

Anti-oxidative stress enzymes in *Pleurotus ostreatus*

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Pleurotus ostreatus is an edible white-rot basidiomycete with medicinal and bioremediation properties. In this research, the activity of key intracellular anti-oxidative stress enzymes, superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX), was investigated and characterized in the mushroom. A *Pleurotus ostreatus* homogenate was centrifuged at 3,000 g for 10 min; the supernatant was centrifuged at 35,000 g for 30 min, and the new supernatant obtained, called "crude extract" was used for our studies. Results showed that up to 7.4 units SOD, 11.7 units CAT and 0.037 units POX were detectable per mg protein in *Pleurotus ostreatus* extract. All three enzymes were sensitive to KCN. Non-denaturing polyacrylamide gel electrophoresis of the extract, followed by activity staining, revealed one SOD band (estimated M.W. 44 kD), one CAT band (estimated M.W. 280 kD) and three POX bands (estimated M.W. 77, 65, and 60 kD, respectively).

Keywords catalase; peroxidase; superoxide dismutase; intracellular anti-oxidative stress enzymes; *Pleurotus ostreatus*

1. Introduction

Reactive oxygen species (ROS) such as H₂O₂, superoxide radical and hydroxyl radical, are normal by-products of cellular respiration and metabolism and, at low concentrations, are useful for cellular physiological functions [1]. However, their overproduction is very detrimental to cell survival. Recent advances in ROS-induced diseases demonstrated that the genetically controlled overproduction of antioxidants prevented the damages produced by drugs that generate ROS [2]. It was also suggested that the consumption of food and produce containing antioxidant products and antioxidant enzymes such as superoxide dismutase (SOD) and catalase, would be very useful in preventing health damage by ROS. The most important enzymes responsible for fighting ROS excess are SODs, catalases and peroxidases.

The oyster mushroom *Pleurotus ostreatus* is an edible white-rot basidiomycete much appreciated for its flavor as a food and for its medicinal and bioremediation properties. The medicinal benefits provided by the mushroom include hypocholesterolemic [3] and antitumor effects [4, 5] as well as improvement of the antioxidant status during ageing [6]. The bioremediation activity of the mushroom includes lignin degradation, polycyclic aromatic hydrocarbons degradation, nitrocellulose degradation and polychlorinated biphenyl (PCB) degradation [7-9]; *Pleurotus ostreatus* has also been shown to release an enzyme with aflatoxin-degradation activity [10]. These bioremediation properties result from the action of antioxidant enzymes secreted by the mushroom in the environment. Although the secreted enzymes have been extensively investigated, much less research has been conducted on the intracellular antioxidant enzymes in *Pleurotus ostreatus*, in spite of the documented beneficial effect of the mushroom on antioxidant status during ageing.

In this research, we identified and characterized the activity of key intracellular anti-oxidative stress enzymes, namely superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX), in *Pleurotus ostreatus*.

2. Materials and Methods

Locally purchased *Pleurotus ostreatus* was homogenized in phosphate buffer 0.1 M, pH 7.0, then centrifuged at 3,000 g for 10 min; the pellet was discarded and the supernatant was centrifuged at 35,000 g for 30 min. The supernatant thus obtained was called "crude extract" and used for our studies.

SOD activity was measured by two different spectrophotometric methods, one based on the inhibition of pyrogallol autooxidation in alkaline solution, and the other based on the inhibition of cytochrome *c* reduction, both methods being described in reference [11]. CAT activity was assayed spectrophotometrically by following H₂O₂ dismutation at 240 nm as described in reference [12]. POX activity was measured by following spectrophotometrically the H₂O₂-mediated oxidation of o-dianisidine at 460 nm as described in reference [13], or that of 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) at 414 nm as described in reference [14].

Non-denaturing gel electrophoresis of the extract was followed by activity staining for each of the enzymes tested. Electrophoresis was conducted in 10% polyacrylamide gels, either at 100 V for 5 h, or at 80 V for 20 h. Staining for SOD activity was described in reference [15]. Staining for CAT activity was performed according to reference [12], except that o-dianisidine was used as the reducing substrate; CAT was revealed as achromatic bands on a brown background. Staining for POX activity was done by incubation, for 30 min, in 50 mM citrate buffer, pH 4.5, followed by a second incubation, for 15 min, in citrate buffer 50 mM, pH 4.5, 16 mM H₂O₂, then rinsing in deionized water and incubation in 50 mM citrate buffer, pH 4.5, containing 1.9 mM o-dianisidine; POX was revealed as orange-brown bands.

