Chemistry of Natural Compounds, Vol. 43, No. 6, 2007

ISOLATION AND PURIFICATION OF LIGNOPEROXIDASE FROM THE MUSHROOM *Pleurotus ostreatus*

Sh. Ya. Mirzaakhmedov,[1] Zh. F. Ziyavitdinov,[1] Z. R. Akhmedova,[2] A. B. Saliev,[1] D. T. Ruzmetova,[1] S. T. Azizov,[3] Dimitrios Fessas,[4] and Stefania Iametti[4]

UDC 577.15+663.15+577.112

The accumulation dynamics of lignolytic enzymes in culture media of the basidiomycetes Panus tigrinus, Pleurotus ostreatus, Fomes fomentarus, *and the micromycete* Aspergillus terreus *were studied during the incubation period. It was found that* Pleurotus ostreatus *is the most active producer of lignoperoxidase enzymes among the studied fungi. Gel filtration and ion-exchange chromatography were used to isolate a homogeneous enzyme with lignoperoxidase activity. The maximum activity was found at pH 2.7 and 29°C. Gel electrophoresis determined the molecular weight (44 kDa).*

Key words: ligninase, *Pleurotus ostreatus*, lignoperoxidase activity, chromatography, electrophoresis.

Higher basidial fungi possess a powerful enzymatic system. In addition to hydrolases, they also form a whole series of lignolytic enzymes belonging to the ligninase subclass, a typical representative of which is peroxide-dependent peroxidase. Despite the discovery of new phenol-oxidizing enzymes, the potential capabilities of fungi of the genus *Pleurotus*, which are active destroyers of lignocellulose substrates, are little studied [1-3]. It was shown [4] that *P. ostreatus* can produce peroxidase on various agricultural plant wastes in Uzbekistan depending on the growth stage and cultivation time and the type of lignocellulose plant wastes included in the nutrient medium as the only carbon source. However, the quantitative content of peroxidase in the culture medium, its isolation and purification, and its properties have not been studied.

Therefore, we present results from a study of the capabilities of *P. ostreatus* to produce ligninoperoxidase.

We made an initial choice from three species of macroscopic fungi, *Panus tigrinus*, *P. ostreatus*, and *Fomes fomentarus*, in addition to the micromycete *Aspergillus terreus*, as producers on wood wastes.

The growth dynamics and kinetic curves for accumulation of peroxidase in culture liquids of the fungi were observed for 1-24 days of cultivation, taking samples daily. It was found that *P. ostreatus* was the most capable biosynthesizer of ligninoperoxidase on the test medium. It began to secrete peroxidase into the culture medium on the third day of cultivation. The growth maximum occurred on the 17-18th days.

The cultures of basidiomycete fungi can be placed in the following order of ability to form peroxidase in medium with wood chips: *P. ostreatus* > *P. tigrinus* > *F. fomentarus* > *A. terreus*. Therefore, we used culture medium of *P. ostreatus* grown for 18 d (at 28-30°C) for further isolation of peroxidase. The yield of culture liquid was 170 mL out of a starting volume of 200 mL. The peroxidase activity of the culture liquid was determined by the literature method [5]. The peroxidase activity in the culture liquid was 233.0 units/170 mL. The amount of protein was determined by the Lowry method and was 54.06 mg/170 mL whereas the specific activity of the enzyme in the culture liquid was 4.31 units/mg protein. Table 1 gives results from the determination of protein and specific activity of the enzyme.

1) Institute of Bioorganic Chemistry, Uzbekistan, Tashkent, fax (99871) 262 70 63, e-mail: ibchem@uzsci.Net; Mirzaakhmedov@mail.ru; 2) Institute of Microbiology, Uzbekistan, 700128, Tashkent, ul. A. Kadyri, 7b; 3) Institute of Electronics, Uzbekistan, 100125, Tashkent, ul. F. Khodzhaeva, 33; 4) University of Milan, DISTAM, via Celoria 2, 20133, Milano, Italy. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 564-566, November-December, 2007. Original article submitted March 29, 2007.

0009-3130/07/4306-0682 ©2007 Springer Science+Business Media, Inc.

