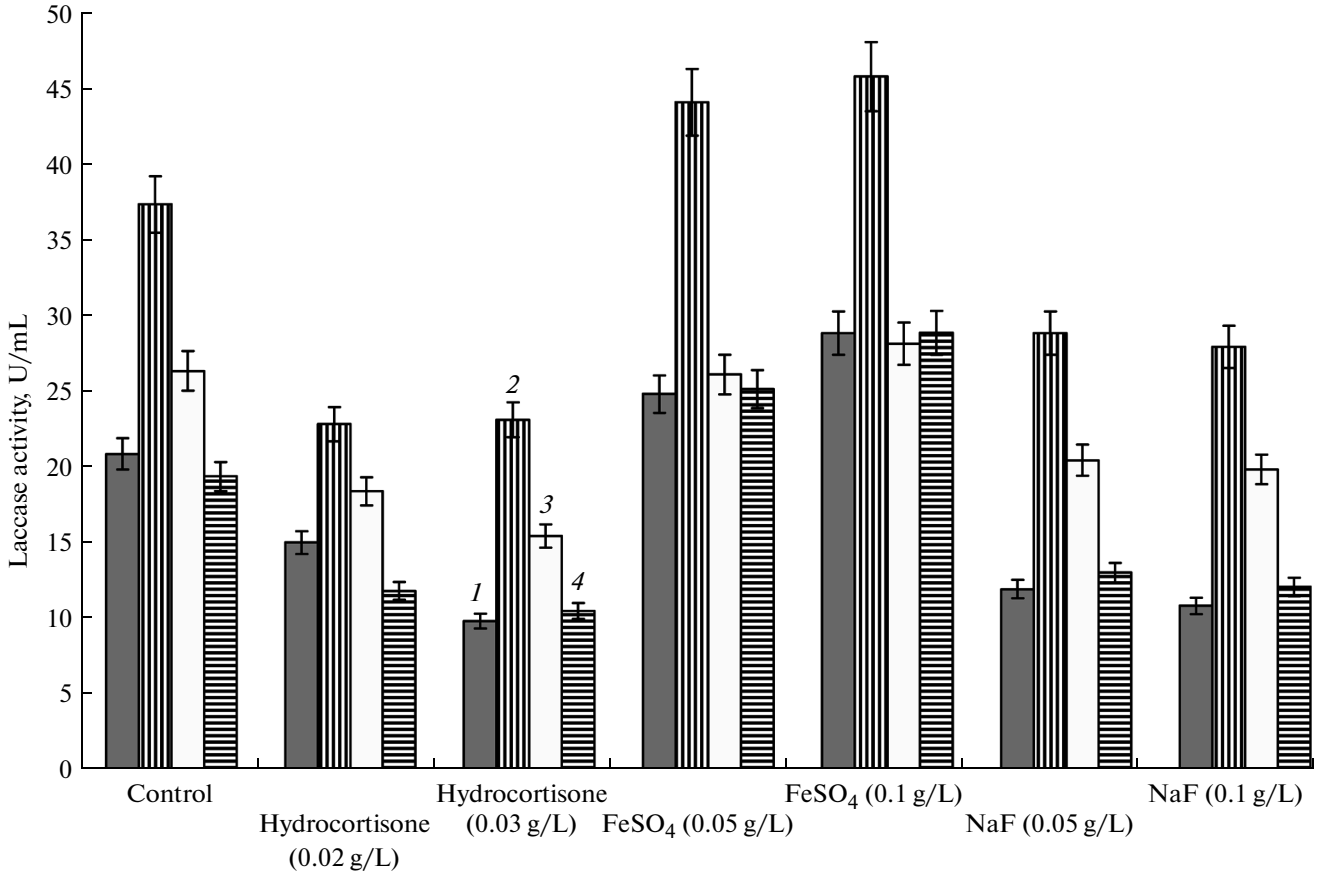

Fig. 2. Influence of the effectors on the laccase activity: 3 days (*1*); 6 days (*2*); 9 days (*3*); and 12 days (*4*).

It was established that the enzyme activities at the initial stage of cultivation with a sufficient content of nutrient elements increased and attained the maximal values at 6–9 days and then decreased due to nutrient depletion (Figs. 2–4). In the presence of hydrocortisone, iron ions, and fluoride ions, the dynamics of changes in the enzyme activities depended on the effector type and concentration.

