ЕКСПЕРИМЕНТАЛЬНІ РОБОТИ

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ISOLATION AND PROPERTIES OF POLYPHENOL OXIDASE FROM BASIDIOCARPS OF *Lactarius pergamenus* Fr. (Fr.) FUNGI

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Fresh juice of basidiocarps of Lactarius pergamenus Fr. (Fr.) fungi was subjected to ion exchange chromatography with used DEAE-toyopearl and CM-cellulose columns, as well as preparative electrophoresis in 7.5% polyacrylamide gels (pH 8.6). Three isoforms of polyphenol oxidase (PPO) were discovered and two isoforms (1-1 and 1-2) were purified with a release of protein 0.42 mg/kg and 0.15 mg/kg of basidiocarps, respectively. These isoforms differ in the mobility at disc-electrophoresis in 7.5% PAGE in alkaline buffer system (pH 8.6). Specific activity of isoform 1-2 is 4.8 times higher than that of the isoforms 1-1. The molecular weight determination by gel chromatography on the Toyopearl HW-55 demonstrated that both isoforms 1-1 and 1-2 have the same 64 ± 2 kDa molecular mass. Electrophoresis in 15% PAGE in the presence of sodium dodecylsulphate and β-mercaptoethanol revealed one band with molecular mass of 64 ± 1 kDa which suggests the presence of one polypeptide chain in the molecule of the enzyme. The enzyme has demonstrated the highest activity at pH 6.0 and temperature +10 ºC, and at +70 ºC the enzyme was inactivated. The PPO activity was the highest in young mushrooms and it decreased with their age and positively correlated with the content of the milky juice. Ortho-aminophenol was most effective among all the tested substrates to determine the activity of PPO (o-, m- and p-aminophenol, catechol, tyrosine, resorcinol, phloroglucinol) and its relative activity was 129% of the activity of catechol. Ascorbic acid was the most effective inhibitor of the polyphenol oxidase activity which was completely blocked at 1 mM concentration, whereas the same concentration of thiourea and sodium sulphite decreased the enzymatic activity by 40-45%. The PPO in L. pergamenus fungi basidiocarps was mainly localized in the mushroom milky juice where its high activity may be associated with protection of basidiocarps against various pathogens.

K e y w o r d s: Lactarius pergamenus Fr. (Fr.), polyphenol oxidase, purification, characteristics.

Polyphenol oxidase (tyrosinase, EC 1.14.18.1) is a metalloprotein that contains copper in its molecule. In the presence of oxygen, this enzyme is able to oxidize the ortho-diphenols to the corresponding quinones. Copper ions that are located in enzyme's active site play an important role in its activity. Splitting-off copper ions (decopperization) leads to the enzyme inactivation [1]. PPO is widely distributed in animal tissues, plants, fungi and bacteria. It is responsible for melanin production in animals and causes browning of damaged plant tissues and plant juice in air [1]. The physiological function of PPO in plants and fungi still remains unclear. It is believed that the enzyme can perform the protective function in embryophytes and fungi. It is proved that o-quinones formed by the enzymatic action protect plants against pathogens and insects. Melanin formed by action of fungi PPO causes sporulation and is involved in fungi virulence [3-5]. PPO activity in basidiomycota fungi has specific characteristics. Study of 67 fungi species showed that PPO activity in the majority of them were low. However, PPO activity in 7 of 8 fungi species of *Lactarius* genus was rather high [6]. These fungi genus differ from

the other by the presence of milky juice which protects them from being eaten by insects, molluscs and warm-blooded animals, as well as protects against microorganisms and other fungi. Some species of *Lactarius*, in particular *Lactarius pergamenus*, contain a milky juice with a very pungent taste that deters animals. While milky juice is present in fungus basidiocarps they are almost never worm-eaten or damaged by molluscs and rodents.

It is known that sesquiterpene aldehydes play an important role in protective system of *Lactarius* fungi [7]. We have found high levels of phthalates in *L. pergamenus* [8]. As it is known, the phthalates (in particular dimethyl-phthalate) repel mosquitoes and are used in creams, aerosols, emulsions, lotions for repelling mosquitoes and ticks (e.g. Diftalar cream, Taiga, Kamarant, Cedar lotion). Probably, phthalates protect fungi against insects. Possibly, the high level of PPO is a component of protection system against pathogens and insects, furthermore the phenolic compounds (which are inherent in plants) were not found in these fungi. There are studies that suggest that *Lactarius* fungi exude this enzyme into the environment [14].

The aim of our study was to investigate alterations in the activity of PPO depending on the "age" of basidiocarps, develop a method of purification of PPO from basidiocarps of *L. pergamenus*, and explore its physical and chemical properties.

