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Proteins from *Punctularia atropurpurascens* with Biotechnological Applications

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Abstract

Basidiomycetes are able to biodegrade waste and xenobiotic molecules through the production of extracellular enzymes. For example, white-rot fungi produce lignin-degrading enzymes which are capable of efficiently decolorizing dye solutions. Many mushrooms also produce lectins, a group of proteins which bind specifically to the carbohydrates in glycoconjugates. Several fungal lectins target their specificities towards oligosaccharides present in mammalian glycoproteins, thus constituting excellent ligands for the preparation of affinity adsorbents useful in isolation and characterization of these glycoproteins. In this study we isolated and characterized two different proteins, a lectin and a laccase, present in extracts from *Punctularia atropurpurascens*. The lectin isolated from the mycelium extract, was immobilized on activated-Sepharose and used to evaluate the interaction with three glycoproteins. The adsorbent was able to efficiently adsorb and elute bovine lactoferrin, constituting a promising tool for the purification of this glycoprotein. *In vitro* experiments revealed that the lectin also exhibited antimicrobial activity against *Aspergillus niger*. Laccase activity was detected in the extracellular extract from *P. atropurpurascens*. This enzyme, in both soluble and immobilized forms, was able to degrade Remazol Brilliant Blue R and Acid Blue 25 dyes. The biological activities found in this fungus demonstrate its potential for various biotechnological applications.

Keywords

Basidiomycete, Laccase, Decolorization, Lectin, Glycoproteins

1. Introduction

Basidiomycetes play an important role in the ecosystem as they are able to biodegrade agricultural wastes and

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several xenobiotic molecules through the production of extracellular enzymes such as lignin peroxidase, laccase and manganese peroxidase. Many reports so far have demonstrated that white-rot fungi such as *Phanaerochaete chrysosporium*, *Pleurotus calyptratus*, *Dichomitus squalens*, *Ischnoderma resinosum* and *Trametes trogii* are capable of efficiently decolorizing pulping effluents and dye solutions with their lignin-degrading enzymes by the oxidation of phenolic groups [1]-[3]. In addition, mushroom extracts have been reported to exhibit antibacterial, antifungal, antiviral, antitumor, hypotensive and hepatoprotective activities and to contain many other bioactive molecules that could be useful for biotechnological purposes [4]. Mushroom extracts contain lectins, a group of proteins with important physiological roles. Lectins are a heterogeneous group of proteins or glycoproteins of non-immune origin that specifically and reversibly bind to the carbohydrate moiety of glycoconjugates. Most lectins play crucial roles in diverse biological processes, particularly in host defense mechanisms, inflammation, and metastasis. They are considered ubiquitous proteins, found in viruses, bacteria, fungi, animal and plants [5]. Owing to their binding specificities, lectins are employed in many biochemical and clinical research areas [6] [7]. Fungal lectins are reported to play an important role in the life cycle of fungi. They have been isolated from the fruiting bodies and also from the mycelia of numerous species of higher fungi [8]. Their biological activities in relation with organisms or animal or human cells include lymphomitogenic activity, immunomodulatory properties, antitumor, antiproliferative, cytotoxic and apoptosis-inducing activities. Examples of these reported activities are those isolated from *Agaricus bisporus*, *Volvariella volvacea*, *Grifola frondosa* and *Tricholoma mongolicum* [9]-[12]. They are also widely used in cytochemical and histochemical analysis and they have found applications in research, for example in the isolation and structural elucidation of cell glycoconjugates, and the monitoring of changes occurring on the surface of cell membranes at various stages of physiological or pathological development. They can be helpful in embryological, microbiological and taxonomic research. Finally, they constitute valuable tools for glycobiological studies in biomedical and cancer research: due to their specificities towards oligosaccharides from mammalian glycoproteins, fungal lectins can be used as ligands in the preparation of affinity adsorbents for the detection, isolation and characterization of mammalian glycoconjugates [13].

The present work aimed at isolating two proteins with different properties and applications, a lectin and an enzyme (a laccase) in extracts from *Punctularia atropurpurascens*. Both proteins were evaluated for their biotechnological applications: the mycelial lectin was used as a ligand for the preparation of affinity adsorbents and it was also tested as an antimicrobial agent against microorganisms, while the extracellular laccase was evaluated for dye degradation.