3. Results

3.1 SOD activity

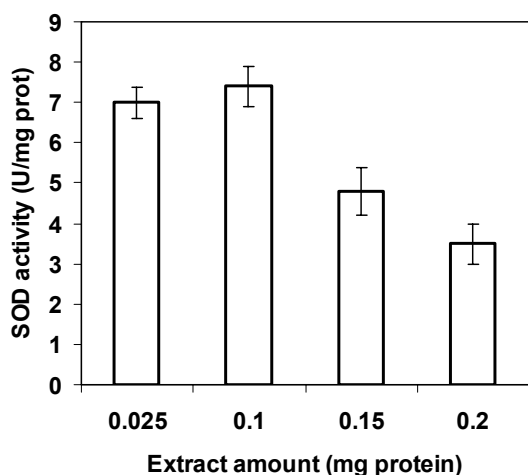


Fig. 1 SOD activity measured in various amounts of *P. ostreatus* extract. The assay was based on the inhibition of cytochrome *c* reduction. Up to 7.4 U SOD were detectable per mg of extract proteins.

In the assay based on the inhibition of cytochrome *c* reduction, SOD competes with cytochrome *c* for the flux of superoxide radical generated by the xanthine-xanthine oxidase reaction. One unit SOD was defined as that amount which caused 50% inhibition of the initial rate of cytochrome reduction. As shown in Figure 1, up to 7.4 ± 0.5 units SOD per mg extract proteins were detectable by this assay.

In the assay based on the inhibition of pyrogallol autooxidation in alkaline solution, SOD scavenges the superoxide radical that acts as a chain-propagating species in the autooxidation. One unit SOD was defined as that amount which caused 50% inhibition of the autooxidation reaction. Up to 10.4 ± 0.8 units SOD per mg extract proteins were detectable by this method, confirming the above results.

SOD activity was undetectable in the presence of 50 mM KCN.

A single band was detectable after electrophoresis of *P. ostreatus* extract proteins in polyacrylamide gel, under non-denaturing conditions, followed by activity staining for SOD, indicating the presence of at least one SOD isoenzyme, with an estimated molecular weight of 44 kD, in the extract (Fig. 2).

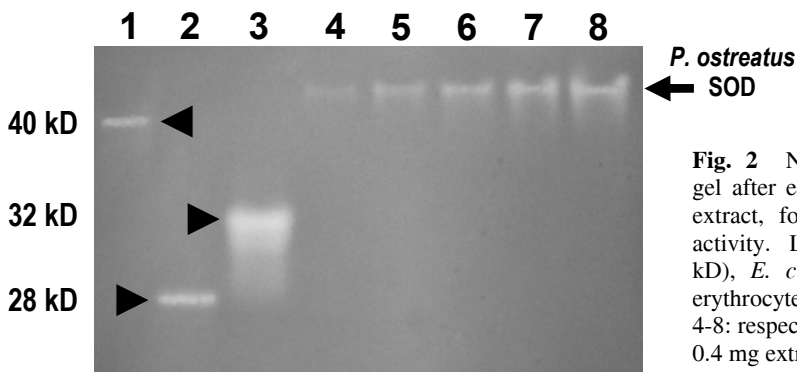


Fig. 2 Non-denaturing polyacrylamide gel after electrophoresis of *P. ostreatus* extract, followed by staining for SOD activity. Lane 1-3: *E. coli* Mn-SOD (40 kD), *E. coli* Fe-SOD (28 kD), human erythrocytes Cu/Zn-SOD (32 kD); lanes 4-8: respectively, 0.05, 0.1, 0.15, 0.25 and 0.4 mg extract proteins.

3.2 CAT activity

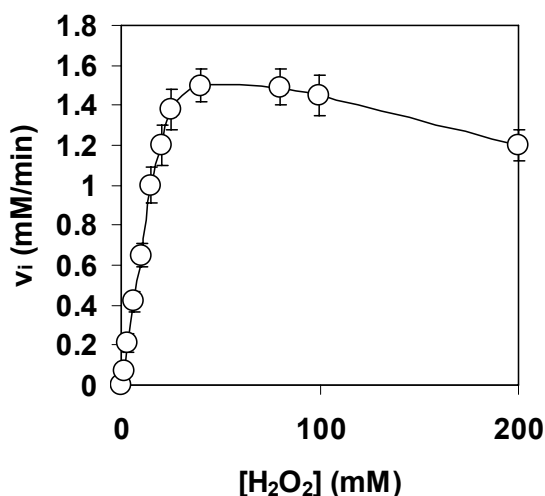


Fig. 3 Effect of H₂O₂ concentration on CAT activity in *P. ostreatus* extract. The hyperbolic plot is characteristic of Michaelis-Menten kinetics. Substrate inhibition was observed for H₂O₂ concentrations above 80 mM.