Materials and Methods

Basidiocarps of *Lactarius* fungi were collected in a mixed forest near the village of Upper Synyovydne Skole county, Lviv region. Collected mushrooms were delivered to the laboratory within twenty-four hours for the analysis. Domestic and imported reagents (analytical grade) were used in the experiments.

Determination of PPO activity and proteins concentrations. PPO activity was determined by the method described in [10, 11] with 0.1 M acetate buffer (pH 6.4) at room temperature (24 °C). Researchers used 1% catechol as a substrate for the oxidation. The reaction mixture contained 0.1 ml buffer, 0.1 ml 1% catechol and 0.025 ml of tested sample. Measurements were carried out on spectrophotometer SF-46 (LOMO, Russian Federation) at 420 nm after 30 sec, 5 min, 10, 15 and 20 min. One unit of activity was defined as the change in absorbance of 0.001 per min. The activity was measured thrice.

Protein concentration in the samples was determined by the Lowry assay [9] with absorbance measurement at 740 nm. Human serum albumin was used as the standard. The purity of the enzyme was determined by disc-electrophoresis in 7.5% polyacrylamide gel in alkaline buffer system (pH 8.6). To determine the localization of the enzyme in the gel column, the last one was cut into small pieces (0.5 cm), each was extracted with 0.1M acetate buffer (pH 6.0) and PPO activity was determined in these extracts. Determined activities were matched to protein bands identified on electrophoregram stained by Coomassie G250.

The molecular weight of the enzyme was determined by gel chromatography on Toyopearl HW-55 column (height – 39 cm, diameter 1.5 cm). Sample (0.8 ml) in 0.1M acetate buffer (pH 6.0) layered on top of the gel. The column void volume was determined by passing solution Blue Dextran (M_w 2000 kDa) through the column. Egg lysozyme (M_w 14.3 kDa), pea lectin (M_w 49 kDa), human serum albumin (M_w 69 kDa), *Helix pomatia* lectin (M_w 74 kDa) and *Laburnum anagyroides* bark lectin (M_w 102 kDa) were used as markers.

Substrate specificity was determined by applying several different substrates such as hydroquinone, resorcin, pyrogallol, catechol, tyrosine, phloroglucinol, and *o*-, *m*- and *p*-aminophenols. Measurements were carried out at 420 nm using 10 mM solutions in 0.1 M acetate buffer (pH 6.0, at room temperature).

The optimum temperature for the enzyme activity was determined by incubating the reaction mixture in the temperature range from 0°C to 70°C. The enzymatic reaction was stopped by adding of three volumes of ethanol to the reaction mixture, followed by measurement of the extinction at 420 nm. The reaction mixture was prepared with 0.2 M phosphate buffers (range of pH from 3.0 to 11.0).

Investigation of the inhibitory effect on PPO activity. The effects of four substances such as ascorbic acid, thiourea, sodium sulfite and citric acid on PPO activity were studied. These compounds, according to the literature, could be inhibitors of the enzyme activity. The substances were tested at concentrations of 1 mM and 10 mM with 0.1 M acetate buffer (pH 6.0). We used 1% catechol as the substrate. All enzyme purification steps were performed at room temperature. Fresh mushrooms (1.3 kg) were ground using household mincing machine,

after that approximately one third of them were placed in a blender and 800 ml of 1% NaCl solution with adding 1.5 g of ascorbic acid. The mixture was homogenized. Homogenate was squeezed through the mesh. The obtained extract was used for extraction of the next portion of mushrooms. After homogenization followed by squeezing extract of all amount of mushrooms, pH of the obtained extract was adjusted to pH 4.0-4.4 by glacial acetic acid. The formed precipitate was removed by centrifugation (2500 rpm, 10 min). Twenty percent sodium hydroxide solution was added to the supernatant to pH 8.2-8.6. The debris was removed by centrifugation (2500 rpm, 10 min). Proteins from the supernatant were precipitated with ammonium sulfate (600 g/l). The precipitate was centrifuged (6000 rpm, 15 min), then dissolved in water. The insoluble part was discarded. Then again proteins were precipitated with ammonium sulfate in the range of 30-85% of its saturation. The resulting precipitate was dissolved in a minimum amount of water and dialyzed against 0.02 M carbonate buffer (pH 8.4). After dialysis, the solution was centrifuged (6000 rpm, 10 min) and the supernatant (≈ 50 ml) was applied to DEAE-toyopearl (60×48 mm) column previously equilibrated with 0.02 M carbonate buffer (pH 8.4). After penetrating of the sample into gel, column was washed with the same buffer. A_{280} and PPO activity by reaction with catechol was determined in the eluate. When absorbance of the eluate became less than 0.1 ($A_{280} < 0.1$), the column was washed with 0.2 M carbonate buffer (pH 8.4). That allowed obtaining an additional amount of the PPO, which, however, was contaminated with the pigment. Fractions which were eluted separately from the column by 0.2 M and 0.5 M carbonate buffer then were combined and salted-out by ammonium sulfate (600 g/l). The obtained fractions were marked as N1 and N2. Their final purification was performed by ion-exchange chromatography on CM-cellulose (Whatman CM 52) column. The fractions were dialyzed against 0.02 M acetate buffer (pH 4.4). Fractions N1 and N2 (separately) were applied to the CM-cellulose column (60×30 mm) equilibrated with 0.02 M acetate buffer. After penetrating of the sample into adsorbent, the column was washed with the same buffer. A_{280} and PPO activity by reaction with catechol was determined in the eluate. The column was washed with 0.2 M acetate buffer (pH 4.4) and 0.5 M acetate buffer (pH 4.4).

