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Isolation of Peroxidase from *Inonotus radiatus* Fungus

Izolacja enzymu peroksydazy z grzyba *Inonotus radiatus*

Изоляция энзима пероксидазы из гриба *Inonotus radiatus*

INTRODUCTION

Investigations on fungal peroxidase carried out so far have mainly aimed to explain the physiological role of the enzyme. It is now recognized, on the grounds of the studies of Trojanowski and Leonowicz (4, 15, 16, 17, 18, 19, 20), Ishikawa et al. (2, 3), and of Lyr (5, 6, 7), that peroxidase and laccase co-operate in degradation processes of lignin molecule. The fungi grown under natural conditions i.e. on trees excrete oxidoreductases, namely laccase and peroxidase into the medium. A few years ago Ishikawa and his co-workers (2, 3) attempted to isolate peroxidase and laccase from *Fomes fomentarius* and *Collybia velutipes* fungi. However, they did not succeed in isolating peroxidase from laccase. Reporting upon the properties of peroxidase preparations being isolated Ishikawa (2) writes: "The purified enzyme preparations, isolated from the mycelia and filtrates of both fungi, exhibited an absorption peak in the region of 410—440 nm on reaction with dithionite, thereby indicating the presence of peroxidase. Furthermore, the preparations also exhibited a weak maximum at 405 nm, indicative of the presence of a heme-containing protein."

Taking the above into consideration an attempt was made to isolate peroxidase from *Inonotus radiatus* fungus which exretes peroxidase, as the only enzyme from oxidoreductase group, into the medium.

MATERIAL AND METHODS

The filtrate of 3-week mycelium culture of *Inonotus radiatus* (Sow. Ex. Fr. P. Karst H.M.J.P.C. No. 4335) was used for the isolation of peroxidase. The fungus mycelium was grown in the liquid medium which consisted in 3% malt extract. The medium was enriched with beech wood meal, which caused an intensive growth of the mycelium and an increased peroxidase biosynthesis (8). The medium was prepared in 500 ml Roux flasks containing 150 ml of basic medium and 5 g of wood meal. After 3 weeks of fungus incubation at 25°, the mycelium was filtered off and the filtrate was used as the starting material for the purification of peroxidase. Peroxidase from horseradish roots of Koch-Light firm was also used in the experiments.

Peroxidase activity was determined by two methods, that of Aurand et al. (9) and of Sequeira et al. (13). p-phenylenediamine was the hydrogen donor in the former method while guaiacol in the latter. Protein content was estimated by the spectrophotometric method (11) and by the procedure of Parnas-Wagner (11). The level of sugars bound with protein was assayed by the method of Wintzler (10).

RESULTS AND DISCUSSION

In the previous report (8) there were described the conditions of *Inonotus radiatus* growth in the liquid medium during which the amount of peroxidase synthetized by the fungus mycelium increased 30 times. In the present experiments the filtrate after 3-week mycelium incubation of *Inonotus raditaus* was used for isolation. The mycelium was discarded because, as compared with the filtrate, it contained only slightly larger amount of peroxidase but markedly larger amount of contaminating proteins, and because of the difficulties in its homogenization.

There were worked out four steps of the purification procedure of peroxidase from the filrate. The filtrate was lyophilized and then dissolved in a small quantity of water. The solution was brought to 100% ammonium sulfate saturation. After the dialysis of saturated peroxidase preparation the filtration was performed on various sephadexes. The best results of purification were obtained on G-100 sephadex. Column size, 2.5×45 cm (Fig. 1). 0.005 M phosphate buffer, $pH=7$ was used for elution. Under these conditions the 20-fold purification of peroxidase preparation was accomplished. Then, before being tranferred to a sephadex column, the peroxidase solution was precipitated by ammounium sulfate for the second time. During precipitation the proteins of lower molecular weight were removed from the peroxidase preparation. During filtration through the sephadex the elution pattern changed in relation to the profile presented in Fig. 1 (Fig. 2). After filtration the 30-fold purification of peroxidase was achieved. Active fractions collected from 20 columns were lyophilized and stored at a low temperature. The