Hydrocortisone and NaF decreased the laccase and Mn peroxidase activities. The secretory peroxidase activity increased in the presence of NaF at the beginning of cultivation and then decreased to the control level on the sixth day. Iron ions exerted a stimulating effect on the activity of all the enzymes.

Hardly any data on the influence of hydrocortisone and NaF on the ligninolytic enzymes are available in the literature. At the same time, the data that show that they (primarily hydrocortisone) are the inhibitors of the PLA_2 activity are available [9, 24–26]. Earlier, we showed that changes in the composition of phospholipids and their metabolites likely to be due to changes in phospholipase activity occurred in *L. tigrinus* mycelium on submerged and solid-phase cultivation [7, 9].

We suggested that the inhibition of the ligninolytic enzyme complex was realized via changes in lipid metabolism at the expense of phospholipases, PLA_2 in particular. We revealed in the course of the work that hydrocortisone and NaF considerably decreased the PLA_2 activity, whereas iron sulfate did not affect this parameter significantly (Fig. 5). Importantly, the inhibitory effect of hydrocortisone and NaF was practically similar. As seen from the data shown, in addition to the medium of these compounds at concentrations of 0.03 and 0.1 g/L, respectively, the enzyme activity decreased by 60–90% in relation to the control after 6–9 days of growth.

In contrast to hydrocortisone and NaF, iron ions did not significantly affect the PLA_2 activity.

We studied the influence of the effectors not only on the phospholipid activity, but also on the quantitative and qualitative distribution of the free fatty acids formed during phosphatidylcholine hydrolysis.