2. Materials and Methods

2.1. Organism

A strain of *Punctularia atropurpurascens* (H2126), grown and maintained on malt extract agar (Oxoid Ltd., Basingstoke, UK), from the collection of the Cátedra de Microbiología General Collection (CCMG) was used.

2.2. Ligninolytic Activity Tests

The presence of lignin-degrading enzymes in the *Punctularia atropurpurascens* strain was determined on solid media, as previously described [14] [15]. The Malt Extract Agar (MEA) medium was supplemented (separately) with 1) 0.02% Remazol Brilliant Blue (RBB, Sigma Chemical Co., St. Louis, MO), 2) 1% tannic acid (Sigma Chemical Co., St. Louis, MO), and 3) 1% gallic acid (Sigma Chemical Co., St. Louis, MO). The diameters of mycelia growth and decolorization or oxidation zones in the plates, were measured along the 10 days of the incubation at 28˚C.

2.3. Preparation of Fungal Aqueous Extracts

The mycelia of *Punctularia atropurpurascens* were grown on MEA. To prepare the extracellular and the mycelial extracts, two 7-day-old culture fungal plugs (diameter, 9 mm) were transferred to 500-ml Erlenmeyer flasks with 200 ml sterile malt extract broth (1.25%, w/v) and incubated at 28˚C at 100 rpm for 12 days. After the fermentation, the broth was centrifuged (15,000 g, 30 min, 4˚C) and the resulting clear supernatant (identified as extracellular extract, EE12) was used for the enzymatic assays. The mycelia were then ground in a mortar with liquid nitrogen and the frozen mycelial powder was suspended in phosphate-buffered saline pH 7.4

(PBS buffer). The resulting suspension was filtered through cheesecloth and the filtrate was centrifuged (15,000 g, 30 min, 4˚C). This mycelial extract was used for isolation of the lectin [16].

2.4. Enzymatic Assays

Laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) activities were determined in the EE12 extract by monitoring the oxidation of syringaldazine [17], MBTH/DMAB [18] and veratryl alcohol [19] as substrates, respectively. The enzyme unit (EU) was defined in each case, as the amount of enzyme that catalyses the formation of 1 µmol of the corresponding product per min.

2.5. Electrophoretic Analysis

Native PAGE was carried out with the Phast System equipment (Pharmacia, Uppsala, Sweden) using 8-25 gradient Phast gels. Proteins were stained using the silver protocol according to the manufacturer's instructions and the laccase activity was detected on the gel by staining with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Isoelectric focusing (IEF) was done using the PhastGel IEF 3-9. A broad pI calibration kit (3-9) was used as standard. Proteins were silver stained according to the manufacturer's instructions (PhastSystem, Pharmacia).

2.6. Optimal Temperature and pH of Laccase Activity

The optimum temperature was determined on EE12 by performing enzyme activity assays at different temperatures between 30˚C and 60˚C. The optimum pH was determined by performing enzyme activity assays in buffers varying from pH 4.0 - 7.0, in the following pH ranges: 100 mM sodium acetate buffer (pH 4.0 - 6.0) and 100 mM phosphate buffer (pH 6.0 - 7.0). Each assay was run in duplicate.

2.7. Dye Decolorizing Activity

The ability of the EE12 extract to enzymatically degrade Remazole Brilliant Blue R (RBBR, Sigma Chemical Co., St. Louis, MO) and Acid Blue 25 (Sigma Chemical Co., St. Louis, MO) dyes was evaluated. Tubes of 3 ml volume containing 0.25 mM dye and 300 µl of EE12 extract in 100 mM acetate buffer pH 4.5, were incubated on a rotation shaker at 37˚C for 48 h. In some cases the reaction was run in the presence of 20 mM $MnSO_4$ and 10 mM H_2O_2. Decolorization was followed spectrophotometrically at 590 nm and the decolorization efficiency (DE) was expressed as percentage of color removal calculated as $C_i - C_r/C_i \times 100$, where C_i and C_r were initial and residual dye concentrations, respectively. Tubes containing dye without enzymes or with heat-inactivated enzymes (at 100˚C for 30 min) were used as controls.