Results and Discussion

PPO of *L. pergamenus* is mainly accumulated in their milky juice where, obviously, this enzyme plays an important physiological role. Six fungi samples at different stages of development were selected for the PPO "age dependent" activity assessment. We took into consideration such criteria as the diameter of the cap, the intensity of milky juice formation during damaging of the mushroom, and the level of its damage (Table 1).

Juice (a mixture of milky and cell juices) squeezed from fungi was purified by centrifugation, and then PPO activity was measured immediately as well as in 2 and 7 days keeping juice at room temperature. Sodium azide (0.5%) was added to the mixture to prevent juice fermentation. The obtained results are presented in Table 2.

It was observed that the concentration of protein in the fungus juice decreased with increasing

Table 1. Morphological characteristics of Lactarius pergamenus basidiocarps selected for determination of PPO activity

No	Cap diameter, mm	Intensity of appearance of milky juice	Damaging level (by worm or mollusks)	Mushroom weight, g	Amount of juice, ml
1	33	4	–	5.57	1.1
2	47	4	–	11.02	4.8
3	77	2	-	23.49	10.0
4	80	1	–	34.13	9.8
5	91	0	5%	33.43	10.6
6	145	0	20%	75.17	31.8

Notes: The intensity: 0 – absence of milky juice on basidiocarps damaged surface, 1 – milky juice appears on the damaged surface after 1-2 min, 3 – large drops of milky juice appear almost immediately on the damaged surface, 4 – milky juice drips from the damaged surface.

Table 2. PPO activity in the extracts from Lactarius pergamenus fungus at different stages of development

No	Average value of PPO activity (unit in ml/ mg of sample weight per min)				Protein concentrations (mg/ml)	Specific activity (U/mg protein)
	One day	Two days	Seven days	Ac		
1	0.934	1.335	1.208	1.159	6.4	0.181
2	1.956	1.837	1.340	1.711	5.1	0.335
3	1.431	1.963	1.074	1.489	4.8	0.310
4	0.987	1.502	0.805	1.098	4.3	0.255
5	0.823	1.401	0.671	0.965	3.9	0.247
6	0.877	1.204	0.537	0.872	3.7	0.236

Notes: No – Number of fungus from Table 1, Ac – average activity for 7 days.

of the size of mushrooms, whereas PPO activity was not the highest in the smallest fungi, but in slightly larger fungi (No 2). Activities of PPO from the larger size fungi (No 3-6) were decreasing. Similar alterations in the specific activity were observed. This indicates that the PPO appears in fungi juice with some delay.

According to published data, majority of PPO from plants and fungi were purified by precipitation with cold acetone [10-12] or ethanol [13]. Further purification was performed by the ion exchange and gel chromatography or their combination [10, 11, 13]. Affinity chromatography on immobilized (through 4-aminobenzoic acid) tyrosine-Sepharose is more effective method [12]. However, this sorbent remains inaccessible.

In our experiments, PPO was purified by extraction with 1% NaCl solution, precipitation of the protein fraction by ammonium sulfate and ion-exchange chromatography on DEAE-toyopearl and CM-cellulose columns. Preparative polyacrylamide (7.5% PAG) disc-electrophoresis with tris-HCl buffer system (pH 8.6) was the final step in the purification procedure. Ascorbic acid was added to the extract to 10 mM for reducing the browning reaction.

A significant reduction in the quantity of protein after precipitation by ammonium sulfate can be attributed to the large quantity of water insoluble precipitate. Obviously, this precipitate also has a protein nature.

The extract after ammonium sulfate precipitation and dialysis against carbonate buffer (0.02 M, pH 8.4) was loaded on DEAE-toyopearl column. The elution profile of PPO activity and the total protein content is shown in Fig. 1.