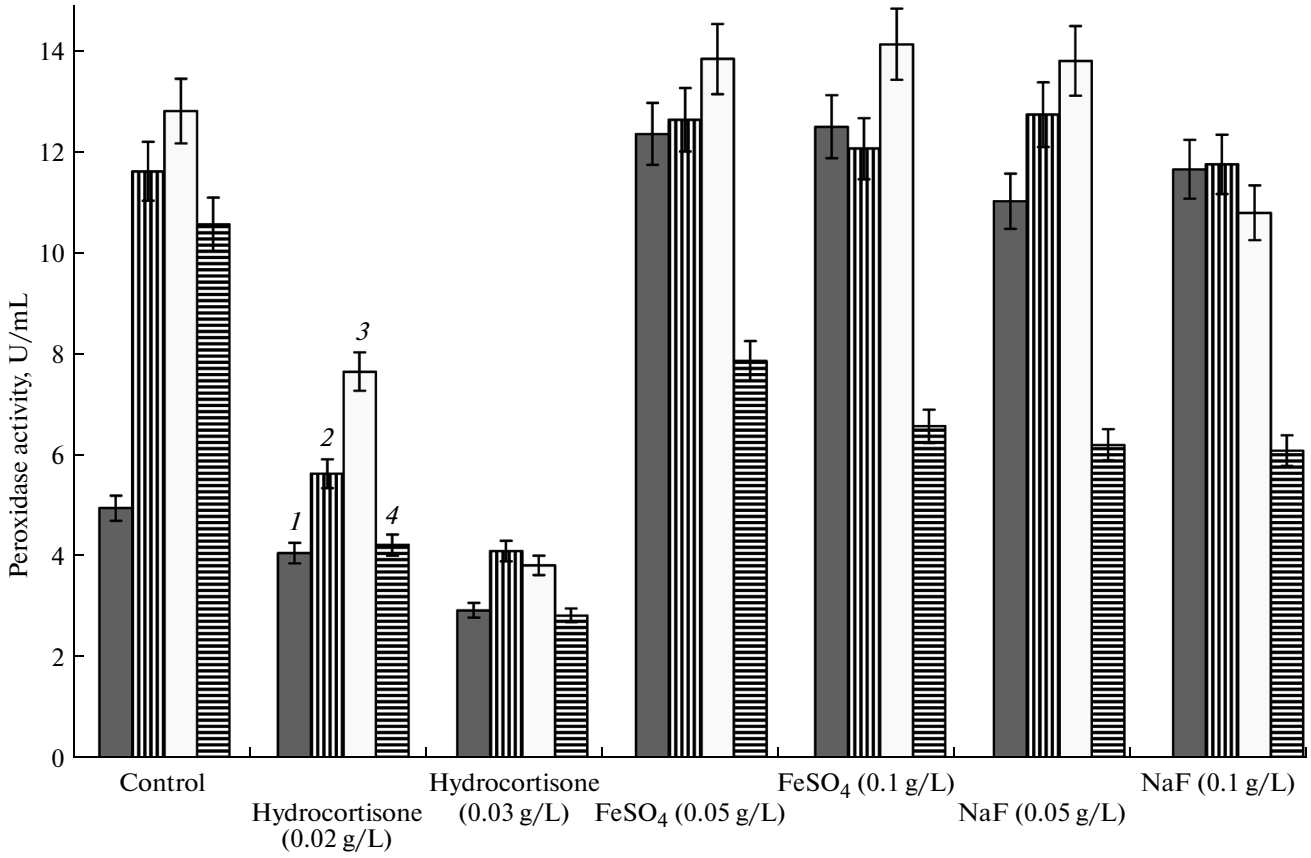

Fig. 3. Influence of the effectors on the peroxidase activity: 3 days (*1*); 6 days (*2*); 9 days (*3*); and 12 days (*4*).

It is seen from the table that PLA_2 activity resulted in the release of a larger amount of identified free fatty acids in the control and in the presence of Fe ions than in the presence of hydrocortisone or NaF. In the control and when Fe ions were introduced, the average nonsaturation coefficients were 0.68 and 0.85, respectively; in the presence of hydrocortisone and NaF, 0.17 and 0.21, respectively. In other words, in the first two cases, more unsaturated fatty acids were released.

It is known from the literature data that growth and development of ligninolytic fungi during solid-phase cultivation on lignocellulose substrates depends, above all, on the activity of the ligninolytic enzyme complex [27]. Basidial fungi actively utilize the substrate, beginning with the surface; the hyphae then penetrate the wood. The character of growth can be assessed visually by the formation of aerial mycelium. After submerged cultivation, we carried out solid-phase cultivation of *L. tigrinus*. When hydrocortisone was used, the inhibition of culture development and the absence of growth of the aerial mycelium was observed. The same picture was observed when sodium fluoride was added.

Thus, a relationship between the activities of ligninolytic enzymes and PLA_2 in the process of fungus growth was found. High PLA_2 activity was found to correlate with higher laccase and Mn peroxidase activities. The inhibitors of the PLA_2 activity decreased both the activity of these enzymes and the growth and development of the fungus on lignocellulose substrates.

The activity of virtually all enzymes of the ligninolytic complex is known to depend on the presence of hydrogen peroxide [28]. In this respect, the investigators share their opinions in relation to lignin peroxidase, secretory peroxidase, and Mn dependent peroxidase. As for laccase, the issue is to be clarified. A number of researchers hold that laccases are altered peroxidase forms and, hence, require hydrogen peroxide; others assign laccase to an independent subclass, which does not require this substrate [29]. In the case of microorganisms, this seems to depend on the species and the conditions of cultivation of a relevant producer. Several laccases—white, yellow, and blue—produced by various microorganisms and differing in their physicochemical properties have been described [20, 30]. In any case, the extracellular ligninolytic

