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Effect of Phenols on the Activity of Peroxidase in Liquid Cultures of *Inonotus radiatus* Fungus

Wpływ fenoli na aktywność peroksydazy w hodowlach płynnych grzyba *Inonotus radiatus*

Влияние фенолов на активность пероксидазы в фильтрате гриба *Inonotus radiatus*

INTRODUCTION

Fungi which belong to *Basidiomycetes* play main part in the degradation of lignin and its phenolic degradation products. Laccase and peroxidase are the enzymes which catalyze these metabolic changes (8, 9, 10, 11, 12, 23). Lignin monomers or dimers are hydrogen substrates or donors for the above enzymes (8, 12, 13).

The products of lignin degradation were found to increase the production of mycelium biomass (4, 7, 17). Robbins et al. (22) reported the stimulation of *Polyporus schweinitzii* growth by ferulic acid.

Some phenols were also found to stimulate the activity of laccase in fungi (1, 2, 3, 5, 6, 17, 18). Leonowicz and Trojanowski (15) showed that Bjorkman's lignin had an inducing effect upon the production of peroxidase and laccase by *Pholiota mutabilis*. Leonowicz (14) demonstrated the stimulating action of ferulic acid on the excretion of laccase and peroxidase by *Pleurotus ostreatus*. This effect was partially reduced by puromycin and chloramphenicol and completely by actionomycin D.

Studies on *Inonotus radiatus* fungus producing peroxidase as the only exoenzyme showed that wood meal caused a 30-fold increase in this enzyme, whereas its water extract rich in phenol compounds brought

a 3-fold increase in the specific activity of exoperoxidase when compared with the control (19). This points out to the inducing effect of water extract from wood meal on the biosynthesis of peroxidase.

In order to find out which of phenol compounds could induce peroxidase activity in *Inonotus radiatus*, further studies were made.

MATERIALS AND METHODS

a) The culture of *Inonotus radiatus* was used in these studies.

b) Experiments with phenols *in vivo*. Lindeberg's mineral medium was used as a basal medium containing (per 1 litre of medium): 1.0 g asparagin, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.47 g KH_2PO_4, 0.48 g Na_2HPO_4, 50.0 μg aneurin, 0.05 g $Ca(NO_3)_2$, 0.0085 g $Mn(CH_3COO)_2$, 0.0032 g $FeCl_3$, 0.0020 g $Zn(NO_3)_2$, 0.0025 g $CuSO_4$. Three variants of the medium differing in glucose centent (1%, 0.5% and 0.1%) were applied.

Experiments were made twice. 100 ml of medium was poured into 300-ml Erlenmayer flasks and sterilized in a Koch's apparatus three times for 45 min at day intervals. Fragments of *Inonotus radiatus* were inoculated into these flasks under sterile conditions. Fungus cultures were incubated at room temperature for 2 weeks. Then, about 4 ml of medium was sterilely poured out from each flask in order to determine peroxidase activity and protein level. The following phenols were then sterilely added to the flasks: vanillic acid M.wt. 168.0 (3-methoxy-4-hydroxybenzoic acid), vanilline M.wt. 152.0 (3-methoxy-4-hydroxybenzoic aldehyde), syryngic acid M.wt. 198.18 (3,5-dimethoxy-4-hydroxybenzoic acid), ferulic acid M.wt. 194.18 (3-methoxy-4-hydroxycinnamic acid), protokatechuic aldehyde M.wt. 138.0 (3,4-dihydroxybenzoic aldehyde), caffeic acid M.wt. 180.0 (3,4--dihydroxycinnamic acid.). Final concentration of phenols was 2×10^{-4} M.