2.8. Reversible Immobilization of EE12 Extract on Concanavalin A-Sepharose

The EE12 extract was applied to a column (diameter 5 mm, height 30 mm) packed with the affinity adsorbent Concanavalin A-Sepharose (2.4 ml of sedimented gel), previously equilibrated with 0.1 M acetate buffer pH 6.0 (adsorption buffer). The column was washed with the adsorption buffer and the bound enzymatic activities (laccase or MnP) were evaluated. A solution of the corresponding substrate, syringildazine (for the case of laccase activity) or MBTH/DMAB (for the case of MnP activity) was loaded onto the column and the bound activity was visualized by the change in the color of the halo produced by the oxidized products (pink or blue, respectively). The column was washed again with the adsorption buffer and elution of the adsorbed material was performed with a 0.45 M methylmannoside solution. Fractions of 1-ml were collected and gel filtered on PD-10 (Sephadex G-25, GE Healthcare) to remove the monosaccharide. The enzymatic activities (laccase or MnP) remaining were determined in these gel filtered eluted fractions, as described above.

In a parallel experiment the EE12 extract was adsorbed on the Con A-Sepharose column as before, and the degradation of a solution of RBBR dye was evaluated. A volume of 800 µl of dye was loaded onto the column and after 20 min, the bound enzymatic activity was visually registered, by the decolorization produced by the oxidized product. The column was washed again with adsorption buffer, elution of the adsorbed material was performed with a 0.45 M methylmannoside solution and the same procedure described above was followed, to determine the enzymatic activity remaining.

2.9. Immobilization of the Lectin from the *Punctularia atropurpurascens* (PAL) Mycelium Extract

The lectin was purified by affinity chromatography on chitosan-Sepharose as described previously [16]. Briefly, the mycelium extract was loaded on the affinity column and the elution was performed with 100 mM N-acetyl-glucosamine in PBS buffer. Purified PAL was coupled to NHS-activated Sepharose via the amino groups, according to the procedure recommended by the manufacturer. An amount of 1.2 g of freeze dried powder was suspended in 1 mM HCl and washed on a sintered glass filter. The lectin solution (0.6 mg/ml) was adjusted to 0.2 M $NaHCO_3$ pH 8.3 buffer, supplemented with 0.5 M NaCl (coupling buffer) and mixed with the gel in an empty PD-10 column. The suspension was rotated end over end for 1 h at room temperature. The excess ligand was washed away on a glass filter with the coupling buffer. The derivative was washed with three cycles of alternating pH consisting of a wash with 0.5 M ethanolamine in coupling buffer, followed by a wash with 0.1 M acetate buffer, pH 4.0 supplemented with 0.5 M NaCl.

The lectin content of the gel was determined by using the bicinchoninic acid (BCA) technique as described by Giacomini *et al*. [20]. Immobilization yield was calculated as the ratio between the amount of immobilized lectin and the amount of the soluble lectin initially applied.

2.10. Affinity Chromatography of Glycoproteins on a PAL-Sepharose Column

A glass mini-column (d = 0.35 cm) was packed with 0.5 ml of PAL-Sepharose and equilibrated with PBS buffer. In individual experiments, volumes of 200 µl of each of the following glycoproteins were applied to the column: bovine lactoferrin (LF), pregnant mare serum gonadotropin (PMSG) and fetuin from fetal calf serum (FT). In each experiment, the column was washed with PBS buffer and a competitive elution was performed with 100 mM N-acetyl-glucosamine in PBS. Fractions of 0.4 ml were collected on microtiter plates (flow rate: 0.5 ml/min) and analyzed for their protein content by recording the A 280 nm.

