Cell Wall-Associated Redox Enzymes in White Rot Fungi

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Many enzymes of white rot fungi involved in wood degradation belong to the class of redox enzymes. The most important are laccase (copper-containing polyphenol oxidase), lignin peroxidase, manganese-dependent peroxidase and manganese-independent peroxidase. However, the role of these enzymes in wood degradation remains unclear and complex redox processes or unknown redox enzymes also may contribute to this process. Several oxidative enzymes secreted by white rot fungi into the environment have been studied in the past, but little attention has been paid to the cell wall-associated redox enzymes. Cell wall-associated laccase activity in the purified cell walls of copper induced cultures of *Trametes versicolor* has been found. Laccases have been extracted by establishing new methods for cell wall purification and for protein release from the cell walls of basidiomycetes.

1. Introduction

The shape of a fungal cell is determined by the cell wall that functions to maintain the integrity of the cell, to protect the cell against the environment and to interact and communicate with the surrounding environment. The cell wall shields the cell against osmotic, chemical, physical and biological injuries. It is involved in growth and morphogenesis, in cell-adhesion and in cell-cell interaction. The cell wall is not inert but a robust, highly elastic and permeable structure (Cabib et al. 1988, Gooday 1995, Latgé and Calderone 2005, Sietsma and Wessels 2005).

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The main components of the fungal cell wall are α- and ß-glucans and chitin that form the cell wall skeleton. In addition, glycoproteins, chitosan, polyuronids, inorganic salts and pigments are found. The skeletal polysaccharides comprise approx. 80% of the cell wall dry matter whilst the protein content varies typically from 3% to 20% (Ruiz-Herrera 1992, Latgé and Calderone 2005, Sietsma and Wessels 2005). Numerous cell wall-associated proteins reveal enzymatic activities. Identified cell wall associated fungal exoenzymes fall into the following biochemical classes, respectively subclasses:

- oxidoreductases
- O-glycosyltransferases
- hexosyltransferases
- aminoacyltransferases
- phosphoric monoester hydrolases.

Of these, hexosyltransferases and O-glycosyltransferases were most often described in an extraprotoplastic location of the cell. The evidences presented for cell wall-association of particular enzymes generally were indirect, i.e., inferred from simple histochemical tests or immunocytochemical analyses or from genome analysis (Rast et al. 2003). Most studies concerning fungal cell wall enzymes were performed on ascomycetous yeasts and filamentous ascomycetes and a few on zygomycetes. Some specific cell wall-associated enzymes detected in these fungi are:

- ß(1-3)-glucanosyltransferase in *Candida albicans* (Hartland et al. 1991)
- acid phosphatase in *C. albicans*, *Aspergillus fumigatus* and *Botrytis cinerea* (Molloy et al. 1995, Weber et al. 1997, Bernard et al. 2002)
- phospho- and lysophospholipases in *C. albicans* (Chaffin et al. 1998)
- chitinase in *Saccharomyces cerevisiae, C. albicans* and *Kluyveromyces* sp. (Iranzo et al. 2002, Bahmed et al. 2002)
- ß-glucosidase in *C. albicans*, *Acremonium persicinum* and *Aspergillus kawachii* (Ram et al. 1984, Iwashita et al. 1999, Pitson et al. 1999)
- trehalase in *C. albicans* and *Neurospora crassa* and in the zygomycete *Mucor rouxii* (Hecker et al. 1973, Ram et al. 1984, Molloy et al. 1995, Lucio et al. 2000)
- ß(1-3)-glucanase in *C. albicans* and *A. persicinum* (Ram et al. 1984, Pitson et al. 1999)
- ß(1-6)-glucanase in *A. persicinum* (Pitson et al. 1999)
- N-acetylhexosaminidase in *C. albicans* and *M. rouxii* (Rast et al. 1991, Molloy et al. 1995).

Most of the cell wall glycoproteins are considered to be catalytically active (Mrsa et al. 1999). Therefore, many other enzymatic activities are expected to be present in the subcellular location of the cell wall that will have to be detected in future work.