The main amount of PPO was eluted from the column with 0.2 M carbonate buffer pH 8.4. Additional enzyme was eluted with 0.5 M buffer (18% of the total enzyme activity, where 100% was set as the quantity of PPO eluted by 0.2 M buffer). This fraction was contaminated with the pigment, however main amount of the pigment was not dissolved in water during the ammonium sulfate precipitation. Thus, a sufficiently pure enzyme fraction was obtained.

PPO fraction eluted from the DEAE-toyopearl column with 0.2 M carbonate buffer was further separated by ion exchange chromatography on CM-cellulose with acetate buffer (Fig. 2).

Two fractions (1-1 and 1-2) were obtained, whilst, the specific activity of fractions 1-2 was ≈2.4 fold higher than the specific activity of fractions 1-1 (Table 3).

PPO fraction 2 obtained by chromatography on the DEAE-toyopearl column was further purified by chromatography on the CM-cellulose column. All the enzyme activity was eluted from the column in the concentration range 0.05-0.2 M acetate buffer (pH 4.4). PPO fractions eluted from CM-cellulose column with 0.02 and 0.2 M acetate buffer were combined and then salted out by the ammonium sulfate. The salt concentration was 600 g/l.

The efficiency of PPO purification is presented in Table 3.

It should be noted that the final yield of PPO in earlier works was very low. For example, the efficiency of PPO purification from ginseng roots by ion exchange chromatography was only 1% [10], from mango peel was 11% [15].

Purification of two fractions by chromatography on DEAE-toyopearl column and purification of two fractions on CM-cellulose column allow suggesting the existence of at least two PPO molecular forms in *L. pergamenus* fungi. To check this hy-

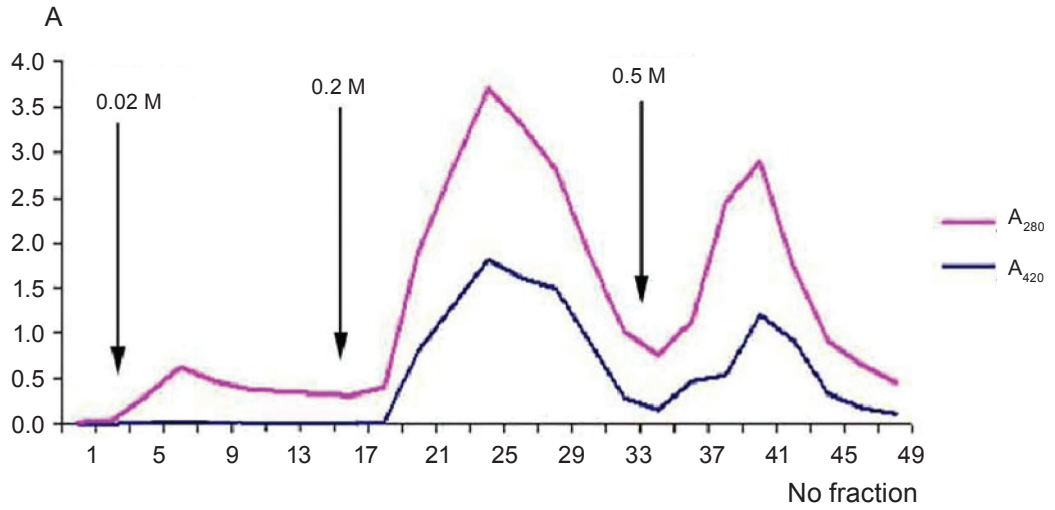

Fig. 1. Purification of PPO of Lactarius pergamenus by chromatography on the DEAE-toyopearl column. Arrowheads indicate the places of applying of 0.002, 0.2 and 0.5 M carbonate buffers (pH 8.4). Fractions volume was 18 ml. PPO activity was determined by reaction with catechol (A_{420}, blue line). Release of protein from column was controlled by measurement of absorbance at 280 nm (A_{280}, red line)

pothesis, disc electrophoresis in 7.5% PAG in alkaline buffer system was performed. Fig. 3 shows the localization of PPO (fraction 1-2) under these conditions of electrophoresis.

Thus, the obtained PPO fractions differ in their specific activity and electrophoretic mobility in alkaline buffer system. This is an evidence of existence of different enzyme isoforms.

Since the resulting PPO band during electrophoresis under the mentioned conditions is localized rather far from major ballast protein group, we concluded that the preparative disc electrophoresis would be effective for further PPO purification.