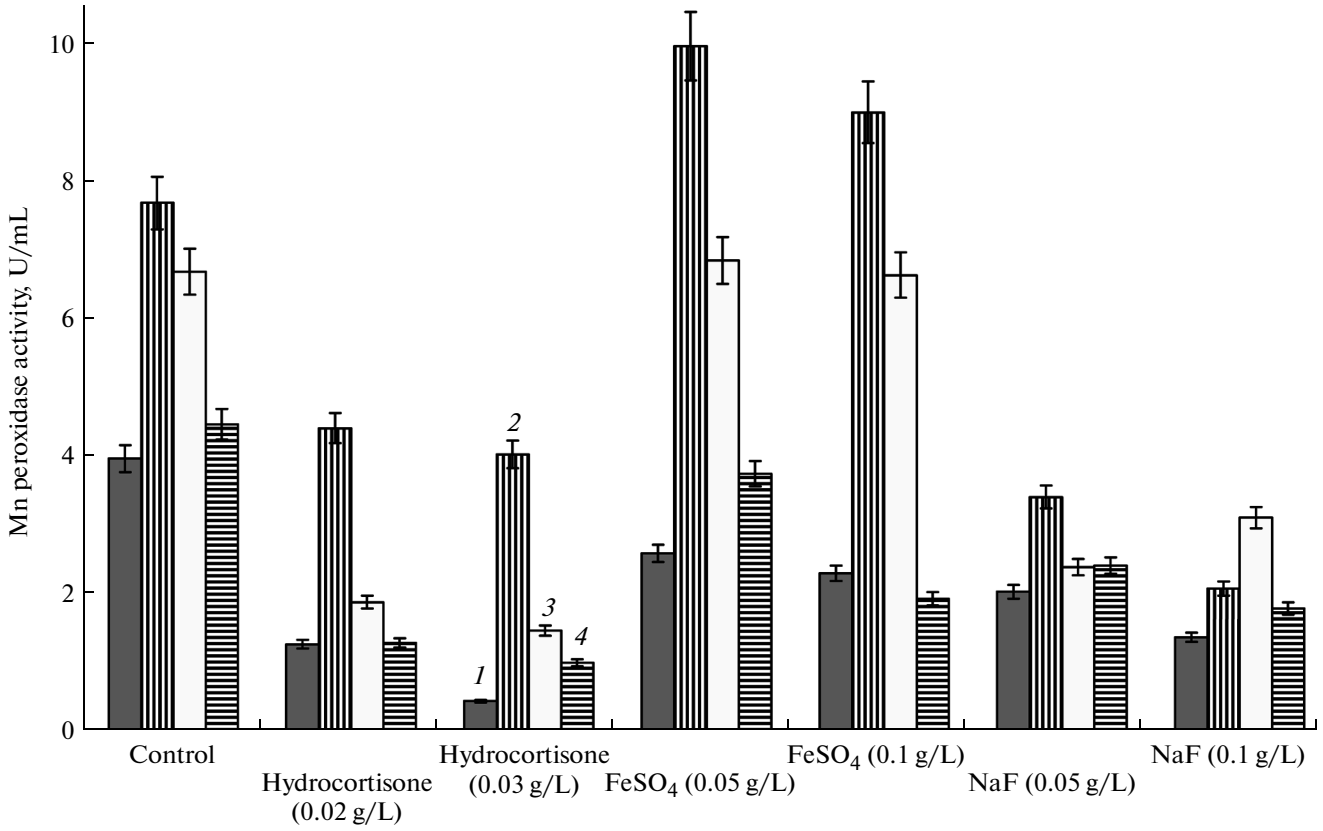

Fig. 4. Influence of the effectors on the Mn peroxidase activity: 3 days (*1*); 6 days (*2*); 9 days (*3*); and 12 days (*4*).

complex is a single ensemble, and individual enzymes depend on each other and on the presence of hydrogen peroxide for efficacy.

The previously conducted studies showed that the phospholipids of wood-decaying fungi, compared to other organisms, contained a large amount of unsaturated fatty acids, primarily those of linoleic acid whose share may account for 60% of the content of all fatty acids [7, 31]. Based on our findings and the literature data, we suggested that the role of PLA_2 was limited to the formation of fatty acids, mainly, unsaturated ones (the sources of peroxide radicals) and to affording their continuous supply to peroxide-generating enzymes (e.g., lipoxygenases). PLA_2 activation occurs on the substrate (wood) surface in the fungal cells, resulting in the release into the perimycelial layer of large amounts of unsaturated fatty acids, which are the preferable substrates for lipoxygenases. Formation of the lipid peroxide radicals oxidizing the aromatic compounds (including lignin) present in the wood, which act as inhibitors of fungus growth, occurs under the action of lipoxygenases and reactive oxygen species. Hydrogen peroxide, which is necessary for the functioning of ligninolytic enzymes, is also formed. Simultaneously, the fungal mycelium secretes the ligninolytic enzyme complex into the extracellular environment. All this results in removal of the barrier determined by the aromatic compounds (resins) and/or makes the latter more available to the action of the ligninolytic enzyme complex. Thus, a more favorable environment is formed around the ligninolytic fungal mycelium for attack and penetration deep into the wood substrate [27].

A decrease in the activity of ligninolytic enzymes in the presence of hydrocortisone seems to be connected with its influence on PLA_2. This influence can be mediated by both inhibiting the PLA_2 activity and decreasing the affinity to unsaturated fatty acids. We showed that a decrease in the ligninolytic enzymatic activities was also observed in the presence of NaF. It is known that, while NaF inhibits glycolysis by blocking enolase [32], it also disturbs lipid metabolism by binding the calcium ions which are needed by virtually all PLA_2 [33].