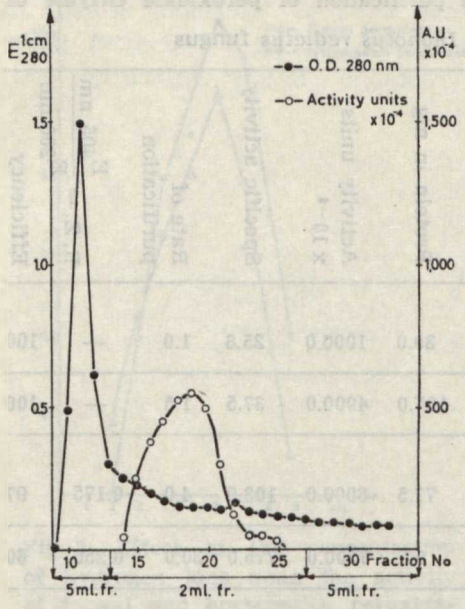

Fig. 1. Elution profile from G-100 sephadex chromatographic column. Column size, 2.5 × 45 cm. Elution system: 0.005 M phosphate buffer, $pH = 7$

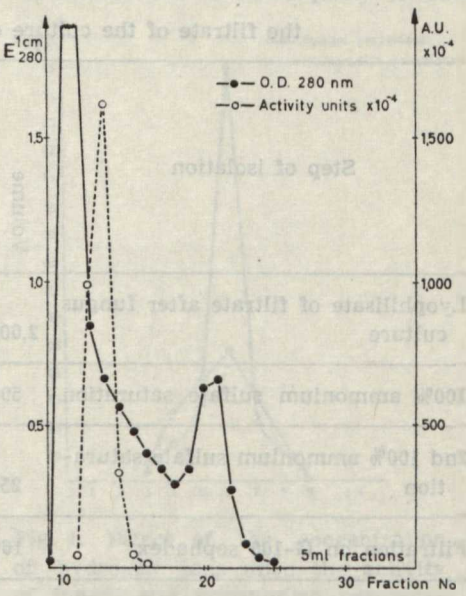

Fig. 2. Elution profile from G-100 sephadex chromatographic column. Column size, 2.5 × 45 cm. Elution system: 0.005 M phosphate buffer, $pH = 7$

results of described steps of the preliminary purification of fungal peroxidase were listed in Table 1.

In order to characterize closer the obtained preparation of fungal peroxidase some analytical procedures were performed. Electrophoresis of the purified preparation of fungal peroxidase was carried out on polyacrylamide gel. The electrophoregram exhibited one main peroxidase band which corresponded to protein band on the gel submitted to parallel electrophoresis (electrophoretic conditions: Tris-glycine buffer, $pH = 8.2$, time 3 hrs., 3 mA). The second peroxidase fraction was faintly visible on gel. Therefore, fungal peroxidase shows small heterogeneity in comparison, for instance, to horseradish peroxidase which, as S h a n n o n et al. (14) reported, possesses 7 isozymes.

Then, there were marked the changes in protein content and there was estimated the content of sugars bound with protein during the succeeding steps of peroxidase purification (Table 2).