2.11. Antimicrobial Activity of PAL

Test organisms used for bioautography assays [21] were *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 11105), *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16404). The purified lectin (4 mg/ml, 50 µl) was applied as small spots on silica plates. Gentamicin (20 µg/ml) and Nystatin (50 IU/ml) were used as positive controls for growth inhibition of bacteria and fungi, respectively. PBS (50 µl) was used as a blank control. The minimum inhibitory concentration (MIC) was defined as the minimum lectin concentration that inhibited the visible microorganism growth. The MIC was determined by the microdilution technique using 100 µl of Mueller-Hinton broth (Difco), 100 µl of two fold dilutions of the extracts (10 mg/ml) and 10 µl of a suspension of the microorganisms (10^8 microorganisms/ml). The trays were incubated (24 h, 37˚) and developed with 0.1% *p*-iodonitrotetrazolium violet (Sigma Chemical Co., St. Louis, MO) solution. Nystatin and gentamicin were used as controls.

3. Results

3.1. Extracellular Enzymes and Dye Degradation

The ligninolytic activity of *Punctularia atropurpurascens* was determined using three different media. Decolorization haloes around the growth areas were observed when the medium was supplemented with RBB (**Figure 1(a)**). The halo diameters increased over the 10 days evaluated and correlated with the increase in the diameter of the mycelia growth during the same period. The oxidation zones, visualized as brown areas in the plates supplemented with tannic (**Figure 1(b)**) and gallic (**Figure 1(c)**) acids, also correlated with the mycelia growth.

3.2. Enzymatic Activity

Extracellular extracts of *Punctularia atropurpurascens* (EE12) showed MnP and laccase activities starting at the fifth day of fermentation (**Figure 1(d)**); no LiP activity was detected. When the EE12 extract was reversibly immobilized on a ConA-Sepharose column, the MnP and laccase activities of the extract were visualized as colored bands on the columns, after the addition of substrates (**Figure 2(a)**). A broad pink zone was observed at approximately 0.5 cm from the top of the column (left) as a result of the laccase activity using syringaldazine as

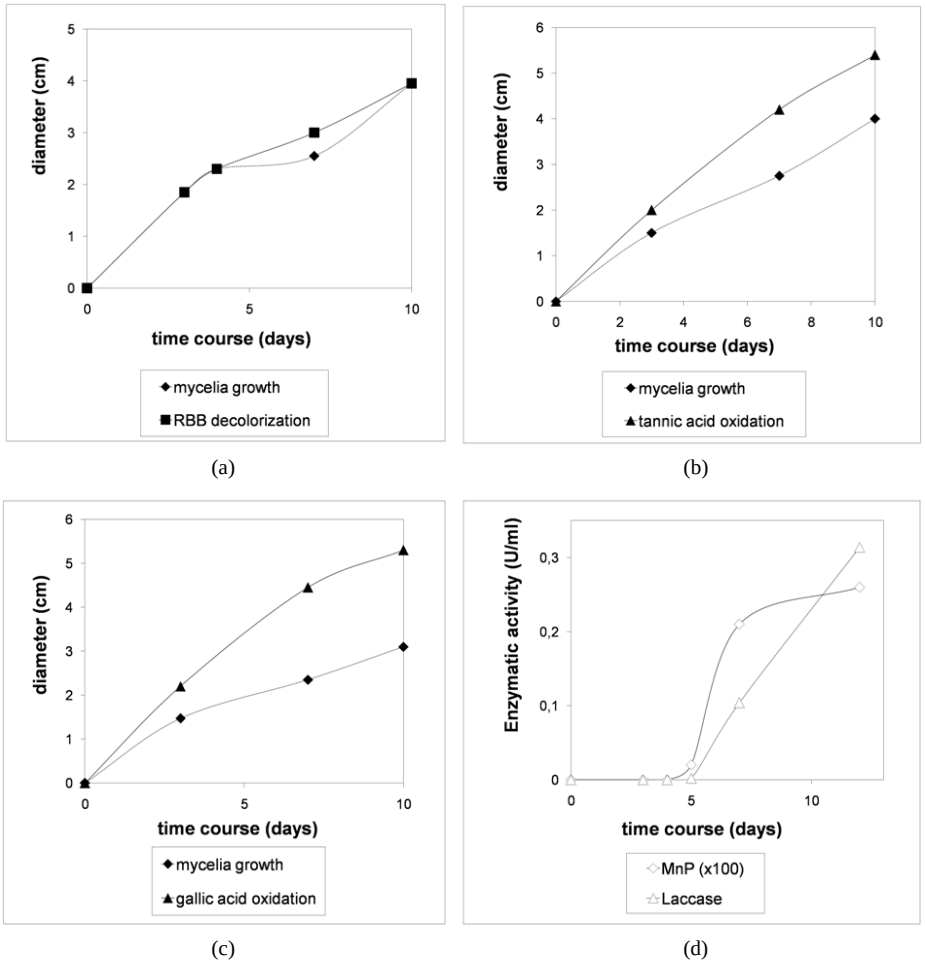
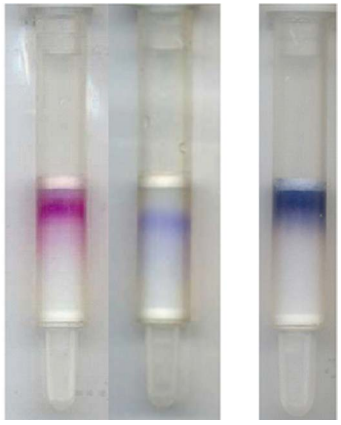