The fungi have a pathway of glucose oxidation (glucose is present as a sucrose constituent in Czapek medium) by glucose oxidase. As a result, gluconic acid and hydrogen peroxide, which is involved in the pro-

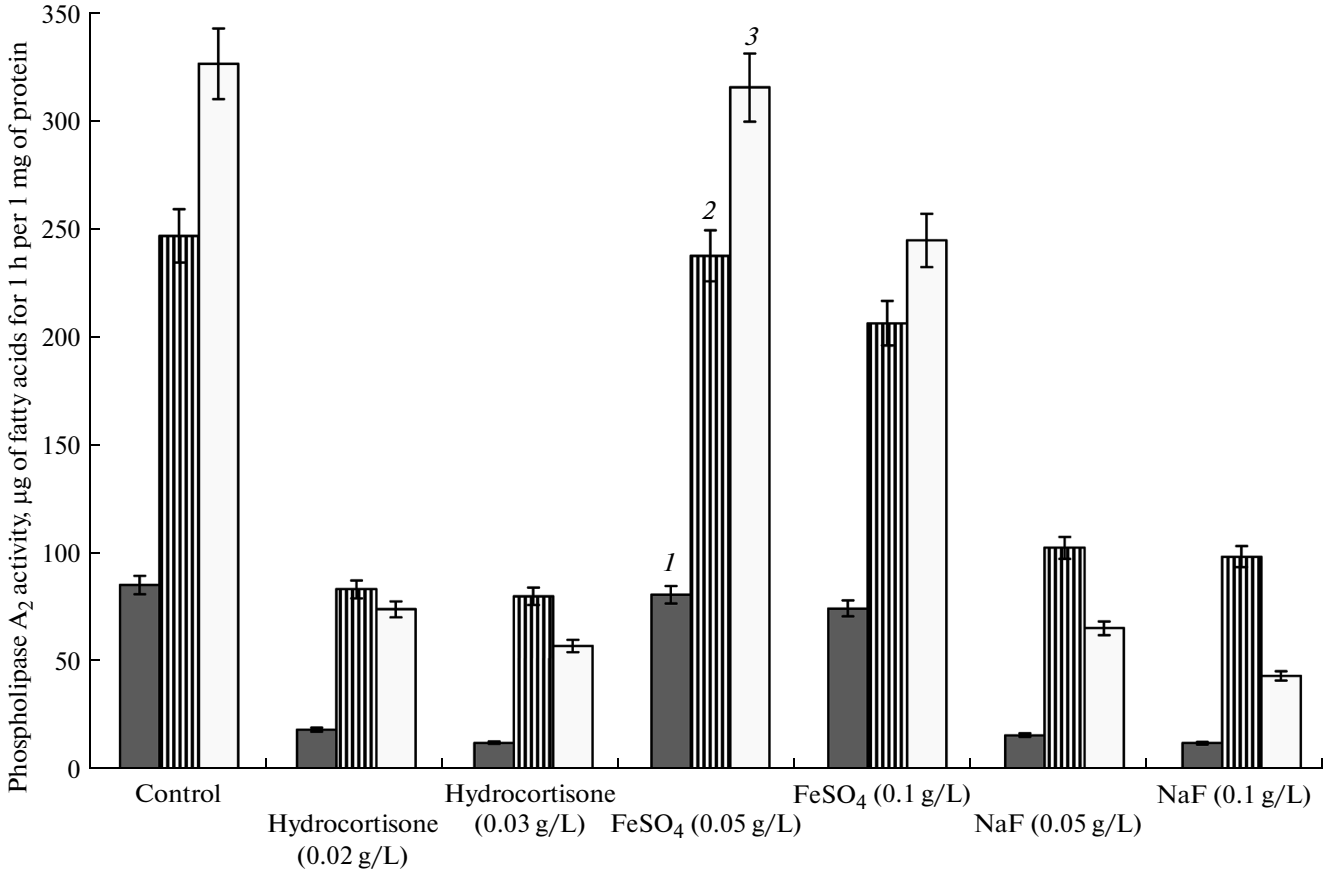

Fig. 5. Influence of the effectors on the phospholipase A_2 activity: 3 days (*1*); 6 days (*2*); and 9 days (*3*).

cesses of lygninolysis by ligninolytic fungi, are formed [34]. It may be suggested that the inhibitory effect of NaF on the activity of ligninolytic enzymes may be realized via one of the above pathways which, in the final analysis, mediate the formation of peroxide radicals and hydrogen peroxide. As shown above, the phospholipase activity invariably increased in the process of cultivation in all the experimental variants. It is possible that, at the initial stages with a sufficient amount of glucose and its sources and a relatively low phospholipase activity, hydrogen peroxide may form through the action of glucose oxidase, whereas at the later stages, it is PLA_2 that makes an increasingly greater contribution as glucose is used up.