The influence of inhibitors on fungal peroxidase is analogous to that on horseradish peroxidase (12), which may suggest the similarity in the structure of both enzymes (Table 3). Argentum nitrate as a spe-

Tab. 1. Comparison of the results of the purification of peroxidase enzyme of the filtrate of the culture of *Inonotus radiatus* fungus

Step of isolation	Volume	Protein in mg	Activity units x 10^{-4}	Specific activity	Rate of purification	R. Z. = $\frac{E_{405\ nm}}{E_{280\ nm}}$	Efficiency
Lyophilisate of filtrate after fungus culture	2,000	39.0	1000.0	25.8	1.0	—	100
100% ammonium sulfate saturation	500	107.0	4000.0	37.5	1.5	—	100
2nd 100% ammonium sulfate saturation	250	77.5	8000.0	103.0	4.0	0.175	97
Filtration on G-100 sephadex	160	9.7	7500.0	775.0	30.0	0.250	60

Tab. 2. Comparison of the results of protein assays according to Parnas-Wagner method (11) and of sugars bound with protein by the method of Wintzler (10) in the preparations of fungal peroxidase

Material	% protein per dry matter	mg% sugars bound with protein	% sugar per 100 mg protein
Medium	5	176	61
Preparation after ammonium sulfate treatment	90	70	5.1
Preparation after filtration on G-100 sephadex	92	0.88	1.78

cific inhibitor of flavine peroxidase was introduced into the experiments with inhibitors. According to Dollin's report (1) flavine peroxidase is inhibited in 100% at $AgNO_3$ concentration = 10^{-4} M. The results of the experiment with $AgNO_3$ presented in Table 3 exclude a possibility of the presence of this kind of peroxidase in the examined material.

While examining the effect of *pH* upon the activity of fungal peroxidase the comparison to horseradish peroxidase was made (Fig. 3, 4).

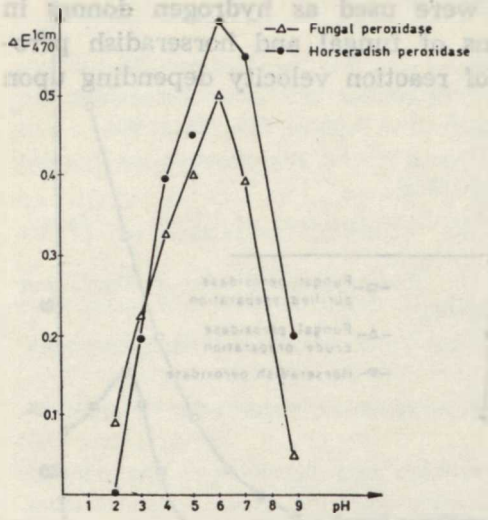

Fig. 3. Effect of the concentration of hydrogen ions upon the activity of fungal and horseradish peroxidases. Guaiacol as hydrogen donor

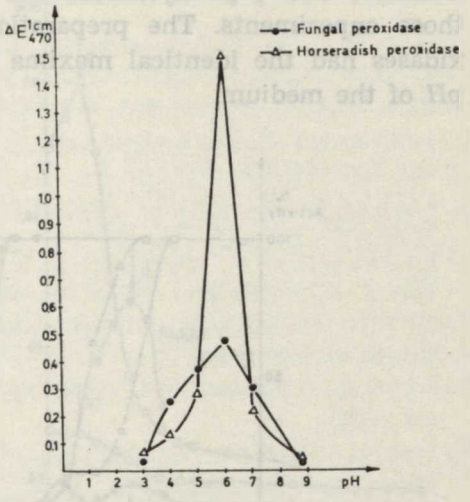

Fig. 4. Effect of the concentration of hydrogen ions upon the activity of fungal and horseradish peroxidases. p-phenylenediamine as hydrogen donor

Tab. 3. Influence of inhibitors on the activity of fungal peroxidase preparation

Inhibitor	Inhibitor concentration in moles	Remaining peroxidase activity in %	% peroxidase inhibition
Hydroxylamine	10^{-4}	25	75
Hydroxylamine	10^{-5}	75	25
Hydroxylamine	5×10^{-6}	96	4
Thiourea	10^{-5}	68	32
Thiourea	5×10^{-6}	95	5
Sodium Diethyl-dithio-carbamate	10^{-5}	0	100
Sodium Diethyl-dithio-carbamate	5×10^{-6}	3	97
Sodium Diethyl-dithio-carbamate	10^{-6}	30	70
2,4-dichlorophenol	10^{-5}	83	17
2,4-dichlorophenol	5×10^{-6}	100	0
Sodium versenate	10^{-5}	100	0
NaCN	10^{-5}	0	100
NaCN	5×10^{-6}	15	85
NaCN	10^{-6}	25	75
NaCN	5×10^{-7}	40	60
$AgNO_3$	10^{-4}	100	0