Experiments with each phenol were made simultaneously in three flasks. *Inonotus radiatus* culture grown in a basal medium without phenols was used as the control. Peroxidase activity and protein content in the culture filtrate were assayed twice for each of the three flasks. Measurements were done after three and four weeks of mycelium growth, that is after a week and after two weeks following phenol addition. The results obtained were the arithmetic mean of six measurements.

c) Experiments with preincubated fragments of *Inonotus radiatus* mycelium with the addition of phenols *in vitro*. Two-week-old *Inonotus radiatus* culture grown in a basal medium was filtered through Buchner's funnel and washed with sterile distilled water on filter paper. 1 g of fresh mycelium mass was then weighted out and placed in 50-ml flasks, each containing 10 ml of Lindeberg's medium. The following phenols were added: vanillic acid, protocatechuic aldehyde, syryngic acid, ferulic acid, caffeic acid. Final concentration of phenols was 2×10^{-4} M.

The control and variants with phenols were repeated three times. Flasks containing the fragments of mycelium and Lindeberg's medium were the control. All flasks were placed in a thermostat at 27°. Peroxidase activity was assayed after 24 and 48 hrs. Basal medium contained 0.5% glucose.

d) Methods. Peroxidase activity was estimated by p-phenylenediamine method after Aurand et al. (21). By this method, a peroxidase activity unit is this amount of the enzyme which changes E_{1cm}^{485} of 1.0 per 1 sec as a result of

p-phenylenediamine oxidation in the presence of H_2O_2. The level of protein from the culture filtrate of *Inonotus radiatus* was assayed spectrophotometrically on VSU-1 spectrophotometer, Zeiss-Jena (20). Dry matter of mycelium was determined after 4 weeks of fungus culture. The mycelium was filtered through Buchner's funnel, placed in Petri's dishes and then dried at 65° up to a dry matter. A final result was the arithmetic mean of mycelium mass from three flasks for each variant with phenol and for control.

Table 1. Dry matter (in g) of 4-week-old mycelium of *Inonotus radiatus* grown in a basal medium containing 1% glucose

Phenol	Experiment 1	Experiment 2
Vanillic acid	0.1092	0.1143
Vanilline	0.1316	0.1087
Protocatechuic ald. . .	0.1053	0.1072
Syryngic acid	0.1088	0.1077
Ferulic acid	0.1032	0.0969
Caffeic acid	0.1197	0.1068
Control	0.1016	0.0897

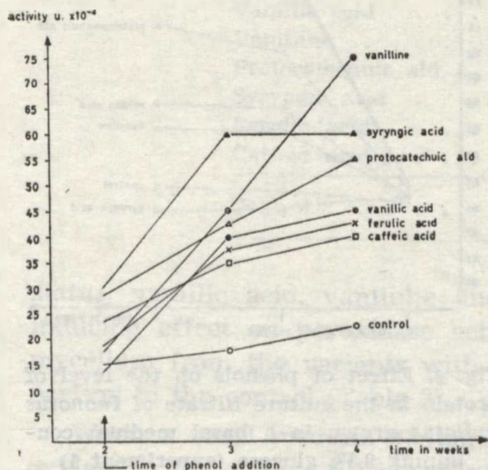

Fig. 1. Effect of phenols on the activity of peroxidase in the culture filtrate of *Inonotus radiatus* grown in a basal medium containing 1% glucose (experiment 1)

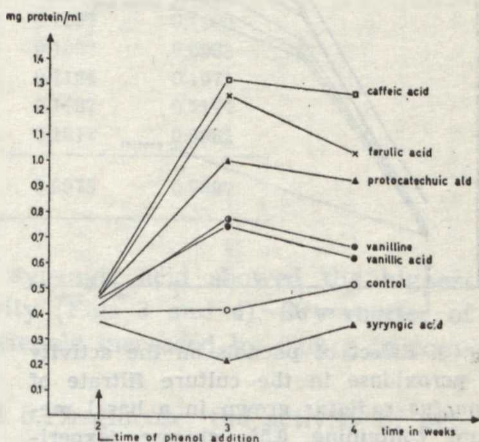

Fig. 2. Effect of phenols on the level of protein in the culture filtrate of *Inonotus radiatus* grown in a basal medium containing 1% glucose (experiment 1)

RESULTS

Experiments *in vivo* designed to determine the effect of phenols on the activity of peroxidase from the culture filtrate of *Inonotus radiatus*

grown in Lindeberg's medium with 1% glucose showed a stimulating action of phenols. The results were illustrated in Fig. 1.