TABLE 1. Purification Stages of Extracellular Lignoperoxidase from *P. ostreatus*

Purification stage	Total activity, units/mL	Total protein, mg	Specific activity, units/mg protein	Degree of purification	Reduction activity, %
Culture liquid	233.0/170	54.06	4.31	-	100
$(NH_4)_2SO_4$ precipitation (40%)	114.2/17.5	18.42	6.2	1.44	49
Desalting (Sephadex G-10)	102.8/36	10.72	9.59	2.23	44.1
Gel filtration (TSK HW 55)	89.5/63	5.69	15.73	3.65	38.4
Ion-exchange chromatography (DEAE-TSK HW 650S)	64.8/40	2.4	27.0	6.26	27.8

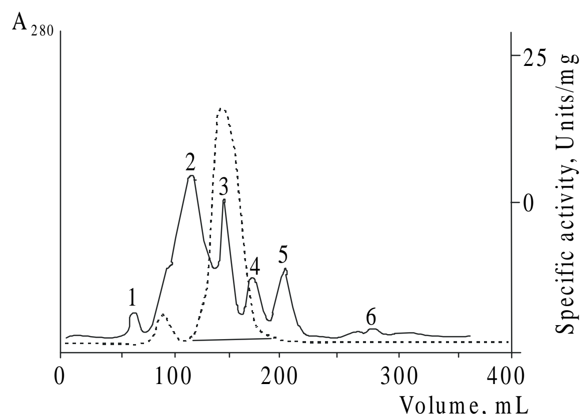

Fig. 1.

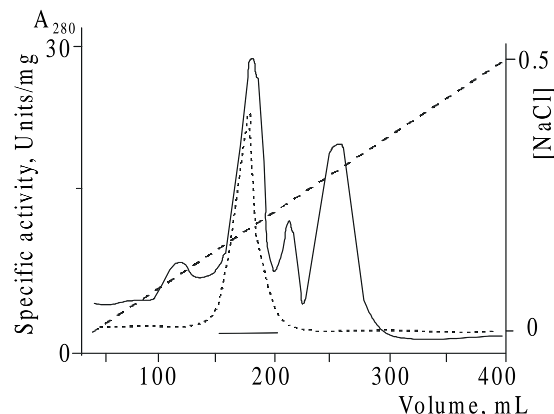

Fig. 2.

Fig. 1. Gel filtration of lignoperoxidase fraction after desalting over TSK HW 55 column (dashed lines denote active fractions).
Fig. 2. Ion-exchange chromatography of combined lignoperoxidase fraction after gel filtration over DEAE-TSK HW 650S column (active fraction denoted by dashed lines).

In the first enzyme purification stage, culture liquid was fractionated by ammonium sulfate at concentration saturations of 20, 40, and 60% (mass/vol). Then, the resulting fractions were desalted by dialysis against distilled water. The protein fraction obtained with 40% saturation of ammonium sulfate had the highest lignoperoxidase activity. The total volume of the active fraction was 17.5 mL; the total peroxidase activity, 114.2 units/17.5 mL; the specific activity, 6.5 units/mg; the amount of total protein, 18.42 mg (1.05 mg/mL). Then, this fraction was desalted over a column of Sephadex G-10 and fractions containing peroxidase activity were combined (36 mL) and lyophilized. Table 1 gives the results from a determination of the peroxidase activity of the desalted fraction after lyophilization and the amount of protein.

Next this fraction was dissolved in sodium acetate (5 mL, 0.01 M) at pH 4.9 and placed on a column packed with TSK HW 55f gel that was equilibrated beforehand. The column was eluted by this same buffer at a flow rate of 30 mL/h. Fractions of 2.5 mL were collected. Figure 1 shows the elution profile of the proteins.

The separation produced six protein fractions, of which fractions 2, 3, and 4 had lignoperoxidase activity. These fractions were combined. The total enzymatic activity was 89.5 units/63 mL whereas the protein concentration was 5.69 mg; the specific activity, 15.73 units/mg protein. This fraction was lyophilized.