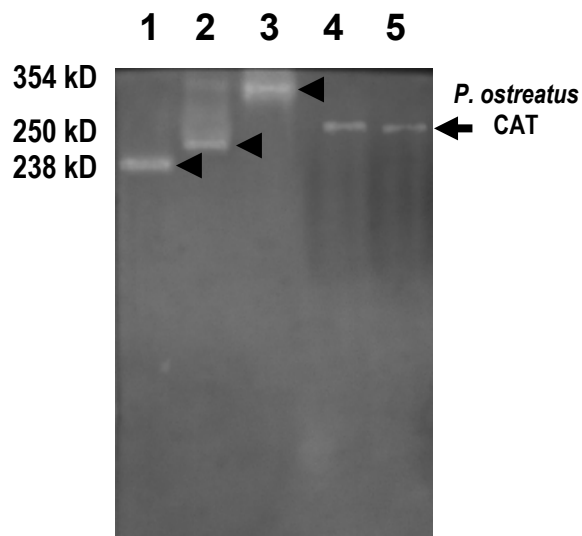


Fig. 4 Non-denaturing polyacrylamide gel after electrophoresis of *P. ostreatus* extract, followed by staining for CAT activity. Lanes 1-3: Guinea pig liver CAT (238 kD), Beef liver CAT (250 kD), *A. niger* CAT (354 kD); lanes 4-5: 0.4 and 0.3 mg extract proteins.

When *P. ostreatus* extract was added to a reaction mixture containing phosphate buffer 0.1 M, pH 7.0 and H₂O₂, the absorbance at 240 nm of the mixture would decrease steadily, indicating H₂O₂ dismutation and the presence of catalase activity in the extract. The enzymatic activity increased as a function of increasing substrate concentration, reaching a maximum value at 40 mM H₂O₂ (Fig. 3). Substrate inhibition was observed for H₂O₂ concentrations above 80 mM (Fig. 3). The maximum activity detected corresponded to 11.7 ± 0.7 units CAT per mg extract proteins (one unit CAT decomposes 1 μ mol H₂O₂/min). The enzymatic activity was inhibited by KCN with an IC₅₀ of 4 mM.

When the extract was electrophoresed in non-denaturing polyacrylamide gel that was subsequently stained for CAT activity, one band was detectable (Fig. 4), indicating the presence of at least one CAT isoenzyme, with an estimated molecular weight of 280 kD, in the extract.

3.3 POX activity

P. ostreatus extract catalyzed the H₂O₂-mediated oxidation of o-dianisidine and ABTS, two of the substrates used by peroxidases. Typical Michaelis-Menten plots were obtained for the variation in reaction velocity as a function of either o-dianisidine (Fig. 5a) or ABTS (Fig. 5b) concentration. The apparent K_m and V_{max} values deduced from the plots were, respectively, 0.06 ± 0.004 mM and 9.4 ± 0.4 μ M/min for o-dianisidine as the varied substrate, and 0.6 ± 0.03 mM and 2 ± 0.05 μ M/min for ABTS as the varied substrate.

