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Secretion profiles of fungi as potential tools for metal ecotoxicity assessment: A study of enzymatic system in *Trametes versicolor*

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ABSTRACT

The relationship between the expression of extracellular enzymatic system and a metal stress is scarce in fungi, hence limiting the possible use of secretion profiles as tools for metal ecotoxicity assessment. In the present study, we investigated the effect of Zn, Cu, Pb and Cd, tested alone or in equimolar cocktail, on the secretion profiles at enzymatic and protein levels in *Trametes versicolor*. For that purpose, extracellular hydrolases (acid phosphatase, β-glucosidase, β-galactosidase and N-acetyl-β-glucosaminidase) and ligninolytic oxidases (laccase, Mn-peroxidase) were monitored in liquid cultures. Fungal secretome was analyzed by electrophoresis and laccase secretion was characterized by western-blot and mass spectrometry analyses. Our results showed that all hydrolase activities were inhibited by the metals tested alone or in cocktail, whereas oxidase activities were specifically stimulated by Cu, Cd and metal cocktail. At protein level, metal exposure modified the electrophoretic profiles of fungal secretome and affected the diversity of secreted proteins. Two laccase isoenzymes, LacA and LacB, identified by mass spectrometry were differentially glycosylated according to the metal exposure. The amount of secreted LacA and LacB was strongly correlated with the stimulation of laccase activity by Cu, Cd and metal cocktail. These modifications of extracellular enzymatic system suggest that fungal oxidases could be used as biomarkers of metal exposure.

1. Introduction

Metal contamination of the biosphere due to human activities has become a major environmental and health problem. The release of metals into the terrestrial ecosystems has led to their important accumulation in biota and often alters biological systems (Akmal et al., 2005; Boularbah et al., 2006). These non-biodegradable contaminants travel up the trophic chains and enter bioaccumulation processes (van Gestel, 2008). Thus, essential metals in excess as well as metals with no biological role can result in adverse effects for living organisms, and consequently on the terrestrial ecosystem functioning. However, the metal assessment of metal effects on biota remains a research subject because of the lack of efficient tools. Before performing studies taking into account the complex properties of soil, it is first necessary to characterize biological responses to a metal stress at the organism level in order to assess their relevance as tools for metal ecotoxicity assessment.

Since fungi represent one of the largest biomass in terrestrial ecosystems, the response of their extracellular enzymatic systems to metals offers promising perspectives for ecotoxicological assessment, providing the mechanisms of metal action are well known. By their ability to adapt their metabolism to various nutrient sources, saprophytic fungi are key colonizers of ecological niches and play a major role in the terrestrial ecosystem functioning (Kjøller and Struwe, 2002; Bouws et al., 2008). These microorganisms produce extracellular enzymes involved in the nutrient mobilization and mineralization of biopolymers. Proteomic studies of the totality of secreted proteins, the secretome, have recently begun to emerge in fungi (Kim et al., 2007). Although a great number of extracellular proteins still remains to be identified, various types of hydrolases and oxidases were characterized. These approaches allowed to show the secretion profiles are modified by a saline stress (Gori et al., 2007; Liang et al., 2007). However, saprophytic fungi considered as the predominant degraders of lignin, have been rather studied for their set of ligninolytic oxidases, such as laccases or peroxidases, for biotechnological, industrial and environmental

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applications (Mougin et al., 2003; Novotný et al., 2009). In spite of these investigations, the characterization of differential expressions of extracellular enzymes under a metal stress is still limited in fungi.

Although fungi have mechanisms for metal tolerance, the activity of their extracellular enzymes can be modulated during metal exposure (Baldrian, 2003). Metals, such as Cu, Zn, Mn or Pb, modify the activity of cellulolytic hydrolases and ligninolytic oxidases in *Pleurotus ostreatus* (Baldrian et al., 2006). Cu is known to increase the extracellular laccase in different filamentous fungi (Crowe and Olsson, 2001; Baldrian, 2003; Baldrian et al., 2006). This oxidase response to metals has been explained at transcriptional level. Collins and Dobson (1997) showed that the stimulation of laccase activity by Cu corresponds to an increase in its mRNA in *Trametes versicolor*. It has also been observed that laccase can be induced as different glycosylated isoforms after exposure to 2,5-xylidine, a well known inducer of fungal laccases (Bertrand et al., 2002a; Kollmann et al., 2005). To our knowledge, such post-translational modifications have not been observed after metal exposures. Furthermore, fungi secrete isoenzymes of oxidases, i.e. enzymes catalyzing the same reactions but exhibiting different sequences in amino acids (Cassland and Jönsson, 1999; Necochea et al., 2005). Thus, differential expressions of enzymes or isoenzymes under a metal stress could provide fungal biomarkers of exposure. By an inoculation strategy, it is feasible to use fungi and their enzymatic system in bioassay studies. For example, hydrolytic and ligninolytic enzyme activities were monitored after inoculation of fungi in soils contaminated by Pb (Kähkönen et al., 2008).

In the present study, we investigated the effect of metals on secretion profiles in an efficient lignin-degrading fungus, *T. versicolor*, at enzymatic and protein levels. The fungus was exposed to essential metals, Zn or Cu, and non-essential metals, Pb or Cd in liquid cultures. Exposures with metal cocktails were performed in order to reflect multiple contaminations, which is rarely realized in the literature. At enzymatic level, we monitored the activities of two main enzyme families involved in the terrestrial ecosystem functioning, hydrolases (acid phosphatase, β-glucosidase, β-galactosidase and N-acetyl-β-glucosaminidase) and ligninolytic oxidases (laccase, Mn-peroxidase). At protein level, a comparative analysis in electrophoresis of total extracellular proteins was carried out to provide an overview of secretion profiles. The laccase secretion was characterized by western-blot and mass spectrometry analyses.