Guaiacol and p-phenylenediamine were used as hydrogen donors in those experiments. The preparations of fungal and horseradish peroxidases had the identical maxima of reaction velocity depending upon *pH* of the medium.

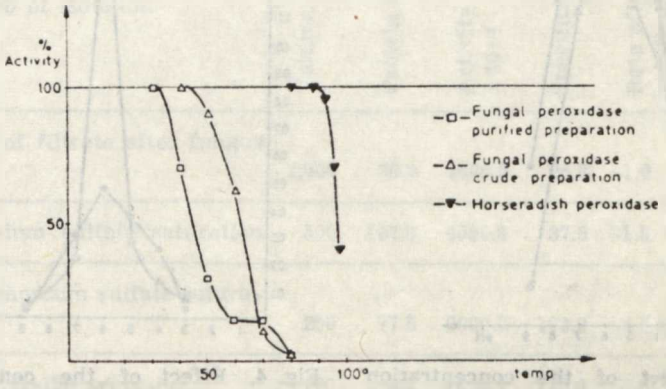

Fig. 5. Effect of temperature upon the activity of fungal and horseradish peroxidases. Time of thermal inactivity, 15 min

During the investigation of the influence of temperature on the activity of fungal peroxidase there were shown the differences in the lability of the enzyme as compared with horseradish peroxidase (Fig. 5).

Then, there were plotted the absorption curves of purified fungal peroxidase in the visible region, and the diagram thus obtained was compared with that of horseradish peroxidase (Fig. 6). The preparations of purified fungal peroxidase were yellow and that was why the absorption spectrum of the enzyme did not exhibit any maximum in the examined region.

On the basis of analogy between the action of horseradish peroxidase and that of fungal peroxidase it was expected that fungal peroxidase also consists of heme-containing protein which is characteristic of horseradish peroxidase. It was thought that the absorption maximum of fungal peroxidase in the Soret band is covered by unidentified yellow substances. An attempt was made to remove the yellow colouring from the preparation of fungal peroxidase. For this purpose the absorption of peroxidase preparation was performed on active charcoal, aluminium oxide and hydroxyloapatite. The relatively best results were obtained by the use of active charcoal. At the same time there was observed a marked decrease in the peroxidase content of such a purified preparation. But then the weak maximum was noted in the region of 400 nm, and after adding sodium dithionite there occurred the shift of maximum

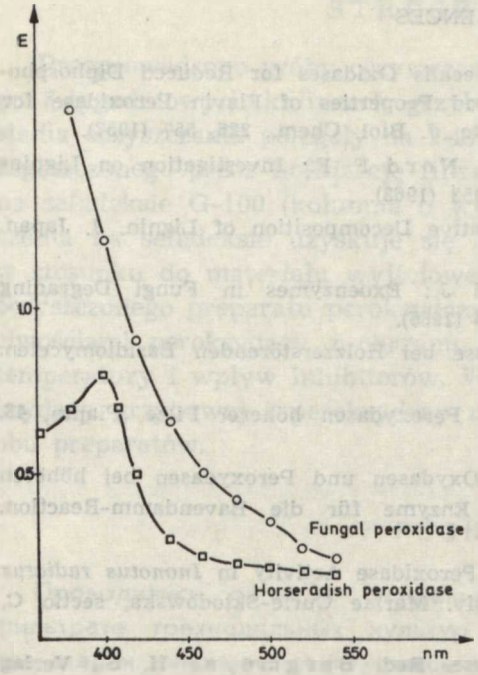

Fig. 6. Absorption curves of fungal and horseradish peroxidases in the visible region