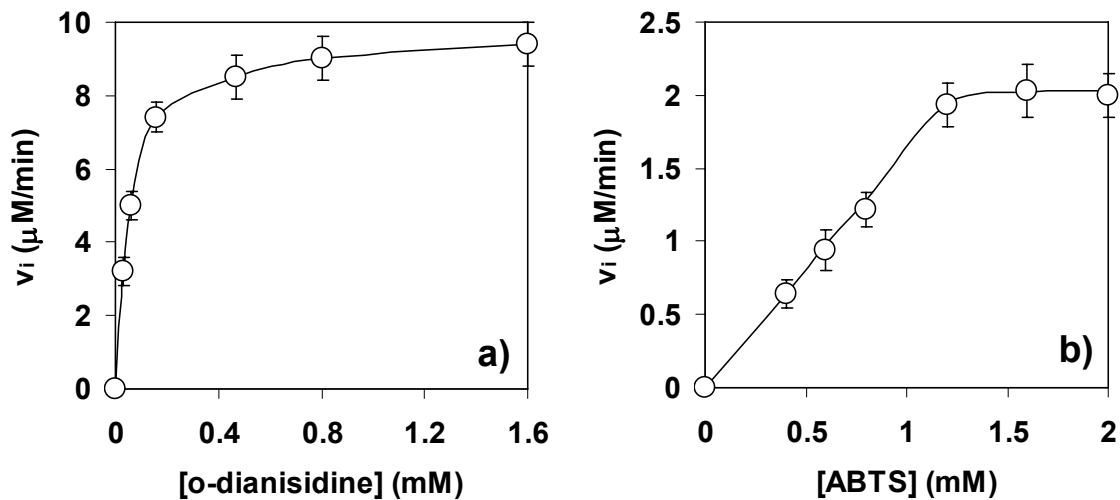


Fig. 5 Effect of o-dianisidine (a) and ABTS (b) concentration on POX activity in *P. ostreatus* extract. The hyperbolic plots are characteristic of Michaelis-Menten kinetics.

The maximum activity detected corresponded to 0.037 ± 0.002 units POX per mg extract proteins when o-dianisidine was used as the reducing substrate, and to 0.032 ± 0.003 units POX per mg extract proteins when ABTS was used as the reducing substrate (one unit POX oxidizes 1 μmol substrate/min).

P. ostreatus peroxidase activity was inhibited by KCN with IC_{50} of 0.1 mM when o-dianisidine was the reducing substrate and 4 mM when ABTS was the reducing substrate.

Electrophoresis of *P. ostreatus* extract in non-denaturing polyacrylamide gel followed by staining for POX activity revealed three bands with estimated molecular weight of 77 kD, 65 kD and 60 kD, respectively (Fig. 6).

4. Discussion

The medicinal properties of edible mushrooms are now under intense investigation. Among the edible mushrooms, *P. ostreatus* has been recognized not only for its beneficial effects on health, but also for its bioremediation properties that have been extensively studied. Various *P. ostreatus* extracts have been found to have distinct medicinal properties [3-6] and even DNA isolated from *P. ostreatus* has been reported to have biotherapeutic potential in mice with solid Ehrlich carcinoma [16]. Although antioxidant properties of the mushroom have been reported, and a number of antioxidant enzymes secreted by the mushroom have been extensively studied, little is known on the intracellular antioxidant enzymes in *P. ostreatus*.

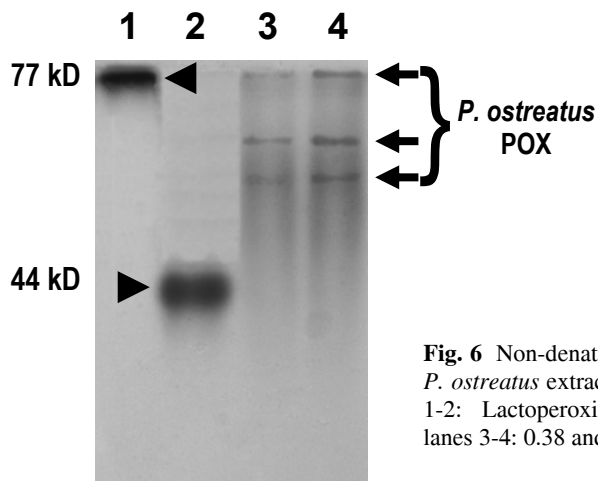


Fig. 6 Non-denaturing polyacrylamide gel after electrophoresis of *P. ostreatus* extract, followed by staining for POX activity. Lanes 1-2: Lactoperoxidase (77 kD), Horseradish peroxidase (44 kD); lanes 3-4: 0.38 and 0.5 mg extract proteins.

In this work, SOD, CAT and POX activities were detected in *P. ostreatus* extract. At least one isoenzyme of SOD with an estimated molecular weight of 44 kD was present; its sensitivity to KCN indicated that it was a Cu/Zn SOD [15]. The SOD activity of the extract, expressed as U/mg protein, was comparable to that found in

extract obtained from dormant saffron corm [11], but roughly 5 times less than that found in *Satureja hortensis* roots [15]. At least one CAT isoenzyme of estimated molecular weight 280 kD was identified; the activity was about half that found in dormant saffron corm extract [12]. As far as POX is concerned, *P. ostreatus* exhibited an activity that was 10 times less than that found in dormant saffron corm using o-dianisidine as the reducing substrate but was about twice that found in dormant saffron corm using ABTS as the reducing substrate [13, 14]. Due to their ability to use a broad range of substrates, numerous peroxidase isoenzymes have been found, particularly in plants. As it appears in this work, at least three POX isoenzymes with estimated molecular weights of 77 kD, 65 kD and 60 kD, respectively, were detectable in *P. ostreatus* extract.

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