2. Material and methods

2.1. Culture conditions and metal exposures

Trametes versicolor ATCC 32745 was grown on a liquid culture medium, containing maltose and ammonium tartarate as carbon and nitrogen sources (Lesage-Meessen et al., 1996). A mycelium mat on agar plugs (10 mm diam.) was inoculated into 10 mL of culture medium in 150 mL Erlenmeyer flasks. Cultures were carried out statically in the dark at 25 °C. After 3 d of incubation, 100 μL of $ZnSO_4$, $CuSO_4$, $CdSO_4$ or $PbCl_2$ sterilized by filtration (0.2 μm pore size membrane) were added into liquid cultures at final concentrations of 0.25 or 1 mM. Experiments with cocktails of four metals in equimolarity were realized at final concentrations of 0.25, 1 and 4 mM. Controls were done without added metal. The experiments were carried out with three independent replicates per treatment.

After 5 d of metal exposure, mycelia were harvested by a nylon screen (40 μm) and dried for 48 h at 80 °C to determine the fungal biomass. The mycelium free culture was filtrated through a 0.2 μm-filter and used for the extracellular enzymatic assays, the quantification of secreted proteins by the Bradford method using bovine serum albumin as a standard (Bradford, 1976) and the protein preparation for electrophoresis.

2.2. Enzymatic assays

Acid phosphatase (EC 3.1.3.2), β-glucosidase (EC 3.2.1.21), β-galactosidase (EC 3.2.1.23), and N-acetyl-β-glucosaminidase (EC 3.2.1.30) activities were assayed using their respective substrates of conjugated p-nitrophenol (Sigma–Aldrich). Assays were carried out in 96-well microplates by mixing 160 μL of substrate solution (25 mM) in citrate/phosphate buffer or CPB (pH 4.5, 0.1 M) with 40 μL of enzymatic samples, followed by incubation at 37 °C. After 45 min, 50 μL of Na_2CO_3 (1 M) was added to stop the reaction. The liberation of p-nitrophenol by enzymatic hydrolysis of the substrate was determined at 405 nm (Keshri and Magan, 2000). The standard solutions of p-nitrophenol were treated in the same way as samples.

Laccase (EC 1.10.3.2) and Mn-peroxidase (EC 1.11.1.14) activities were measured respectively by monitoring the oxidation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic) acid at 420 nm in CPB (0.1 M, pH 3.0) (Wolfenden and Willson, 1982) and the oxidation of 4-(4-hydroxy-3-methoxyphenyl)-3-buten-2-one at 334 nm in CPB (0.1 M, pH 5.0) in the presence of $MnSO_4$ and H_2O_2 (Paszczynski et al., 1986). Solutions of enzymes were added to a final volume of 1 mL.

Activities were measured in triplicate and expressed in $U\,g^{-1}$ dry weight of fungal biomass. One unit of activity was defined as the amount of enzyme that catalyzed 1 μmol substrate in 1 min. Controls performed from boiled enzymatic extracts to inactive the activities were treated in the same way as the samples.

2.3. Extracellular protein preparation for electrophoresis

The extracellular filtrates were concentrated 100-fold by an Amicon 10 kDa filter at 4 °C and resuspended in 10 mL of Tris–HCl buffer (pH 6.8, 50 mM) twice to remove salts. For protein precipitation, cold acetone with 0.07% β-mercaptoethanol (−20 °C) were added in concentrated protein solutions (3:1 v/v). After mixing and incubation of 30 min at −20 °C, the samples were centrifuged at 14 000*g* for 10 min. The pellets were washed with cold acetone and then air-dried. The dry protein pellets were dissolved in 200 μL of a solution containing 2% SDS, 8 M urea and 50 mM Tris–HCl (pH 6.8) and incubated 24 h at 4 °C. The proteins were separated by SDS–PAGE (poly-acrylamide gel electrophoresis) using 4% stacking gel and 12.5% separating gel at 110 V with Mini-protean electrophoresis cell (Biorad) and proteins were stained using the Coomassie Blue standard method (Biorad). Glycosylated isoforms of laccases induced by exposure of *T. versicolor* to 2,5-xylidine were purified as described by Bertrand et al. (2002b) and used as controls for the laccase characterization. The molecular weights were estimated by comparison with a commercial protein mixture (Precision plus protein standards, Biorad). Electrophoresis gels were done from three independent sets.

2.4. Protein characterization by western-blot and LC–MS/MS

The proteins were electro-transferred (0.8 mA per cm^2 of gel) from the electrophoresis gel to a nitrocellulose membrane using a Trans-blot cell (Biorad). Detection of laccase on the membrane was performed with rabbit anti-bodies in combination with goat anti-rabbit Ig conjugated with alkaline phosphatase (Jolivalt et al., 2005). The intensity of revealed spots was quantified by imager (ImageQuant, Amersham) and related to the dry weight of fungal biomass. Western blots were done in triplicate.

Bands of interest were excised from SDS–PAGE. In-gel digestion was performed with the Progest system (Genomic Solution)