Figure 1. Ligninolytic activity of *P. atropurpurascens*. Fungal growth (♦) and decolorization (■) on MEA supplemented with RBB (a). Fungal growth (♦) and oxidation (▲) on MEA supplemented with tannic acid (b) and gallic acid (c). MnP (◊) and laccase (△) activities of the EE12 extract (d).



(a) (b)

Figure 2. Enzymatic (a) and dye decolorizing (b) activities of EE12 adsorbed on Con A-Sepharose. (a) Laccase (left) activity determined with syringaldazine and MnP (right) activity determined with MBTH/DMAB; (b) RBBR dye (the blue band disappears after 20 minutes of incubation).

substrate. In the case of the MnP activity, a tenuous and diffuse blue zone was observed at approximately 1 cm from the top of the column (right) produced by the oxidation of the MBTH/DMAB substrate. These results demonstrate that both enzymes present in the EE12 are active in the gel. As the interaction with the Con A lectin is mediated by alpha-D-mannopyranosil or alpha-D glucopyranosyl or sterically related residues in glycoconjugates, this also reveals that these enzymes are glycosylated. Moreover, the reversibility of the lectin-glycoenzyme interaction was confirmed when the enzymes were eluted from the column with the methyl mannoside. After gel filtration on PD-10 columns to eliminate the competitive carbohydrate, the enzymes were still active (data not shown).

3.3. Electrophoretic Analysis

The PAGE profile of the extracellular extract EE12 showed the presence of a defined protein band coincident with the migration front and a retarded diffused and smeared zone (**Figure 3(a)**, right). When the gel was stained with the enzymatic substrate ABTS (**Figure 3(a)**, left) two colored bands are observed, the main active one is coincident with the silver stained band. The other green band (just below the main band), does not coincide with any protein band in the silver stained gel.

There was no change in the specific stain when the gel was submerged in a $MnSO_4$ and H_2O_2 solution (data not shown). These results may indicate the presence of at least two laccases in the extracellular extract. IEF analysis of the EE12 extract showed a clear band of pI lower than 3.0 and a smeared band centred at pI 5.2 (**Figure 3(b)**, left). However, the stain with ABTS indicated that only this latter band was revealed by the specific detection (**Figure 3(b)**, right).

3.4. Optimal Temperature and pH of MnP and Laccase Activities

Temperature profiles of MnP activity showed the optimum temperature at 50˚C, while two peaks at 35˚C and 50˚C of laccase activity were detected (**Figure 4(a)**). The pH profile (**Figure 4(b)**) showed maximum activity at 4.5 for MnP and 6.0 for laccase.