In the basidiomycetes, there are some well characterized structural cell wall proteins such as hydrophobins (Wösten 2001, Walser et al. 2003, Peddireddi et al. 2005) and galectins (Walser et al. 2003, 2004, 2005). However, in comparison to ascomycetes much less is known about the enzymes residing in cell walls of basidiomycetes. Many cell wall associated enzymes will be responsible for basic reactions in cell wall synthesis (Latgé and Calderone 2005, Sietsma and Wessels 2005). Others will contribute to breakdown of growth substrates.

Particularly in the wood inhabiting wood, oxidative and hydrolytic enzymes for lignocellulose degradation might be expected to reside in the fungal cell wall. For example, presence of laccase has been demonstrated by coprecipitation of gold particles with a phenolic laccase substrate in the outer sphere of the hypha of *Pycnoporus cinnabarinus* (Jones et al. 1999). Various redox- and hydrolytic enzymes have also been localized by immuno-gold labeling to the cell wall in e.g. *Phanerochaete chrysosporium*, *Trametes versicolor*, *Rigidoporus lignosus*, *Phellinus pini*, *Lentinula edodes, Volvariella volvacea,* and *Phlebia radiata* (see review by Daniel 1994). In *P. chrysosporium*, presence of lignin peroxidase and manganese peroxidase (MnP) was detected both within degraded wood and on the surface of the fungal hypha (Ruel et al. 1991, Srebotnik et al. 1988). MnP secreted by *Ceriporiopsis subvermispora* whilst growing on wheat straw has been shown to be entrapped within the hyphal polysaccharide sheath due to direct interaction of the enzyme with polyglucan. Diffusion of the enzyme towards the lignocellulosic substrate was restricted to the extend of the hyphal polysaccharide network (Ruel et al. 2003). Laccases entrapped within the exopolysaccharide shields of *C. subvermispora* and *R. lignosus* hyphae and within the cell walls in *P. radiata* have also been reported (Nicole et al. 1992, Ruel et al. 2003). Furthermore, aryl-alcohol oxidase, pyranose oxidase, cellulases, glucanases and xylanase have been detected by gold-immunolabeling in association with the cell wall or the extracellular glucan sheath in wood degrading fungi (e.g. Barrasa et al. 1998, Cai et al. 1999).

Whilst immunolabelling proved to be very helpful for specific enzyme localization and monitoring of mobility within substrate, antisera prepared

against purified enzymes do not allow detection of unknown enzymes and may not respond to modified forms of the enzymes. Efficient and reliable methods for purification of cell walls and isolation of proteins from hyphal sheaths and from cell walls are therefore needed. In ascomycetes, commonly applied methods to obtain pure of cell wall fractions employ homogenization of the fresh material, followed by salt, detergent and/or enzyme treatment of the pure cell walls to isolated the associated enzymes or other proteins (Bruneau et al. 2001, Pitarch et al. 2002). In basidiomycetes, reports are so far few. From sporocarps of the edible basidiomycete *Agaricus bisporus*, a total of 19% of cell wall-associated phenoloxidase activity was liberated by NaCl extraction steps whilst up to 48% was set free by digitonin treatment. In this case, enzymes were obviously non-covalently bound to the cell wall (Sassoon and Mooibroek 2001). In the human pathogen *Cryptococcus neoformans*, cell wall-associated laccase activity retained in the cell wall after sequential extraction with salt, urea and SDS. However, about 40% of the activity was released from intact cells or cell wall fractions upon enzymatic treatment with glucanases suggesting a strong association of the enzymes to the cell wall carbohydrate via hydrolysable bonds (Zhu et al. 2001, 2003).