After preparative electrophoresis in alkaline buffer system (pH 8.6), the enzyme was eluted from PAG with 1% NaCl solution, centrifuged and filtrated. Dialysis against saturated ammonium sulfate solution was used for protein concentration. The specific activity of the sample obtained using this method was determined. The electrophoresis

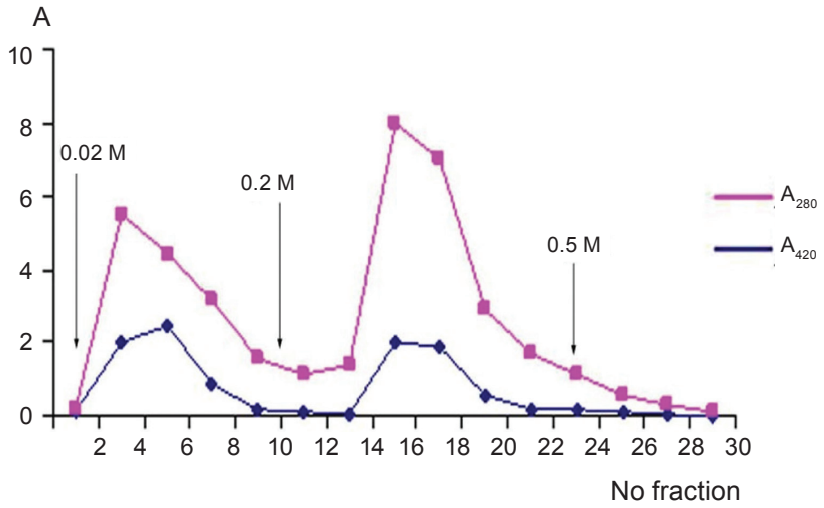

Fig. 2. Separation of PPO (fraction 1-1) of Lactarius pergamenus by chromatography on CM-cellulose. Arrowheads indicate the places of applying of 0.002, 0.2 and 0.5 M acetate buffers (pH 4.4). Fractions volume was 15 ml. PPO activity was determined by reaction with catechol (A_{420}, pink line). Protein release from column was controlled by measurement of absorbance at 280 nm (A_{280}, blue line)

T a b l e 3. Efficiency of PPO purification on different steps

Fraction	Volume, ml	Total protein, mg	Total activity, units	Specific activity, u/mg	Yield (%) by proteins	Yield (%) by activity	Purification degree
Crude extract	1,485	4,752	9,801	2.06	100	100	1
After correlation of pH	1,390	4,587	10,008	2.18	96.5	101.1	1.05
Precipitate after salting out	50	270	3,100	11.48	5.63	31.3	5.56
Purification using DEAE-toyopearl							
Fraction I	37	148	2,220	15.0	3.11	22.7	7.30
Fraction II	31	27.9	419	15.0	0.59	4.27	7.24
Purification using CM-cellulose							
Fraction 1-1	5.0	35.0	390	11.14	0.73	3.98	5.45
Fraction 1-2	4.0	14.0	368	26.28	0.29	3.75	12.94
Fraction 2	1.1	0.73	30.25	41.4	0.049	0.423	8.63
Purification by preparative disc electrophoresis							
Fraction 1-1	0.2	0.11	3.2	29.11	0.07	3.21	45.85
Fraction 1-2	2.5	0.5	70	140.0	0.03	3.32	110.66

in 15% PAG in the presence of 0.1% sodium dodecylsulfate was used for the determination of the molecular weight of the polypeptide chains. The results are presented in Fig. 4.

It is evident that both PPO isoforms (under denaturing conditions) migrated with the same rate during electrophoresis, which is an evidence of the equality of their molecular weight. The molecular weight of the polypeptide chains of both enzyme isoforms is 64 ± 1 kDa.

The determination of molecular weight of fraction 1-2 of PPO from *L. pergamenus* using chromatography on Toyopearl HW-55 column in the presence of proteins-markers with known molecular weight allow assuming that the molecular weight of fraction 1-2 is 64 ± 2 kDa (Fig. 5). This indicates that the enzyme consists of one polypeptide chain.

The effect of temperature on PPO activity is presented in Fig. 6. The highest activity of PPO from *L. pergamenus* was observed at 10 °C. As the temperature was increasing, the activity of PPO was decreasing and the enzyme was inactivated at 70 °C. The slight alteration in absorption could account for catechol oxidation by oxygen from the atmosphere.