3.5. Dye Decolorizing Activity

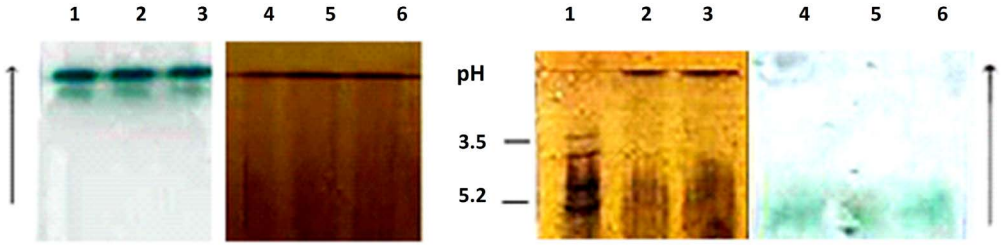
The ability of the enzymes of the extracellular extract EE12 to degrade Remazole Brilliant Blue R (RBBR) and Acid Blue 25 was evaluated. Both dye solutions were almost completely decolored (90%, calculated as indicated in Methods) after 48 h of incubation while no changes in the decolorization efficiency were observed after the addition of Mn and H_2O_2 suggesting that the laccase, but not the MnP, activity may be responsible for the decolorization effect. The decolorization of the RBBR dye was also evaluated using the enzymes immobilized on Con A-Sepharose. In this case, the enzymatic activities of the adsorbed EE12 extract was evaluated by visualizing the disappearance of the initial blue band present in the column (**Figure 2(b)**). The colored band disappeared after 20 minutes of incubation (data not shown) indicating that the enzyme retained its activity when adsorbed on the affinity gel.

3.6. Synthesis of PAL-Sepharose and Interaction with Glycoproteins

The lectin was purified from the *P. atropurpurascens* mycelium extract (PAL) as previously reported [16] and then immobilized on NHS-activated Sepharose. The resulting PAL-Sepharose adsorbent contained 0.8 mg PAL/ml sedimented gel, determined by the BCA optimized protocol (as described in Materials and methods) using a calibration curve ($y = 0.024x + 0.0025$, $r^2 = 0.994$). The chromatographic behaviors of three glycoproteins (LF, PMSG and FT) were evaluated on columns packed with PAL-Sepharose. The adsorbent displayed high selective binding of LF, as 60% of the applied material was eluted from the column, but there was no interaction with FT or PMSG (**Figure 5**).

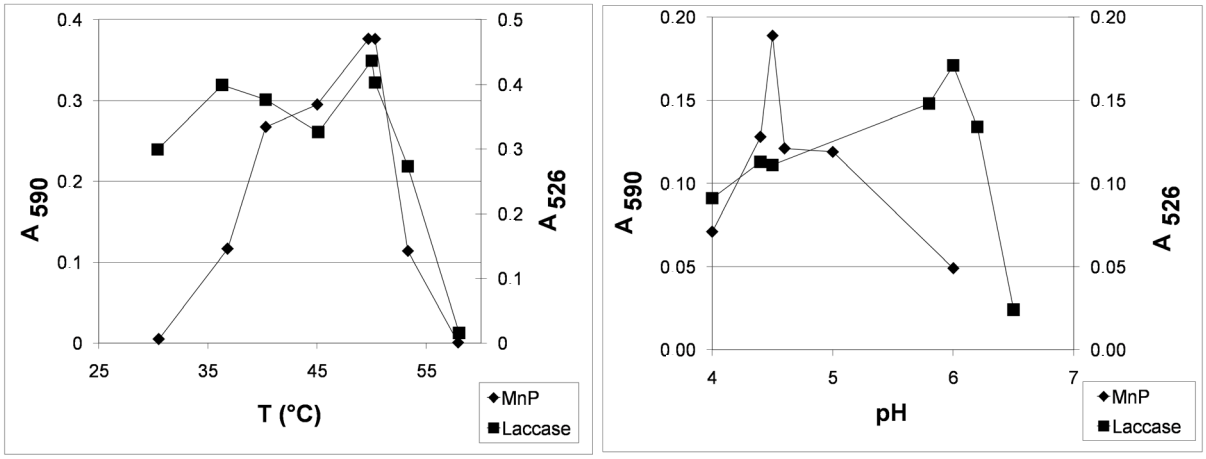
3.7. Antimicrobial Activity of *P. atropurpurascens* Lectin

The purified lectin (PAL) was evaluated against four microorganisms: *S. aureus*, *E. coli*, *C. albicans* and *A. niger*. Under the experimental conditions PAL was not effective at inhibiting the growth of *E. coli*, *S. aureus* or *C. albicans*. However, a clear inhibition halo was obtained against *A. niger*. The MIC against *A. niger* was determined as 0.08 mg of PAL/ml.