2. Materials and Methods

Trametes versicolor strain Nr. 6 from our institute collection, were cultivated on Basidiomycete-Medium (BSM, Hüttermann et al. 1973) with 1% agar. Liquid standing cultures were prepared by inoculation of 100 ml BSM medium in 500 ml flasks and cultivated for 14 days at 25°C in the dark. Laccase secretion was induced after 7 days of cultivation by 0.5 mM 2,5-xylidine (Fahreus et al. 1967) or 150 µM $CuSO_4$.

Mycelia were separated from liquid supernatant by filtration. Culture medium remaining after filtration of mycelium was centrifuged for 20 min at 3200 g and proteins precipitated with four volumes of 10% trichloroacetic acid (TCA) in acetone (w/v) and kept at -20 °C overnight. Protein pellets were separated by centrifugation at 16.000 g for 20 min, washed four times with acetone and air dried. Supernatant protein samples for native PAGE were concentrated using PES-Vivaspin concentrator 10.000 MWCO (Vivascience, Hannover, Germany). Mycelia were grinded in liquid nitrogen and

pure cell wall fractions were obtained after several washing steps and sorbitol gradient purification (Dwivedi et al., in preparation).

Laccase activities were determined at room temperature by monitoring the oxidation of 5.0 mM ABTS at 420 nm in 120 mM sodium acetate buffer pH 5.0 (Matsumura et al. 1986). One unit of enzyme activity (IU) represents the amount of laccase that oxidized 1 µmol substrate per minute. Amounts of total protein were determined by using Pierce Coomassie Plus Reagent (Perbio, Germany) using bovine serum albumin (BSA) as a standard.

For 2D-electrophoreisis protein samples were solubilised in loading buffer containing 8 M urea, 4% (w/v) CHAPS 50 mM DTT, 5% (v/v) Triton X100 and 0,67% (v/v) ampholyte buffer. In the 1st horizontal dimension, isoelectric focusing was performed using 18 cm IPG-strips pH range 3-10 (Amersham) according to the manufacture's protocol. In the 2nd dimension, proteins were separated on 12% PAGE using Ettan DALTsix (Amersham). Gels with native proteins were washed for 30 minutes with 100 mM sodium acetate buffer pH 5.0 and stained with 6.9 mM α-naphthol and 8.4 mM of N,N,N',N'-tetramethyl-p-phenylenediamine (TMDA) to visualize the laccase activity (Sterjiades et al. 1993). To visualize all proteins, gels were silver-stained (Blum et al. 1987). For peptide identification by LC-MS analysis (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS), proteins spots were excised from gels and in gel-digestion with trypsin was performed at 58°C according to Havlis et al. (2003).

3. Results

In liquid culture with or without suitable inducers, *T. versicolor* secretes numerous extracellular proteins belonging to the class of redox oxidases (e.g. laccases) (Xiao et al. 2003), lignin-peroxidases, Mn-dependent and Mn-independent peroxidases (Collins et al. 1999), cellobiose dehydrogenases (Roy et al. 1996) and possible other enzymes important for lignocellulose degradation. Proteins in supernatants of standing BSM cultures of *T. versicolor* strain 6 were separated by 2D-electrophoresis. About 180 protein spots were detected. Most of them had a low pI-value and molecular weights in the range from 40 to 130 kDa (Fig. 1). Spots were eluted from gels, digested by

trypsin and analysed by LC-MS. Amongst other proteins, various peroxidases and a laccase were identified (Dwivedi et al., unpublished).

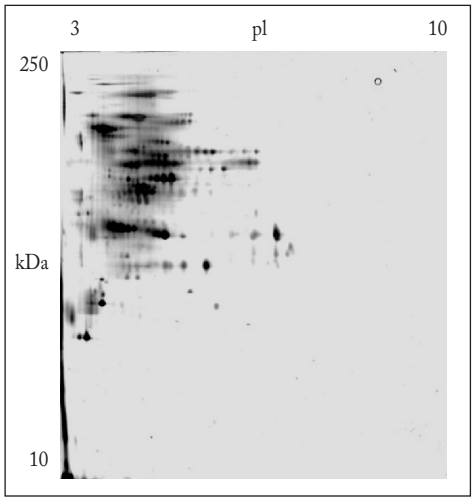

Figure 1. 2D-SDS-PAGE gel showing proteins from supernatants of standing *Trametes versicolor* BSM cultures at day 14 of cultivation

