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Isolation and Partial Characterization of an Extracellular Low-Molecular Mass Component with High Phenoloxidase Activity from *Thermoascus aurantiacus*

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An extracellular low-molecular mass component (LMMC) with catalytic properties was isolated from liquid cultures containing wheat bran of ascomycete thermophilic *Thermoascus aurantiacus.* The partially purified LMMC showed very high activity with typical phenoloxidase substrates in the absence of hydrogen peroxide at acidic pH (2.8). However, in this pH range, the phenoloxidase (PO) activity was quickly lost. The LMMC showed a high optimum temperature (80°C) and an elevated thermostability. The molecular mass of the component estimated by gel filtration chromatography was 530 Da. IR and ^{1}H- and ^{13}C-NMR spectra indicated the presence of hydroxamic acid moiety. Qualitative determination of metal ions by several techniques revealed the presence of mainly iron associated with this structure. Iron may be the responsible for the ability for catalyze oxidation reactions, such as o-dianisidine oxidation, by the LMMC. These results suggested the existence of a hydroxamate-type metal-binding component, most likely hydroxamate siderophore. In addition, the chrome azurol S (CAS) universal assay for noncomplexed siderophores detection revealed the production of these compounds by *T.aurantiacus* in solid and liquid media.

***Key Words: Thermoascus aurantiacus;* phenoloxidase; hydroxamate siderophores.**

The Brazilian strain of fungus *Thermoascus aurantiacus,* which belong to ascomycete class, was previously related as a degrader of *Eucalyptus grandis* wood and producer of phenoloxidase activity (1). The PO activity of *T. aurantiacus* was induced by presence of different lignocellulosic substrates in liquid medium, particulary by wheat bran. Crude extracts containing this PO activity oxidized various substrates at low pH (3.0) and in absence of hydrogen peroxide (2). Moreover, the crude extracts of *T. aurantiacus* with high PO activity showed hardwood kraft pulp bleaching activity (3).

For many years, the wood decay process by fungi was associated almost exclusively with lignocellulolytic enzymes production. However, recent studies by electron microscopy have shown that fungal enzymes are too large to penetrate into the cell wall in the early stages of decay. Thus, the hypothesis that low-molecular mass agents may initiate the breakdown of both cellulose and lignin was proposed (4,5). The production and isolation of low-molecular mass agents (approx. 1,000 Da) was recently related in the brown-rot fungi *Gloeophyllum trabeum* and *Tyromyces palustris* and the white-rot fungi *Irpex lacteus* and *Phanerochete chrysosporium*, all belong to the basidiomycete class (6–8). These low-molecular agents were glicopeptides which containing Fe(II) and catalyzes the reaction that reduces O_2 to $HO^{\cdot}$ in the presence of electron donors. Goodell et al. (9) isolated and characterizated a family of low-molecular mass phenolate compounds from *G. trabeum*, some of which showed ability to both chelate Fe (III) and reduce it to Fe (II). The radical species generated by these compounds seem to be involved in the initial stages of wood decay by white and brown-rot fungi (10,11).

In order to elucidate the nature of the unusual high PO activity present in the cultures of ascomycete *T. aurantiacus,* when grown in liquid medium containing wheat bran, the partial purification through chromatography of an extracellular component was carried out. Some kinetic and physico-chemical properties of a partially purified component with high PO activity were studied.

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MATERIALS AND METHODS

Microorganism and culture conditions. Stock cultures of ascomycete thermophilic *Thermoascus aurantiacus* (Brazilian strain) were cultivated and maintained on solid Czapek and potato-dextrose agar media. For PO activity production, the fungus was grown on modified Czapek liquid medium (pH 6.0) containing wheat bran (1.5% w/v) as previously described (2). Flasks containing 200 mL of culture medium were inoculated with two agar-mycelium discs taken from stock plates and incubated without shaking at 48 ± 1°C during 10–15 days or until maximum PO activity production. The supernants with high PO activity collected from each culture flasks were combined and used as initial material for isolation of the extracellular component with PO activity. Distilled and deionized (dd) water was used in all experiments.

Assay of phenoloxidase activity. The PO activity was assayed by a modification of Szklarz et al. (12) in the filtered supernatants (Millipore membrane 0.45 μm) or eluted fractions of the chromatography columns using 1.0 mM o-dianisidine as substrate in 50 mM citrate-phosphate buffer (pH 2.8). The PO activity was expressed as the μmol of oxidized substrate per minute (IU).

Isolation of extracellular component with PO activity. In order to remove the brown color of the cultures of *T. aurantiacus* grown on wheat bran, the insoluble polymer polyvinilpyrrolidone (PVP) was directly added to filtrates. After 30 min under gentle stirring, the PVP was removed from the mixture by filtration or centrifugation. The filtrates collected were concentrated by ultrafiltration (UF), using a MINITAN System with a 10 kDa cutoff membrane. However, a great part of the PO activity passed through this membrane and approx. 90% activity was detected in the filtrate (Filtrate I $<$ 10 kDa). A second UF with the filtrate I using a 1 kDa cutoff membrane was carried out. Again, all PO activity was detected in the filtrate (Filtrate II $<$ 1 kDa) and very little activity was detected in the retentate. Filtrate II was lyophilized, dissolved in double distilled (*dd*) water and subsequently applied on a Sephadex G-10 column (1.5 $\times$ 50 cm). The column was equilibrated and eluted with *dd* water (pH 6.2) and 2.5 mL fractions were collected at a flow rate of 0.21 mL min^{-1}. The column exclusion volume (Vo) was determined with blue dextran (2×10^6 Da). The fractions were assayed for PO activity and absorbance at 280 nm (A_{280}) was measured. Fractions which showed high PO activity were pooled, lyophilized and applied again on the same column. This procedure was repeated until a sufficient quantity of the fraction containing the PO activity was pooled, lyophilized and used in posterior characterization studies. The lyophilized residue showed a yellow pale coloration.