(a) (b)

Figure 3. Electrophoresis analyses of the extracellular extract (EE12) from *Punctularia atropurpurascens*. (a) PAGE analysis, right: silver stained; left: stained with ABTS reagent; (b) IEF analysis, left: silver stained; right: stained with ABTS reagent.



(a) (b)

Figure 4. (a) Optimal temperature. (♦) MnP activity; (■), laccase activity; (b) Optimal pH. (♦) MnP activity; (■) laccase activity.

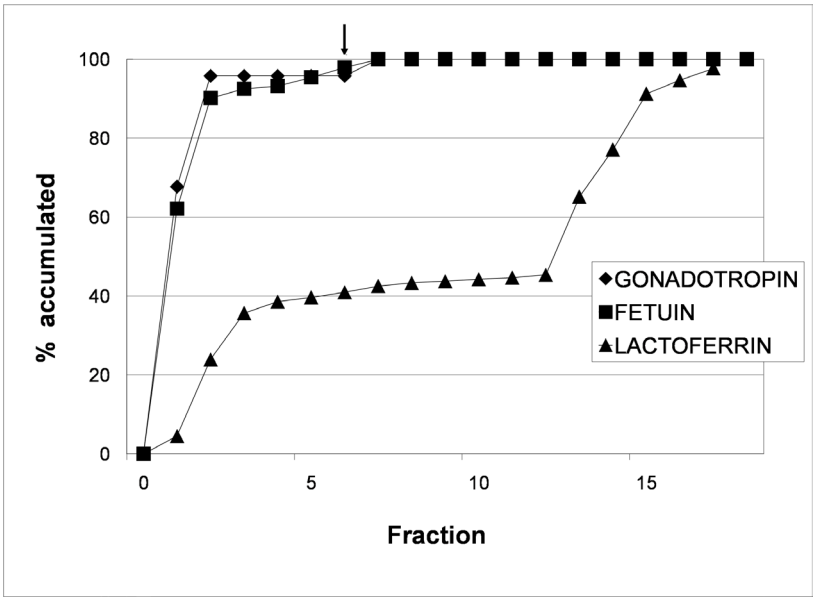


Figure 5. Affinity chromatography of glycoproteins on PAL-Sepharose. Profiles for lactoferrin (▲), gonadotropin (♦) and fetuin (■). The arrow indicates the start of the elution step with 100 mM N-acetyl glucosamine in PBS.

4. Discussion

Recently, there has been increasing interest in studying the lignin-modifying enzymes of a wide array of white rot fungi with the aim of improving lignin-degrading systems, useful for different biotechnological purposes [22].

This study explored the ability of the extracellular extract from *P. atropurpurascens* to decolorize dyes. Using plate assays for both RBB decolorization and tannic/gallic acid oxidation, we showed that the ligninolytic system is present during fungal growth. At the same time, MnP and laccase activities were detected in the extracellular extract during the fungal growth phase. Previous reports for other fungi described the production of MnP or laccase during the stationary state [23] [24]. The onset of the production of ligninolytic enzymes during the fungal growth phase is a positive trait when considering enzyme production or activity necessary for industrial processes. The profiles for optimal pH and temperature for manganese peroxidase indicate the presence of only one enzyme. Its optimum temperature was 50˚C and optimum pH was 4.5. In the case of the laccase activity the profiles are consistent with the presence of at least two isoenzymes, as has been previously reported for *Phanaerochaete chrysosporium* [23]. Optimum laccase activity was found at 35˚C and 50˚C and optimum pH was 6.0. Most of the reported MnP enzymes possess optimum temperatures ranging from 25˚C to 35˚C but values as high as 60˚C have also been reported [25] [26]. However, in the case of laccases the reported optimum temperatures are between 50˚C and 80˚C [27], thus the presence of a laccase isoenzyme in the extracellular extract of *P. atropurpurascens* with an optimum temperature of 35˚C is an interesting finding.