The effect of pH on PPO activity is represented in Fig. 7. Optimum pH for activity of PPO from *L. pergamenus* was found to be 6.0 and the activity

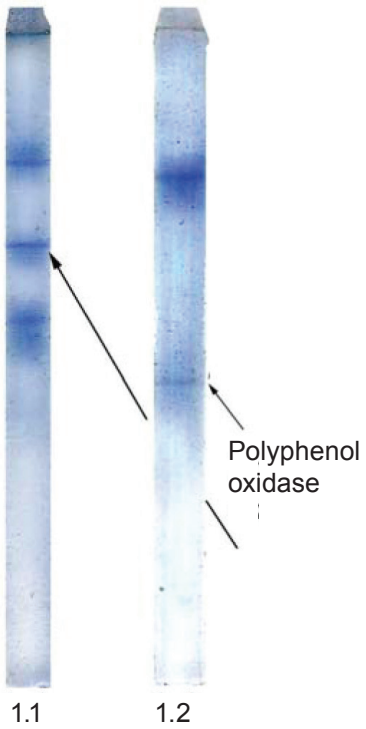

Fig. 3. Determination of PPO Lactarius pergamenus localization by disc electrophoresis in 7.5% PAG in alkaline buffer system: 1.1 – fraction 1-1, 1.2 – fraction 1-2 (after enzyme purification by ion exchange chromatography

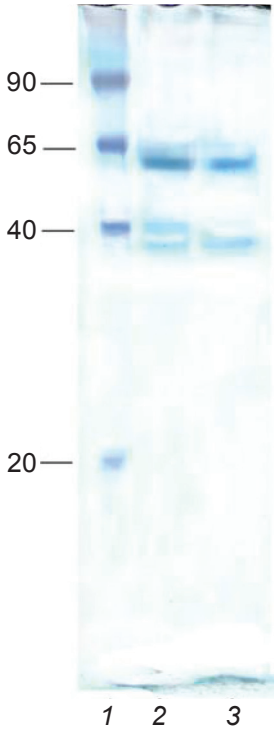

Fig. 4. Electrophoresis in 15% PAAG in the presence of sodium dodecyl sulfate of PPO Lactarius pergamenus fractions 1-1 and 1-2: 1 – protein-markers with indicated molecular weights; 2 – fraction 1-1; 3 – fraction 1-2

did not alter significantly (from 100 to 87%) when the pH dropped from 6.0 to 4.0.

Experiments with short-run acidation and alkalization of the medium containing PPO from *L. pergamenus* exhibit that the enzyme was stable at acidation to pH 1.8 and alkalization to pH 13.4 without a significant reduction in catalytic activity. The stability of PPO in an acidic medium is evident from the fact that after electrophoresis in PAG and staining of enzyme with Coomassie G-250, gel color cannot be preserved in 7% acetate acid and after 2-3 days it disappears.

The relative activities of the various substrates for PPO are represented in Table 4.

The effects of some substances on the activity of PPO from *L. pergamenus* are shown in Table 5.

Substances such as ascorbic acid, sodium sulphite, thiourea and citric acid exhibit an inhibitory effect in the experiments using catechol as a substrate in acetate buffer (pH 6.0) medium, but the rate of inhibition by these substances varies.

Thus, ascorbic acid is the most potent inhibitor of PPO, which completely inhibited its activity at a

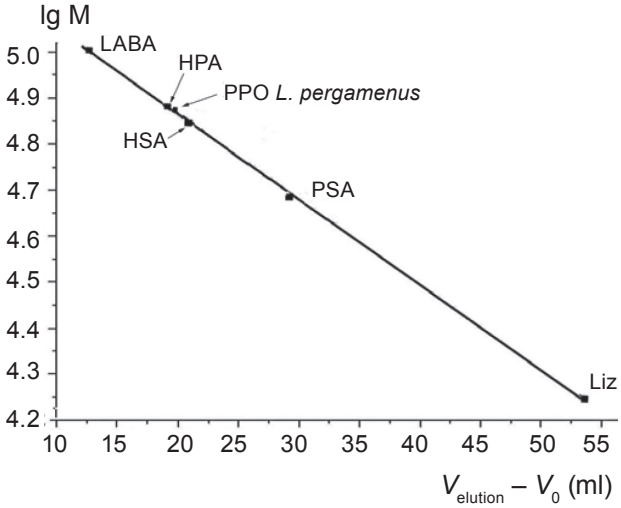

Fig. 5. Molecular weight determination of PPO Lactarius pergamenus fractions 1-1 and 1-2 by chromatography on the Toyopearl HW-55 column. Velution – volume of eluent in which respective protein was released from column: Liz – egg lysozyme, PSA – Pisum sativum lectin, HSA – human serum albumin, HPA – Helix pomatia lectin, LABA – a bark lectin from Laburnum anagyroides, PPO L. pergamenus