In the next purification step, the lyophilized active fraction obtained in the previous step was dissolved in sodium acetate (10 mL, 0.01 M, pH 4.9) and separated by ion-exchange chromatography over a column of DEAE-TSK HW-650S that was previously equilibrated with this same buffer. Figure 2 shows the elution profile of the proteins.

Elution of proteins using a linear gradient of NaCl (0-0.5 M) produced four fractions. It was found that lignoperoxidase was eluted from the column in the second fraction. The total activity was 64.8 units/40 mL; the protein content, 2.4 mg; the specific activity, 27 units/mg protein. Table 1 gives the separation steps and the purification results.

The molecular weight of the lignoperoxidase from *P. ostreatus* was determined by the Laemmli method as 44 kDa.

Thus, the use of classical methods to purify proteins produced a sample of lignoperoxidase with specific activity 27.0 units/mg and a degree of purification of 6.26 times.

EXPERIMENTAL

Sorbents G-10, TSK HW-55f, and DEAE-TSK HW-650S (Pharmacia, Sweden, and Toyo Soda Company, Japan) were used. PAAG was prepared as before [6]. Fungi *P. tigrinus*, *P. ostreatus*, *F. fomentarus*, and *A. terreus* were taken from the culture collection of the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan. The other inorganic salts were purchased from Sigma Chemical Company.

Cultivation Conditions. A seed culture of fungi was maintained on slanted agar gel. Analytical samples were taken every 12 h. Fungi were cultivated in medium containing plant wastes such as pulp from cotton wastes and birch chips of various concentration (2-5%) and particle size with added 7% gel (5%). Cultivation was carried out in Ehrlenmeyer flasks (750 mL) with nutrient medium (200 mL) on rockers rotating at 240 rpm at 30°C for 3-21 d. Peroxidase was separated from culture liquid of *P. ostreatus* grown for 18 d (at 28-30°C). Fungal biomass was separated from culture liquid first by centrifugation at 8000 rpm for 30 min and then by filtering the supernatant through a nitrocellulose filter with pore diameter 0.4 μm.

Enzyme activity was determined by the Gudkova method [5].

Protein Precipitation. Proteins were isolated from culture liquid filtrate by sulfate precipitation at 40% saturation.

Desalting. Proteins were placed on a column of Sephadex G-10 (2.6 × 50 cm) and eluted with distilled water at a flow rate of 30 mL/h. Optical density of the eluted proteins was measured at 280 nm. Then, active fractions were combined and lyophilized.

Gel Filtration. Lyophilized enzyme (10.72 mg) was dissolved in sodium acetate (5 mL, 0.01 M, pH 4.9) and placed on a column (1.6 × 125 cm) of TSK HW-55f previously equilibrated with this same buffer. Proteins were eluted from the column at flow rate 30 mL/h with detection at 280 nm.

Ion-exchange Chromatography. The active fraction (5.69 mg) from the previous step was placed on a column of DEAE-TSK HW-650S (1.6 × 15 cm) that was equilibrated with sodium acetate (0.01 M, pH 4.9). Proteins were eluted using a linear gradient of NaCl (0-0.5 M) at flow rate 60 mL/h with detection at 280 nm.

Gel Electrophoresis. Electrophoresis was carried out by the Laemmli method [6].

Protein Determination. Protein was determined by the method of Lowry and Rosenbrough [7].

ACKNOWLEDGMENT

We thank INTAS foundation, project No. 04-82-7253, for financial support.

REFERENCES

1. R. L. Crawford and D. L. Crawford, *Enzyme Microb. Technol.*, **6**, 434 (1984).
2. H. S. Becker and A. P. Synytsin, *Biotech. Lett.*, **15**, 3, 289 (1993).
3. I. Hatakka, *FEMS Microbial Rev.*, **13**, 2-3, 125 (1994).
4. Z. R. Akhmedova, *Khim. Prir. Soedin.*, 739 (1995).
5. L. V. Gudkova, *Enzymes in Medicine, Food Industry, and Agriculture* [in Russian], Naukova Dumka, Kiev (1968), 172-174.
6. K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
7. O. H. Lowry and N. J. Rosenbrough, *J. Biol. Chem.*, **193**, 265 (1951).