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The secretome of *Trametes versicolor* grown on tomato juice medium and purification of the secreted oxidoreductases including a versatile peroxidase

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ABSTRACT

The present work was carried out with the aim to analyze the secretome of *Trametes versicolor* BAFC 2234 grown on tomato juice medium supplemented with copper and manganese. *T. versicolor* BAFC 2234 was selected among diverse wood dwelling agaricomycetes from Argentina by its ability to cause a strong white rot on hardwood and in addition to show high tolerance toward phenolic compounds. A considerable number of the identified proteins were related to the degradation/modification of lignocelluloses. Hydrolases, peroxidases and phenoloxidases were the most abundant enzymes produced under the above-mentioned culture conditions. The lignin-modifying oxidoreductases laccase, manganese peroxidase (MnP) and versatile peroxidase (VP) were successfully purified – the latter for the first time from *T. versicolor*. The native VP protein has a molecular mass of 45 kDa and an isoelectric point of pH 3.7. The study clearly shows that complex plant-based media being rich in phenolics, such as tomato juice, can stimulate the secretion of a broad set of extracellular lignocellulolytic enzymes. Using such natural products as fungal culture media may give the opportunity to investigate plant biomass decomposition as well as the biodegradation of organic pollutants in an environment close to nature.

1. Introduction

White-rot fungi are unique in their ability to degrade all polymeric components of wood and other lignocellulosic substrates. Interest in this group of fungi and their lignocellulolytic enzyme system has increasingly spurred by their biotechnological potential, among others by the degradation of numerous environmental pollutants (Gao et al., 2010). Proteomic analysis is a powerful, modern tool that can provide a systematic understanding of events at the molecular level and clarify complex and divergent physiological mechanisms involved in degradation processes. It is defined as the global assessment of cellular proteins expressed in a particular biological state (Kim et al., 2007); therefore culture conditions might influence protein expression. The analysis of the extracellular proteome can offer new clues either for the general understanding of fungal degradation and/or for potential industrial applications of the secreted proteins (Barreiro et al., 2011).

Phenoloxidases and peroxidases are among the most common extracellular proteins secreted by white-rot fungi. *Trametes versicolor* is an effective lignin-degrader and known to secrete high amounts of the phenoloxidase laccase (EC 1.10.3.2) (Jönsson et al., 1997). In addition, various peroxidases have been shown to be secreted by this fungus, amongst them manganese peroxidase (MnP, EC 1.11.1.13) and lignin peroxidase (LiP, EC1.11.1.14) are the best known (Johansson et al., 1993). Versatile peroxidase (VP, EC 1.11.1.16) is also a ligninolytic peroxidase but with MnP–LiP hybrid properties and thus capable of oxidizing both the typical substrates of MnP (Mn^{2+}) and LiP (veratryl alcohol). Its hybrid molecular structure that provides multiple binding sites is the basis for the oxidation of different substrate types spanning a wide range of redox potentials, including low- and high-redox potential compounds as well as compounds, which other peroxidases are not able to oxidize efficiently (e.g. the dye Reactive Black 5). This peroxidase

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was first observed in members of the genera *Pleurotus* and *Bjerkandera* in 1999 and then purified and further investigated (Camarero et al., 1999; Martínez, 2002; Ruiz-Dueñas et al., 2001, 2009). VP was later also detected in species from the genus *Panus* (Lisov et al., 2007). Kim et al. (2005) reported VP production by *T. versicolor* but, as far as we know, this *T. versicolor* VP was not purified up to now.

Basidiomycetous white-rot fungi are versatile and robust organisms having an enormous potential for biodegradation of various recalcitrant materials including toxic chemicals (Gao et al., 2010). These fungi are capable of mineralizing even xenobiotic compounds due to the nonspecific nature of their extracellular oxidative enzymatic system that naturally completely degrades complex polymeric materials of phenolic origin such as lignin or humic substances (Asgher et al., 2008). A few strains of the genus *Trametes* were even reported to productively degrade phenol and its derivatives (Carabajal et al., 2012; Chakroun et al., 2012; Grey et al., 1998; Yemendzhiev et al., 2008).

The herein studied strain of *T. versicolor*, BAFC 2234, was selected among a number of white-rot fungi from Argentina owing to its tolerance toward phenolic compounds and its ability to use phenol as sole carbon source (Carabajal et al., 2012). The objectives of this work were (i) to analyze the secretome of *T. versicolor* BAFC 2234 grown on tomato juice medium using a proteomic approach and (ii) to partially purify its secreted lignin-modifying enzymes laccase, MnP and VP.

2. Materials and methods

2.1. Fungal strain

Twenty-five Argentinean basidiomycetous fungi were tested for their tolerance toward phenol during growth on agar plates. At a phenol concentration of 10 mM, *T. versicolor* (strain BAFC 2234), still reached 45–50% of the growth rate observed in the absence of phenol. Fungal strains that were capable of growing in the presence of 10 mM phenol were then cultivated on GA agar plates (Levin and Forchiassin, 2001) supplemented with other phenolic compounds, namely 2-methoxyphenol, 2,6-dimetoxyphenol, or 3,4,5-trihydroxybenzoic acid (gallic acid). Again *T. versicolor* strain BAFC 2234 showed the highest growth rate and was therefore selected for further studies (Carabajal et al., 2012). The fungus is deposited in the Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires (BAFC). Stock cultures were maintained on 2% malt extract agar (MEA) at 4 °C.