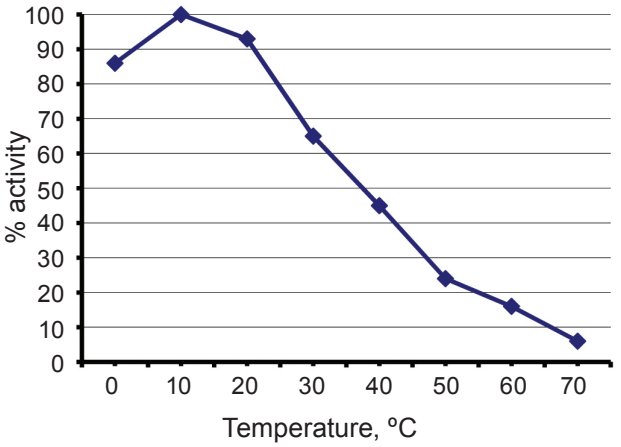

Fig. 6. Effect of temperature on PPO L. pergamenus activity

concentration of 1.0 mM, while thiourea and sodium sulfite at the same concentration inhibited PPO activity only partially. Citric acid exhibited a weak inhibitory effect at concentration of 10.0 mM and slight increase the enzyme activity was observed at a concentration of 1.0 mM.

Thus, the highest PPO activity was observed in basidiocarps of *L. pergamenus* fungi with small

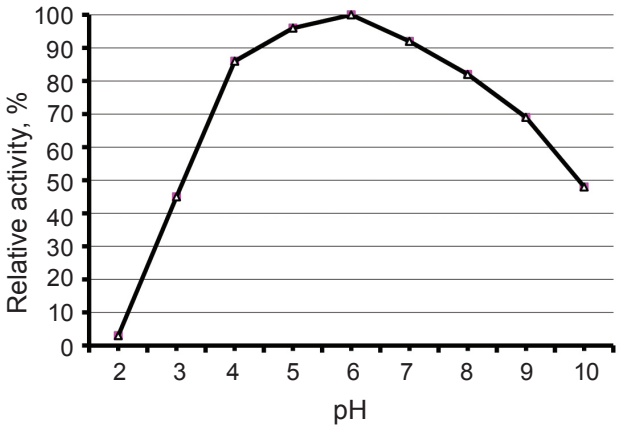

Fig. 7. Effect of pH on PPO L. pergamenus activity

Table 4. Substrate specificity of PPO Lactarius pergamenus

Substrate (10 mM)	Relative activity of enzyme (%)
Catechol*	100
o-aminophenol	129
m-aminophenol	69.3
p-aminophenol	50.6
Tyrosine	16
Resorcinol	1.3
Phloroglucinol	1.3

*Hydroquinone and pyrogallol relative activities in comparison with catechol were of zero level.

size of the mushroom cap and with high intensity of milky juice production. The enzyme activity was decreasing with increasing diameter of the mushroom cap and with reduction of milky juice content.

PPO purification was performed by using ion exchange chromatography on the DEAE-toyopearl and CM-cellulose columns. Three fractions of the enzyme with the specific activities that correlated as 11:26:41 were obtained. PPO isoforms content decreased in accordance to the increase of their specific activity.

The molecular weight of purified PPO determined by gel chromatography on Toyopearl HW-55 column is 64 ±2 kDa. Electrophoresis in 15% PAAG in the presence of SDS showed that the molecule of the enzyme consists of a single polypeptide chain with the same molecular weight (64 ±2 kDa).

Table 5. Effect of inhibitors on the activity of PPO from Lactarius pergamenus

Inhibitor	Catalytic activity (%)
Ascorbic acid	
1 mM	0
10 mM	0
Thiourea	
1 mM	54.5
10 mM	0
Sodium sulphite	
1 mM	59.1
10 mM	0
Citric acid	
1 mM	113.7
10mM	90.9

The highest activity of PPO from *L. pergamenus* was observed at 10 °C. A rise in the temperature leads to a decrease in enzyme activity and the activity was lost at temperature above 70 °C owing to irreversible inactivation.

The activity of PPO from *L. pergamenus* has optimum pH 6.0. It was revealed that the enzyme activity slightly altered (from 100 to 87%) at lower pH from 6.0 to 4.0

Catechol, *o*-, *m*-, *p*-aminophenols and tyrosine can serve as the substrates for PPO from *L. pergamenus*. Relative activity of tyrosine was only 16%. Hydroquinone and pyrogallol activities in comparison with catechol were at zero level.

Ascorbic acid was the most potent inhibitor of PPO from *L. pergamenus*. It inhibited enzyme activity completely at a concentration of 1.0 mM. Thiourea and sodium sulfite used at the same concentrations (1.0 mM) inhibited enzyme activity only partly.

ОДЕРЖАННЯ ТА ВИВЧЕННЯ ВЛАСТИВОСТЕЙ ПОЛІФЕНОЛОКСИДАЗИ З БАЗИДІОМ ГРИБА *Lactarius pergamenus* Fr. (Fr.)