After phenol addition, the level of protein also rose in the filtrate, when compared with its initial value (Fig. 2). Dry matter of mycelium increased, on an average, by 0.03 g in comparison to the control (Table 1).

An increase in the peroxidase activity was higher than the increase in protein content in the culture filtrate (Figs 1 and 2). On this basis, it can be assumed that phenols have a selective effect on the biosynthesis of peroxidase and its excretion into the medium.

Of the six phenols tested, vanillic acid, vanilline and syryngic acid were found to have the highest inducing effect. This suggests that this effect of phenols on peroxidase activity depends upon their chemical structure, since methoxyphenols affect peroxidase activity to a greater extent than o-diphenols.

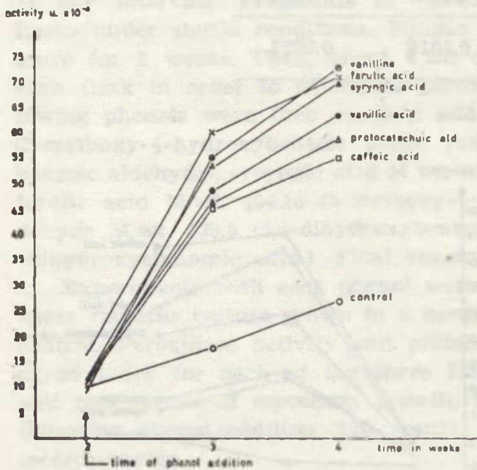

Fig. 3. Effect of phenols on the activity of peroxidase in the culture filtrate of *Inonotus radiatus* grown in a basal medium containing 0.5% glucose (experiment 1)

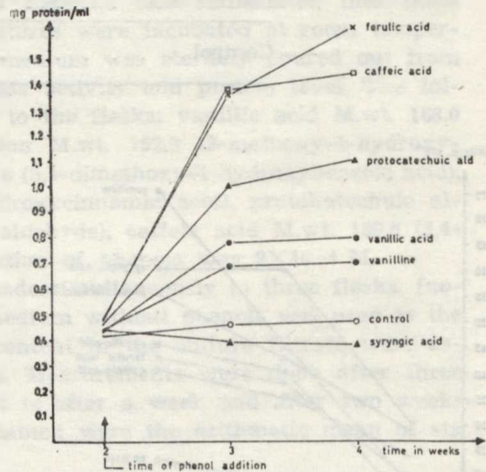

Fig. 4. Effect of phenols on the level of protein in the culture filtrate of *Inonotus radiatus* grown in a basal medium containing 0.5% glucose (experiment 1)

A stimulating action of phenols on the activity of peroxidase was also observed in experiments in which Lindeberg's medium contained 0.5% glucose (Fig. 3). The level of protein from the culture filtrate increased as well (Fig. 4). However, the stimulation of peroxidase activity in comparison to the increase in protein was higher than in the previous experiments in which the basal medium contained 1% glucose.

Considering the level of protein in culture filtrates of *Inonotus ra-*

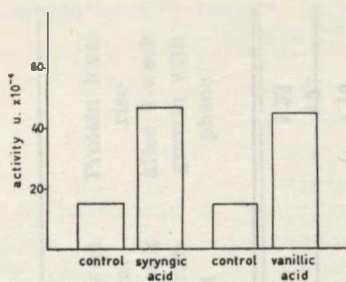

Fig. 5. Peroxidase activity level in experiments with preincubated fragments of *Inonotus radiatus* mycelium after 48-hr incubation with syryngic and vanillic acids. Basal medium contained 0.5% glucose