The PAGE analysis revealed the presence of two laccase isoenzymes while the IEF analysis showed the presence of only one active band at pI 5.2. The literature reports the presence of laccase isoenzymes which differ in their pIs, in the fungi *Flavodon flavus* [28] and *Ceriporiopsis subvermispora* [29]. This is not the case of the *P. atropurpurascens* laccase isoenzymes of the EE12 extract. These isoenzymes are also able to degrade the RBBR and Acid Blue 25 dyes. This is in accordance with previous reports describing the ability of other fungi to degrade RBBR and Acid Blue dyes [30]-[33]. Also, the reported correlation between ligninolytic enzyme production and decolorization, was confirmed with *P. atropurpurascens*. This is not surprising, given the structural similarity between the most commercially important dyes and lignin (sub)structures, making them amenable to transformation by ligninolytic enzymes, particularly laccases [34].

The extracts prepared from *Punctularia atropurpurascens* mycelia produced a lectin with specificity towards chitosan compounds. Previous results showed the interaction of soluble PAL with the following mammalian glycoproteins: bovine lactoferrin (LF), fetuin from fetal calf serum (FT) and PMSG [16]. These results encouraged us to produce an affinity adsorbent using this lectin as ligand. The resulting PAL-Sepharose was used to study the interaction with the mentioned glycoproteins by analyzing their chromatographic profiles. Fetuin, a plasma glycoprotein widely distributed in mammals, is extensively used as a model protein for structural analyses and research related to the biological properties of glycoproteins. The PMSG is synthesized and secreted by specialized cells derived from the fetal trophoblast. It is a unique member of the gonadotropin family with follicle stimulating and luteinizing activities. Finally, LF plays important biological roles and antitumoral, antimicrobial, anti-inflammatory and immunoregulatory properties have been attributed to this glycoprotein. Among the three analyzed glycoproteins only bovine lactoferrin was efficiently adsorbed and subsequently eluted from the PAL-Sepharose column, making this lectin a promising tool for structural studies of this glycoprotein.

The *in vitro* experiments revealed that the crude extract and the purified lectin from *P. atropurpurascens* both exhibited antimicrobial activity against *Aspergillus niger*. The MIC of the purified PAL was 0.08 mg/ml, similar to the values reported for lectins from other sources and against other microorganisms [35]. Moreover, it has been reported that plant lectins specific to chitin also show antifungal activity [36]. The reported chitin specificity of PAL [16] may be involved in the interaction with the fungal cell wall, leading to inhibition of *A. niger* growth. Like many natural products of microbial origin that are applied in human health therapies, antifungal proteins may be successfully exploited not only in agriculture but also in medicine. In view of the increasing development of microbial resistance and the adverse reactions elicited by some of the currently available drugs, fungal proteins with antifungal activities like those reported here, may have potential as alternative therapeutic products. As the number of reports dealing with this type of proteins is increasing, there are now more options for the prevention of human and plant diseases [4]. These properties demonstrate the potential of basidiomycetes and in particular of *P. atropurpurascens* for many biotechnological applications.

5. Conclusion

In this study we isolated and characterized two different proteins, a lectin and a laccase, present in extracts from *Punctularia atropurpurascens*. The lectin isolated from the mycelium extract, was immobilized on activated-Sepharose and used to evaluate the interaction with three glycoproteins. The adsorbent was able to efficiently adsorb and elute bovine lactoferrin, constituting a promising tool for the purification of this glycoprotein. Future studies with this immobilized lectin may include its interaction with other glycoproteins as well as its application for the purification of lactoferrin from complex samples. *In vitro* experiments revealed that the lectin also exhibited antimicrobial activity against *Aspergillus niger*. Laccase activity was detected in the extracellular extract from *P. atropurpurascens*. This enzyme, in both soluble and immobilized forms, was able to degrade Remazol Brilliant Blue R and Acid Blue 25 dyes. Further work will include a more exhaustive characterization of this protein. The reported biological activities found in this fungus demonstrate its potential for biotechnological applications.

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