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Із свіжозібраних базидіом *Lactarius pergamenus* Fr. (Fr.) методами іонообмінної хроматографії з використанням DEAE-toyopearl, КМ-целюлози та препаративного електрофорезу в 7,5%-му поліакриламідному гелі (ПААГ) (pH 8,6) виявлено три, з яких очищено дві ізоформи поліфенолоксидази (ПФО) (1-1 та 1-2) з виходом за протеїном відповідно 0,42 та 0,15 мг/кг базидіом. За диск-електрофорезу в 7,5%-му ПААГ при pH 8,6 ізоформи відрізняються рухливістю. Питома активність ізоформи 1-2 у 4,8 раза вища за активність ізоформи 1-1. Молекулярна маса, визначена гель-хроматографією на Toyopearl HW-55 ізоформ 1-1 і 1-2 була однаковою і становила 64 ± 2 кДа. Електрофорез у 15%-му ПААГ у присутності додецилсульфату натрію і β-меркаптоетанолу виявив одну зону з Мм 64 ± 1 кДа, що свідчить про наявність одного поліпептидного ланцюга у молекулі ензиму. Найвищу активність ензим виявляв при pH 6,0 і 10 ºС, а при 70 ºС відбувається інактивація ензиму. Активність поліфенолоксидази є найвищою в молодих грибів і знижується з їх віком, позитивно корелюючи із вмістом в них молочного соку. Орто-амінофенол виявився найефективнішим серед усіх випробуваних субстратів за визначення активності ПФО (*о*-, *м*- і *п*-амінофеноли, пірокатехін, тирозин, резорцин, флороглюцин) і його відносна активність становила 129% від активності пірокатехіну. Найефективнішим інгібітором активності ПФО була аскорбінова кислота, яка повністю блокувала активність у концентрації 1 мМ, тоді як у присутності такої самої концентрації сульфіту натрію та тіосечовини активність ензиму знижувалась лише на 40–45%. ПФО в базидіомах гриба *L. pergamenus* переважно локалізована у молочному соці гриба, де її висока активність може бути пов'язана з участю у захисті базидіом від патогенів.

Ключові слова: *Lactarius pergamenus* Fr. (Fr.), поліфенолоксидаза, очистка, властивості.

ПОЛУЧЕНИЕ И СВОЙСТВА ПОЛИФЕНОЛОКСИДАЗЫ ИЗ БАЗИДИОМ ГРИБА *Lactarius pergamenus* Fr. (Fr.)

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Из свежесобранных базидиом *Lactarius pergamenus* Fr. (Fr.) с помощью методов ионообменной хроматографии с использованием DEAE-toyopearl, КМ-целюлозы и препаративного электрофореза в 7,5%-ом полиакриламидном геле (pH 8,6) выявлено три изоформы полифенолоксидазы (ПФО) (1-1 и 1-2), из которых две очищено с выходом по протеину соответственно 0,42 и 0,15 мг/кг базидиом. При диск-электрофорезе в 7,5%-ом ПААГ в щелочной буферной системе (pH 8,6) изоформы отличаются подвижностью. Удельная активность изоформы 1-2 в 4,8 раза выше активности изоформы 1-1. Молекулярная масса, установленная гель-хроматографией на Toyopearl HW-55 изоформ 1-1 и 1-2 была одинаковой и составляла 64 ± 2 кДа. Электрофорезом в 15%-ом ПААГ в присутствии додецилсульфата натрия и β-меркаптоэтанола обнаружена одна зона с Мм 64 ± 1 кДа, что свидетельствует о наличии одной полипептидной цепи в молекуле энзима. Наивысшую активность энзим проявлял при pH 6,0 и 10 ºС, а при 70 ºС инактивировал. Самая высокая активность полифенолоксидазы у молодых грибов с возрастом снижалась, положительно коррелируя с содержанием в них млечного сока. Орто-аминофенол оказался наиболее эффективным среди всех испытуемых

субстратов при определении активности ПФО (*о*-, *м*- и *п*-аминофенолы, пирокатехин, тирозин, резорцин, флороглюцин) и его относительная активность составляла 129% от активности пирокатехина. Эффективным ингибитором активности ПФО была аскорбиновая кислота, которая полностью блокировала активность в концентрации 1 мМ, тогда как при наличии такой же концентрации сульфита натрия и тиомочевины активность энзима снижалась лишь на 40–45%. Полифенолоксидаза в базидиомах гриба *L. pergamenus* преимущественно локализована в млечном соке гриба, где ее высокая активность может быть связана с участием в защите базидиом от патогенов.

Ключевые слова: *Lactarius pergamenus* Fr. (Fr.), полифенолоксидаза, очистка, свойства.

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