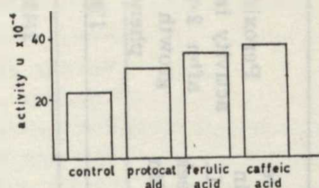

Fig. 6. Effect of protocatechuic aldehyde, ferulic and caffeic acids on the activity of peroxidase in experiments with preincubated fragments of *Inonotus radiatus* mycelium. Basal medium contained 0.5% glucose

Table 2. Dry matter (in g) of 4-week-old mycelium of *Inonotus radiatus* grown in a basal medium containing 0.5% glucose

Phenol	Experiment 1	Experiment 2
Vanillic acid	0.1188	0.1026
Vanilline	0.1197	0.1180
Protocatechuic ald.	0.1007	0.0985
Syryngic acid	0.1124	0.1071
Ferulic acid	0.1187	0.1174
Caffeic acid	0.1017	0.0982
Control	0.0975	0.0897

diatus, vanillic acid, vanilline and syryngic acid showed the highest inducing effect on peroxidase activity (Figs 3 and 4). Dry matter of mycelium from the variants with phenols increased by 0.02 g in comparison to the control (Table 2).

When a basal medium contained 0.1% glucose, the activity of peroxidase in the culture filtrate of *Inonotus radiatus* changed insignificantly under the influence of phenols (Table 3). On the other hand, the increase in protein was very high. It seemed possible that under such conditions (low content of glucose) autolysis of the fungus cells occurred (Table 4). Dry matter of mycelium was very small (Table 5).

Experiments *in vitro* with preincubated mycelium fragments showed that syryngic acid and vanillic acid caused, on an average, a 2-fold increase in the activity of peroxidase after 48-hr incubation when compared with the control (Fig. 5). Protocatechuic aldehyde ferulic acid and

Table 3. Effect of phenols on the activity of peroxidase in the culture filtrate of *Inonotus radiatus* grown in a basal medium containing 0.1% glucose

No. of experiment	Phenol	Peroxidase activity in $\times 10^{-4}$ A.U. before phenol addition	Peroxidase activity in $\times 10^{-4}$ A.U. after 1-week growth with phenol	Peroxidase activity increase after 1-week growth with phenol	Peroxidase activity in $\times 10^{-4}$ A.U. after 2-week growth with phenol	Peroxidase activity increase after 2-week growth with phenol
1+2*	Vanillic acid	0.018	0.026	1.44	0.026	1.44
	Vanilline	0.015	0.026	1.74	0.030	2.00
	Protocatechuic ald.	0.017	0.023	1.35	0.023	1.35
	Syryngic acid	0.020	0.025	1.25	0.026	1.30
	Ferulic acid	0.020	0.032	1.60	0.035	1.75
	Caffeic acid	0.019	0.021	1.10	0.021	1.10
	Control	0.017	0.023	1.35	0.021	1.23

A.U. — activity units.

* Results of experiments 1+2 are the arithmetic mean of 6 simultaneous tests.

Table 4. Effect of phenols on the level of protein in the culture filtrate of *Inonotus radiatus* grown in a basal medium containing 0.1% glucose

No. of experiment	Phenol	Protein level in mg/ml before phenol addition	Protein level in mg/ml after 1-week growth with phenol	Protein level rise after 1-week growth with phenol	Protein level in mg/ml after 2-week growth with phenol	Protein level rise after 2-week growth with phenol
1+2*	Vanillic acid	0.123	0.460	3.72	0.440	3.57
	Vanilline	0.136	0.564	4.15	0.581	4.28
	Syryngic acid	0.090	1.049	11.20	1.164	12.90
	Protocatechuic ald.	0.088	0.501	5.70	0.592	6.75
	Ferulic acid	0.060	1.251	20.80	1.139	19.00
	Caffeic acid	0.053	1.153	21.70	1.150	21.70
	Control	0.119	0.139	1.16	0.119	1.00

* See Table 3.

