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Enhanced extracellular laccase activity as a part of the response system of white rot fungi: *Trametes versicolor* and *Abortiporus biennis* to paraquat-caused oxidative stress conditions

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Abstract

Effects of paraquat dichloride (PQ) on the laccase (LAC) activity and some biochemical parameters of *Trametes versicolor* and *Abortiporus biennis* strains belonging to white rot *Basidiomycetes* fungi were examined. PQ water solution was added to 10-day-old stationary cultures cultivated on a liquid medium. Having measured the activity of extracellular laccase during the first 120 h, we found that the addition of 25 μM paraquat to *T. versicolor* and 20 μM paraquat to *A. biennis* cultures significantly stimulated the LAC activity in comparison to the control value (without PQ). Native PAGE gel analysis demonstrated that no new isoforms of laccase appeared in the presence of PQ stress. The increase of LAC activity was connected with dry weight loss. Enhanced activity of extracellular superoxide dismutase was observed during the first 48 h after PQ application in both investigated strains. The PQ-treatment also caused an evident increase of catalase activity, formaldehyde level and depletion of glutathione in *T. versicolor* as well as in *A. biennis* mycelia.

Keywords: Laccase; White rot *Basidiomycetes*; Oxidative stress response; Paraquat

1. Introduction

Laccases (LAC; benzendiol:oxygen oxidoreductases; EC 1.10.3.2.) are glycoproteins belonging to the blue multicopper oxidases which are widely distributed in all forms of life. These enzymes are able to oxidize both organic and inorganic substrates with concomitant reduction of oxygen to water [1,2]. The wood degrading fungi are the best-known laccase producers in nature. Fungal laccase can degrade lignin in the absence of other ligninolytic enzymes like lignin peroxidase and manganese peroxidase. The high oxidative ability of laccase draws a lot of attention and can be potentially used in industry, including biodegradation of environmental pollutants, pulping and bleaching processes in the pulp and paper industry as well as decolorization of synthetic dyes or herbicide degradation [3]. Besides their action in degradation processes, laccases in fungi are believed to be involved in melanin synthesis or fungal morphogenesis. It seems that apart from that function LAC may also play an important role in general fungal stress response to changes of temperature or cadmium treatment [4,5]. Although the oxidative stress defense mechanisms have been very often described in the recent literature, very few works estimate the possibilities of adaptive stress response in the white rot *Basidiomycetes*. Fungi like other living organisms are sensitive to various types of stress factors including temperature, nutrient starvation, exposure to heavy metal ions, the availability of water or treatment with xenobiotics [4,6].

Paraquat (1,1′-dimethyl-4,4′bipyridinium dichloride hydrate, methyl viologen dichloride) is an effective contact herbicide widely used for a non-selective control of weeds in many countries. PQ is an ionic redox-cycling substance which catalyzes the overproduction of superoxide anion radicals (SORs) and consequently other oxygen active species

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generated through Fenton-like reactions [7]. The organic contaminants resulting from the decay of paraquat can be adsorbed onto soil, especially on clay, which is the main component of the mineral fraction of soils. As a consequence the bioaccumulation of PQ increases significantly. Therefore in recent years the concentration of herbicides and their residues in water sources, food and soil have risen and may cause multiple toxicological and environmental problems [8–10]. Because of its prooxidative character, paraquat is commonly used in laboratories as an oxidative stress conditioning factor.

It is a well-known fact that organisms have developed an efficient antioxidative protection system which consists of enzymatic and non-enzymatic elements [11]. In response to various types of stressors there appears a general stress response which is activated by some intracellular signals such as abnormal or denaturated proteins, enhanced production of reactive oxygen species (ROS) or others [5]. White rot *Basidiomycetes* are known as the most efficient lignin degraders using oxidative processes involving ROS, especially the extremely reactive hydroxyl radicals (HO•) [12]. The decomposition reactions have been catalyzed by special enzymes, including the above described laccase [13].