As shown above, the introduction of $FeSO_4$ insignificantly increased the activity of the ligninolytic enzymes but sharply enhanced the peroxidase activity over the first 24 h of cultivation. Since peroxidases are true heme-containing enzymes, this is probably linked to an increase in the level of the enzyme activity. At the same time, $FeSO_4$ decreased the PLA_2 activity compared to the control, although to a lesser degree than hydrocortisone and iron fluoride. The unsaturation coefficient of the fatty acids formed under the action of PLA_2 was, however, even higher than in the control. The biosynthesis of fatty acids in almost all the organisms ends in the formation of saturated acids, which then undergo a series of desaturation and elongation reactions with the formation of fatty acids. Since desaturases are iron-containing enzymes [35], we may suggest that an increase in the concentration of iron ions in the medium influences the synthesis of polyunsaturated fatty acids, which are regulators of the PLA_2 activity in fungi. As a result, the enzyme affinity to polyunsaturated fatty acids decreases against the background of a decline in general activity.

Thus, the results obtained provide for a better understanding of the biochemical processes underlying ligninolysis and of the mechanisms of involvement of lipids and their metabolites in the processes of biodegradation of lignocellulose substrates by ligninolytic fungi. This, in turn, will allow us not only to purposefully modify these substrates but also to develop new means of biological protection of the goods made of wood.

Distribution of phosphatidylcholine fatty acids influenced by *L. tigrinus* PLA_2 in the presence of the effectors

Fatty acids	Fatty acids (μg/mg lipids)			
	control	NaF	hydrocortisone	$FeSO_4$
$C_{4:0}$ Butanoic acid	0.73 ± 0.04	–	–	–
$C_{10:0}$ Capric acid	0.13 ± 0.01	–	0.1 ± 0.01	–
$C_{12:0}$ Lauric acid	0.02 ± 0.001	–	–	–
$C_{13:0}$ Tridecanoic acid	0.44 ± 0.02	0.78 ± 0.04	0.61 ± 0.03	0.1 ± 0.01
$C_{14:0}$ Myristic acid	0.82 ± 0.04	0.39 ± 0.02	0.93 ± 0.05	–
$C_{15:0}$ Pentadecanoic acid	2.12 ± 0.11	0.68 ± 0.03	3.70 ± 0.19	2.25 ± 0.11
$C_{16:0}$ Palmitic acid	0.65 ± 0.03	4.23 ± 0.21	3.16 ± 0.16	1.29 ± 0.06
$C_{17:0}$ Margaric acid	13.59 ± 0.68	0.79 ± 0.04	0.25 ± 0.01	10.34 ± 0.52
$C_{18:0}$ Stearic acid	1.69 ± 0.08	–	0.19 ± 0.01	0.18 ± 0.01
$C_{20:0}$ Arachic acid	–	–	0.24 ± 0.01	0.67 ± 0.03
$C_{22:0}$ Behenic acid	–	1.29 ± 0.06	–	–
$C_{14:1}$ Myristoleic acid	0.23 ± 0.01	–	0.26 ± 0.01	0.14 ± 0.01
$C_{15:1}$ Pentadecainic acid	2.17 ± 0.11	–	–	1.80 ± 0.10
$C_{16:1}$ Palmitoleic acid	9.57 ± 0.48	1.11 ± 0.05	0.54 ± 0.030	9.12 ± 0.46
$C_{17:1}$ Margaroleic acid	1.76 ± 0.14	–	0.25 ± 0.01	0.30 ± 0.02
$C_{18:1}$ Oleic acid	–	0.18 ± 0.01	0.41 ± 0.02	0.29 ± 0.01
$C_{18:2}$ Linoleic acid	–	–	0.46 ± 0.02	–
$C_{18:3}$ α,γ-Linolenic acid	–	–	–	0.67 ± 0.03
$C_{20:2}$ Gondoinic acid	–	0.40 ± 0.02	–	0.28 ± 0.01
Saturated fatty acids	20.21 ± 1.01	8.17 ± 0.41	11.18 ± 0.56	14.82 ± 0.74
Unsaturated fatty acids	13.71 ± 0.72	1.69 ± 0.08	1.88 ± 0.10	12.60 ± 0.63
Unsaturation coefficient	0.68 ± 0.03	0.21 ± 0.01	0.17 ± 0.01	0.85 ± 0.04

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