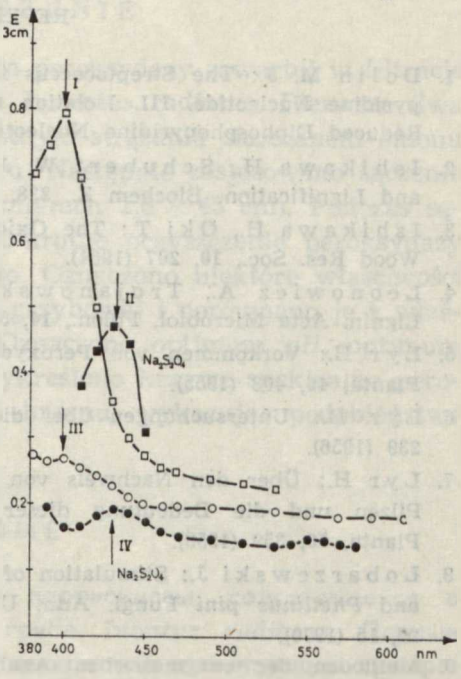

Fig. 7. Absorption curves of fungal and horseradish peroxidases in the visible region. The changes in the absorption spectra under the influence of sodium dithionite ($Na_2S_2O_4$). I — horseradish peroxidase, II — horseradish peroxidase after $Na_2S_2O_4$ reduction, III — fungal peroxidase, IV — fungal peroxidase after $Na_2S_2O_4$ reduction

to 430 nm (Fig. 7). Fungal peroxidase spectrum, as illustrated in Fig. 7, would be the evidence of hemoproteid structure of the enzyme.

The yellow colouring of purified preparation of fungal peroxidase may be attributed to the formation of enzyme-chinone complexes. The rise of such complexes seems to be indicated by the elution profile of fungal peroxidase on the sephadex, on which yellow substances together with peroxidase protein are eluted from the column.

The investigations on further steps of the purification of fungal peroxidase are continued.

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STRESZCZENIE

Przeprowadzono próby oczyszczania peroksydazy zawartej w filtracie po 3-tygodniowych kulturach grzyba *Inonotus radiatus*. Pierwsze dwa stadia oczyszczania polegały na 2-krotnym strącaniu siarczanem amonu zagęszczonego przez liofilizację filtratu. Następnie zastosowano sączenie na sefadeksie G-100 (kolumna o wymiarach 2,5 × 45 cm). Podczas sączenia na sefadeksie uzyskuje się 30-krotne oczyszczenie peroksydazy w stosunku do materiału wyjściowego. Oznaczono niektóre właściwości oczyszczonego preparatu peroksydazy grzybowej i porównano je z właściwościami peroksydazy z chrzanu. Oznaczono optimum *pH*, optimum temperatury i wpływ inhibitorów. Wykreślono krzywe spektralne peroksydazy grzybowej i peroksydazy z chrzanu, wykazując podobieństwo obu preparatów.

РЕЗЮМЕ

Проводились попытки очищения пероксидазы, содержащейся в фильтрате трехнедельных культур гриба *Inontus radiatus*. Первые две стадии очищения заключались в двукратной преципитации сульфатом аммония лиофилизованного фильтрата. Затем проводилась фильтрация на колонке Sephadex G 100 (размеры колонки 2,5 × 45 см). Этой фильтрацией достигается 30-кратное очищение пероксидазы по отношению к начальному материалу. Определили некоторые свойства очищенного препарата грибной пероксидазы и сравнили их со свойствами пероксидазы из хрена. Определили оптимум *pH*, оптимум температуры и влияние ингибиторов. Вычерчены спектральные кривые грибной пероксидазы и пероксидазы из хрена, которые показывают сходство обоих препаратов.

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