Table 5. Dry matter (in g) of 4-week-old mycelium of *Inonotus radiatus* grown in a basal medium containing 0.1% glucose

Phenol	Dry matter
Vanillic acid	0.0370
Vanilline	0.0311
Protocatechuic ald. . .	0.0379
Syryngic acid	0.0335
Ferulic acid	0.0317
Caffeic acid	0.0329
Control	0.0380

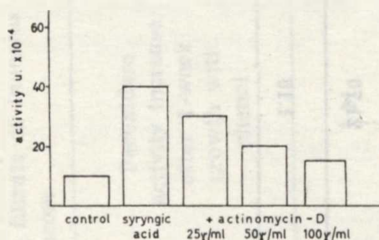

Fig. 7. Effect of actinomycin D on the reduction in syryngic acid inducing action on the activity of peroxidase in experiments with preincubated fragments of *Inonotus radiatus* mycelium. Basal medium contained 0.5% glucose

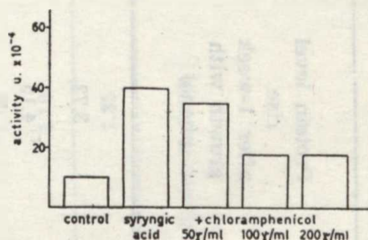

Fig. 8. Effect of chloramphenicol on peroxidase activity induced by syryngic acid in experiments with preincubated fragments of *Inonotus radiatus* mycelium. Basal medium contained 0.5% glucose

caffeic acid stimulated the activity of peroxidase to a smaller extent under the same conditions (Fig. 6).

The increase in peroxidase activity was higher in the experiments *in vivo* (Figs 1 and 3) than in those *in vitro* (Figs 5 and 6). This could be explained by a longer period of mycelium growth in the presence of phenol effectors in the experiments *in vivo*.

Further studies aimed at revealing which stage of peroxidase biosynthesis is affected by phenols. Experiments were made with preincubated fragments of *Inonotus radiatus* in a basal medium containing 0.5% glucose with the addition of syryngic acid (final concentration = 2×10^{-4} M) and such antibiotics as actinomycin D at 25, 50 γ/ml concentration and chloramphenicol at 50, 100, 200 γ/ml. Syryngic acid was chosen because of its highest effect stimulating the activity of peroxidase in the previous experiments.

Actinomycin D and chloramphenicol added to the medium inhibited the inducing effect of syryngic acid. Actinomycin at 50 γ/ml reduced

its stimulating effect by 66.5% and at 100 γ/ml by 83.4%. The same concentrations of chloramphenicol reduced by 16% and 75%, respectively (Figs 7 and 8).

DISCUSSION

Experiments *in vivo* revealed a relationship between the amount of glucose in the medium and the inducing effect of phenols. At 1% and 0.5% glucose concentrations, the addition of phenols increased several times peroxidase content in the culture medium. Stimulation of peroxidase activity by phenols did not occur at 0.1% glucose content, which was due to low carbon content. The effect of phenols on the increase in mycelium biomass, as reported by Flaig et al. (7), was not observed in our experiments since we used lower phenol concentrations (average one — 0.03%). Whereas this effect was observed by Flaig et al. at 0.1% concentration of phenols. It is noteworthy, that only some of the phenols tested (vanillic acid, vanilline, syryngic acid) had a selective effect on the increase in peroxidase content in the culture filtrate of *Inonotus radiatus*. The inducing effect of phenols on peroxidase activity was maximal at 0.5% glucose concentration.

The results of experiments *in vivo* were confirmed by those *in vitro*. In experiments with syryngic acid, actinomycin D and chloramphenicol, the observed inhibitory effect on the stimulation of peroxidase might point to participation of syryngic acid as a positive effector in the biosynthesis of peroxidase by *Inonotus radiatus* mycelium.