Laccase activities in the BSM culture supernatants were 42 ± 2.4 mU/ml. Laccase activity staining of 2D-PAGE gels showed a single spot at pI 3.5 ± 0.4 (not shown). Upon addition of 150 μM $CuSO_4$ and 0.5 mM 2,5-xylidine, laccase activities in culture supernatants raised to 260 mU/ml and 1.6 U/ml, respectively (Fig. 2). In 2D-gelelectrophoresis, both with copper and with 2,5-xylidine more protein spots were detected in silver-staining but laccase activity staining identified only the one spot known already from non-induced cultures (Fig. 3).

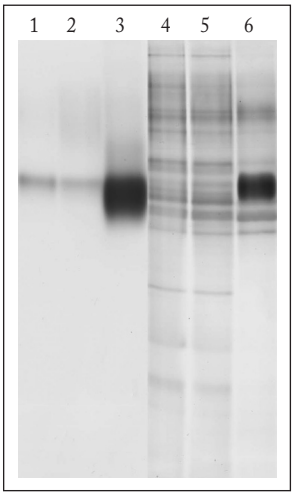

Figure 2. Native PAGE zymograms of laccases (lanes 1-3) and corresponding silver-stained proteins on 12 % PAGE (lanes 4-6). Equal sample volume of supernatant from 14 days-old *Trametes versicolor* cultures were loaded (1, 4: non-induced; 2, 5: copper-induced; 3, 6: 2,5-xylidine-induced)

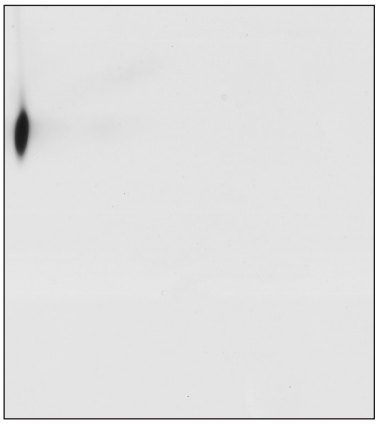

Figure 3. Native 2D-gel-electrophoresis of supernatant proteins from a 2,5-xylidine-induced, 14 days-old culture of *Trametes versicolor* stained for laccase activity

Laccase activity was also measured with mycelium of the cultures. Laccase activities per g mycelial fresh weight were 0.26 U, 0.60 U and 2.70 U in non-induced, copper-induced and 2,5-xylidine-induced cultures, respectively. In disrupted and washed mycelium, about a third of this activity remained in the cell wall fraction. Various buffers, salt concentrations, reducing agents, and cell wall-hydrolyzing enzymes were tested alone or in combination to extract the native enzymes. In the best case, 77% of enzymatic activity was

released from the cell walls. Two laccase activity bands were detected after separation of released enzymes by native 1D-gels, one of which seems to correspond to the enzyme found in large quantities secreted in the culture supernatant after 2,5-xylidine induction (Fig. 4).

Figure 4. Laccases from 2,5-xylidine-induced culture supernatant of *Trametes versicolor* (1) and isolated from the cell wall fraction of the mycelium from the same culture (2)

4. Conclusions

Our work shows that *T. versicolor* strain 6 secretes various oxidizing enzymes into culture media but also retains enzymatic activity in the cell walls. We have established protocols for cell wall purification and for release of enzymes from the cell walls of the species. Currently, we modify the protocols for optimal use in other basidiomycetes such as *Pleurotus ostreatus*, *Irpex lacteus* and *Coprinopsis cinerea*. Moreover, we proceed with biochemical characterization of the isolated cell wall-associated laccases from *T. versicolor*. Additionally, we characterize the enzymes in their natural cell wall bound form and compare the data with those obtained for laccases from the culture media.

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