The main goal of our study was to observe the defensive biochemical behavior of two selected strains of white rot fungi under PQ-mediated oxidative stress conditions. The study has compared a well-known laccase producer-*Trametes versicolor* [14] with *Abortiporus biennis*, which has a low level of constitutive laccase activity. Our first aim was to determine the changes of extracellular LAC activity in both PQ-treated fungal strains. The activity of extracellular manganese peroxidase (MnP; EC 1.11.1.13)—another important ligninolytic enzyme was also measured in the investigated strains. Because the highest levels of LAC as well as MnP were usually detected during the secondary metabolism phase (idiophase) [15], 10-day-old (idiophasic) cultures were used for experiments included in the present report. The next step of our investigations was to estimate whether the presence of herbicide really causes overproduction of SOR (superoxide stress) in stressed fungal cultures. Apart from the experiments mentioned above, changes in the level of glutathione (GSH), phenolic compounds as well as the activity of catalase (CAT; EC 1.11.1.6) and superoxide dismutase (SOD; EC 1.15.1.1) as well-known antioxidants were also determined in both strains. In the present report, we also analyzed the concentration of formaldehyde (FA) in the mycelia as well as in the culture fluid. It appears that FA is one of the molecules considered in initial phases of biotic and abiotic stress responses, often described as a marker. The measurable level of demethylation and transmethylation reaction products dramatically increases in the case of disease stress [16,17].

2. Materials and methods

2.1. Strain, media, and growth conditions

Trametes versicolor (L. ex Fr.) Pil and *A. biennis* (Bull. ex Fr.) Sing, were obtained from the culture collection of the Department of Biochemistry, M. Curie-Skłodowska University in Lublin, Poland. Fungal cultures were maintained on 2% (w/v) malt agar slants. For inoculation, fungal agar plugs (approximately 0.5 cm^2) were cut and put into the basal medium, prepared according to Fahreus and Reinhammar [18] (for *T. versicolor*) and Lindeberg [19] (for *A. biennis*). Cultures were cultivated in static glass flasks at 25 °C, until the mycelium occupied the whole surface of the liquid. The fungal mycelium was collected and homogenized in a Warning Blender. After inoculation with 2.5% (v/v) of homogenizing material, the stationary cultivated, experimental cultures were incubated in 25 ml Erlenmayer flasks with 10 ml of mediums described above at 25 °C for 10 days.

2.2. Oxidative stress conditions

The 10-day-old idiophasic cultures were treated with different concentrations of a water solution of paraquat (the range from 5 to 50 μM). The final concentration of PQ was established experimentally for each fungus separately basing on the extracellular laccase activity, the main lignin-modifying enzyme. The following values were obtained: for *T. versicolor* 25 μM, for *A. biennis* 20 μM as final concentration. Stock solution was prepared just before the experiments and sterilized by ultrafiltration. Portions of herbicide were added directly to the culture medium. Time of the beginning of idiophase was determined according to Jennings [20].

2.3. Preparation of fungal material

All measurements were recorded periodically every 120 h after chemical treatment. At the time of collection, mycelia were separated from the culture fluid, washed out with distilled water and homogenized in phosphate buffer (pH 7.4) by a glass, motor-driven Potter's homogenizer at 4 °C. After centrifugation (15 min, 10,000*g*) the crude extract of mycelia was divided into two portions. The first portion was kept at 4 °C and used for enzymatic measurements (less than 2 h). The other part was frozen and kept for non-enzymatic determinations. Extracellular fluid of paraquat-treated as well as control fungal cultures was filtered and used for assays. Dry weight of mycelia was assayed after 24 h incubation at 80 °C.

2.4. Enzymes activity assay

2.4.1. Determination of laccase activity

Laccase (LAC) activity was determined basing on the oxidation of syringaldazine (4-hydroxy, 3,5-dimethoxybenzaldehyde) [21]. The reaction mixture contained 0.1 ml of the enzyme sample, 0.5 ml of 0.1 M citrate-phosphate buffer (pH 4.8 for *T. versicolor* and pH 5.4 for *A. biennis*) and 0.35 ml of distilled water. The reaction was started by the addition of 0.05 ml of 0.5 mM syringaldazine solution at 25 °C. The changes of absorbance were recorded at 525 nm. The specific activity of laccase was calculated with the excitation coefficient for product of 65,000 $M^{-1} cm^{-1}$ and expressed in nanokatals per milligram of protein.