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STRESZCZENIE

Wpływ fenoli na aktywność peroksydazy w filtracie hodowli grzyba *Inonotus radiatus* badano w doświadczeniach *in vivo* i *in vitro*. W obu przypadkach stwierdzono indukujący wpływ fenoli w stężeniu 2×10^{-4} M na aktywność peroksydazy. Doświadczenia *in vivo* przeprowadzano na dwutygodniowych kulturach grzyba, wzrastających na pożyce mineralnej Lindeberga różniącej się zawartością glukozy (1, 0,5, 0,1%).

Badano wpływ następujących fenoli: kwasu wanilinowego, waniliny, aldehydu protokatechusowego, kwasu syryngowego, kwasu ferulowego, kwasu kawowego. Pomiary aktywności peroksydazy, zawartości białka w przesączu przeprowadzano przed dodaniem fenoli oraz po tygodniu i dwu tygodniach wzrostu z odpowiednim fenolem.

Stwierdzono, że przy zawartości 0,5% glukozy w pożyce podstawowej przyrost aktywności peroksydazy w porównaniu z przyrostem poziomu białka w przesączu był wyższy niż w wariancie z dodatkiem 1% glukozy w pożywce. Spośród przebadanych fenoli metoksyfenole wykazywały większy indukujący wpływ na aktywność peroksydazy niż o-dwufenole.

W doświadczeniach *in vitro* obserwowano również stymulujące działanie fenoli na aktywność peroksydazy po 48 godz. inkubacji. Kwas syryngowy, kwas wanilinowy w stężeniu 2×10^{-4} M powodowały średnio dwukrotny wzrost aktywności peroksydazy w stosunku do kontroli. Kwas ferulowy, kwas kawowy i aldehyd protokatechusowy wykazywały w doświadczeniach z preinkubowanymi skrawkami mycelium niewielki wpływ.

Dodanie aktynomycyny i chloramfenikolu, które hamują biosyntezę białka, powodowało zmniejszenie indukującego wpływu kwasu syryngowego na biosyntezę peroksydazy w doświadczeniach z preinkubowanymi skrawkami mycelium *Inonotus radiatus.*

РЕЗЮМЕ

В экспериментах *in vivo* и *in vitro* исследовали влияние фенолов на активность пероксидазы в фильтрате гриба *Inontus radiatus.* В обоих случаях при концентрации фенола 2×10^{-4} М установили увеличение активности пероксидазы. Опыты *in vivo* проводили на двухнедельных культурах гриба, выращиваемых на минеральной среде Линдеберга, с разным содержанием глюкозы (1, 0,5, 0,1%).

Исследовали влияние следующих фенолов: ванилиновой кислоты, ванилина, протокатехусового альдегида, сиринговой кислоты, ферульной кислоты. Активность пероксидазы и содержание белка в фильтрате измеряли перед добавлением фенолов и в 1 и 2-недельной культуре гриба, выращенной с соответствующим фенолом.

Установили, что при 0,5% содержании глюкозы в питательной среде Линдеберга увеличение активности пероксидазы, по сравнению с увеличением содержания белка в фильтрате, было большим, чем в варианте с 1% глюкозы в питательной среде. Из исследованных фенолов большим стимулирующим влиянием на активность пероксидазы обладали метоксифенолы, чем о-дифенолы.

После 48 час. инкубации с фенолами в исследованиях *in vitro* в срезах мицелиев также наблюдали стимулирующее действие фенолов на активность пероксидазы. Сиринговая и ванилиновая кислоты при концентрации 2×10^{-4} M вызывали увеличение активности пероксидазы по сравнению с контролной в среднем в 2 раза. Ферульная и кофейная кислоты и протокатехусовый альдегид в исследованиях *in vitro* проявляли слабое стимулирующее действие.

Добавление актиномицина и хлоромицетина, которые задерживают синтез белка, в опытах *in vitro* со срезами мицелия *Inontus radiarus* уменьшало стимулирующее влияние сиринговой кислоты на биосинтез пероксидазы.

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