2.2. Production of ligninolytic enzymes

Ligninolytic enzymes of *T. versicolor* were produced in a 30-L stirred-tank bioreactor (Biostat B; Braun Biotech International GmbH, Melsungen, Germany). Twenty liter of the complex liquid medium TJM (tomato juice medium) consisting of eco-tomato juice (Albi & Co., Germany) and distilled water (50:50, v/v) was used as growth medium. The inoculum consisted of 1 L of a homogenized fungal suspension pre-cultured in 500-mL Erlenmeyer flasks containing 200 mL of TJM on a rotary shaker at 100 rpm and 24 °C for 10 days. To ensure the stimulation of laccase and MnP production by the fungus, $CuSO_4$ and $MnCl_2$ were added 60 h after inoculation (final concentration 250 μM and 500 μM, respectively). Samples (1 mL of the culture liquid) were taken one or two times per day, and the ligninolytic enzyme activities and pH were measured. Fermentation was carried out under following conditions: 120 rpm, 4 L min^{-1} aeration rate and 28 °C; the pH was not regulated. Whole cultures were harvested after 7 days, filtrated and used for subsequent purification and secretome studies.

2.3. Enzyme purification

Fungal biomass was removed by filtration (filter GF6; Whatman, Dassel, Germany) and the cell-free culture liquid obtained was concentrated and dialyzed by repeated ultrafiltration at 11 °C (10-kDa cutoff; Pall-Filtron, Dreieich, Germany). A minor part of this concentrated extracellular enzyme mix was analyzed using proteomic methods (see below). To obtain laccase and peroxidases, the major part of the crude extract was further purified by up to four steps of fast protein liquid chromatography (FPLC) using anion exchange separation media. In the first step, extracellular proteins were separated on Q-sepharose® (strong anion exchanger, column: 16 mm × 100 mm, GE Healthcare, Freiburg, Germany) and eluted with a linear gradient of 0–0.8 M NaCl in 10 mM sodium acetate buffer (pH 5.5) at a flow rate of 5 mL min^{-1}. Fractions containing laccase/peroxidase activities were pooled, concentrated, dialyzed against 10 mM sodium acetate (pH 5.5–7.0) with 10 kDa Vivaspin concentrators (Sartorius Stedim Biotech; Göttingen, Germany) and loaded onto a Mono-Q® column (10 mm × 100 mm, GE Healthcare, Freiburg, Germany).

Bound proteins were eluted with 10 mM sodium acetate buffer containing 2 M NaCl at a flow rate of 6 mL min^{-1} (2nd step, sufficient for laccase separation). Fractions containing peroxidase activity were further purified by loading them onto a Mono-S column (10 mm × 100 mm, GE Healthcare, Freiburg, Germany) that was eluted with 10 mM sodium acetate buffer containing 2 M NaCl at pH 4.5. After the respectively last purification step, the final fractions of laccase, MnP and VP were pooled, concentrated and stored at −20 °C. The elution of proteins was monitored at 280 nm (total protein) and 407 nm (heme).

Part of the VP fraction obtained was further purified by semi-preparative HPLC-SEC with an HPLC system (Agilent 1200 series, Waldbronn, Germany) equipped with a Biosep-SEC-S-2000 column (300 mm × 7.8 mm, Phenomenex) under isocratic conditions (flow rate, 1 mL min^{-1} at 25 °C; solvent: aqueous mixture of 50 mM sodium acetate and 100 mM sodium chloride, pH 6.5). SDS-PAGE was performed to verify the purity of the enzyme preparations (12% NuPAGE Bis-Tris gel; Invitrogen, Karlsruhe, Germany). Analytical isoelectric focusing (IEF) was carried out using IEF precast gels (3–10 pH; Invitrogen). After electrophoretical separation, the gel was stained and protein bands were visualized with the Colloidal Blue Staining Kit (Invitrogen). Single protein bands of interest were gel-excised and further analyzed by nano LC–MS/MS (see below).

Protein concentration was determined by the method of Bradford using the Roti®-Nanoquant Protein Assay Kit (Roth, Karlsruhe, Germany) with serum albumin as the standard.

2.4. Secretome and peptide analysis by nano LC–MS/MS

The secretome of *T. versicolor* as well as single purified enzymes (gel excised) were investigated by shotgun LC–MS/MS. In total, 4 μg of protein lysates were diluted with 20 mM ammonium bicarbonate (ABC) to a final concentration of less than 1 M urea. Samples were reduced (2.5 mM DTT for 1 h at 60 °C) and alkylated (10 mM iodacetamide for 30 min at 37 °C). Proteolysis was performed overnight using trypsin (Promega, Madison, WI) with a ratio of 1:25 at 37 °C. The tryptic digestion was stopped by adding acetic acid at a final concentration of 1%, followed by desalting and purification using ZipTip-μC 18 tips (Millipore, Billerica, MA). Proteolytically cleaved peptides (400 ng) were separated prior to mass spectrometric analyses by reverse phase nano HPLC on a 15 cm Acclaim PepMap100-column (C18 3 μm, 100 Å) using an EASY-nLCProxeon system (Thermo Scientific, Waltham, MA) at a constant flow rate of 300 nl min^{-1}. Separation was achieved using a non-linear gradient of 70 min with 0.1% acetic acid, 2% acetonitrile in water (solvent A) and 0.1% acetic acid in 100% acetonitrile (solvent B). Separated