After verifying that the fraction containing PO activity passed through 10 and 1 kDa membranes, we tested an alternative procedure where the cultures previously treated with PVP and lyophilized, were directly applied on the Sephadex G-10 column (1.5 $\times$ 50 cm). The column was equilibrated and eluted with 25 mM of Tris-HCl buffer, pH 7.5. Two main fractions with PO activity were obtained in this manner. The first, of high molecular mass, eluted on Vo of the column and showed a very low PO activity, and the second eluted in a low molecular mass region and showed very high PO activity. Fractions were collected and assayed as described above. The peak with PO activity eluted in the same volume as those obtained through the above procedure.

Characterization of partially purified low molecular mass component. Some kinetics and physicochemical properties of the partially purified and lyophilized LMM-component with PO activity resuspended in *dd* water were studied. The optima temperature and pH were determined as described previously with the crude extract of *T. aurantiacus* (2). Thermostability was determined by preincubation of LMMC at different temperatures or, when the pH-stability was studied, the component was preincubated in 50 mM citrate-phosphate buffer at different pH values. In both experiments, 1 mM o-dianisidine was used as substrate. Apparent Km values were calculated for o-dianisidine and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrates using ENZFITTER software (ELSEVIER BIOSOFT). Other substrates were assayed under the same conditions as o-dianisidine, as described above.

The relative molecular mass of the component with PO activity dissolved in *dd* water was estimated by gel-filtration using a Sephadex G-10 column (1.5 $\times$ 50 cm) and *dd* water as eleuent. The column was calibrated using isobutiryl coenzyme A (837.7 Da), glutathione oxidized (610 Da), flavin mononucleotide (FMN) (455.4 Da) and riboflavin (376.4 Da) as standards.

The protein content of the LMMC with PO activity was determined by the Lowry (13) and Bradford (14) methods with bovine serum albumin as the standard. Amino acid composition of the LMMC with PO activity was determined by ion-exchange chromatography on AMINOCHROM OE-914 apparatus after hydrolysis of the lyophilized residue in 6 N HCl for 24 h at 104°C.

Some structural properties of the component with PO activity were determined through spectroscopic techniques. ^{13}C and ^{1}H-NMR spectra of LMMC lyophilized and dissolved in DMSO-d_6 were obtained on a VARIAN GEMINI spectrometer at 300 Mhz. Chemical shifts are given in the δ (parts per million) scale with TMS as an internal standard. Infrared (IR) spectrum was taken over the range of 4000-500 cm^{-1} in solid KBr pellets on a PERKIN ELMER 1430 instrument. Signals or bands obtained in the NMR or IR spectra of LMMC were assigned with reference to the literature (Silverstein et al., 1981). Qualitative analysis for presence of metal in the compound was researched through techniques such as plane grating spectroscopy and X-Ray Fluorescence. Ultraviolet and visible spectra were measured with a BECKMAN DU-70 spectrophotometer over the range of 200–700 nm.

Siderophore assay. The universal siderophore detection assay, described by Schwyn and Neilands (15), was used to show siderophore production during the growth of *T. aurantiacus* in solid and liquid media. For detection in solid medium, the CAS reagent was added to modified agar-Czapek (2) without iron in the trace solution. *T. aurantiacus* was inoculated in the plates containing this medium and incubated at 48°C until reaction appeared. An orange halo surrounding a fungus colony after 6 days of growth indicated a positive CAS reaction. For detection in liquid medium, the fungus were grown under the same conditions as for PO activity production. Samples were harvested at different incubation times and 0.5 mL of filtered culture were mixed with 0.5 mL of CAS solution and the absorbance at 630 nm (A_{630}) was read after 12 h of incubation. The A630 obtained using CAS solution is inversely proportional to the concentration of siderophores (15). The fractions containing PO activity eluted from Sephadex G-10 column and the partially purified component also were tested by CAS liquid assay.

RESULTS

Partial Purification

To elucidate the component responsible for the high PO activity present in extracellular extracts of *T. aurantiacus,* the isolation and partial purification of the component with catalytic activity from wheat bran cultures was carried out. The isolation procedure included a concentration step by ultrafiltration (UF) through 10 kDa cutoff membrane. However, more than 90% of PO activity passed across of this membrane and consequently a second UF using a 1 kDa cutoff membrane, was carried out. Almost all PO activity could be detected in the filtrate $<$ 1 kDa, eliminating the possibility of ligninolytic enzyme or other protein to be responsible by PO activity of *T. aurantiacus,* since these kind

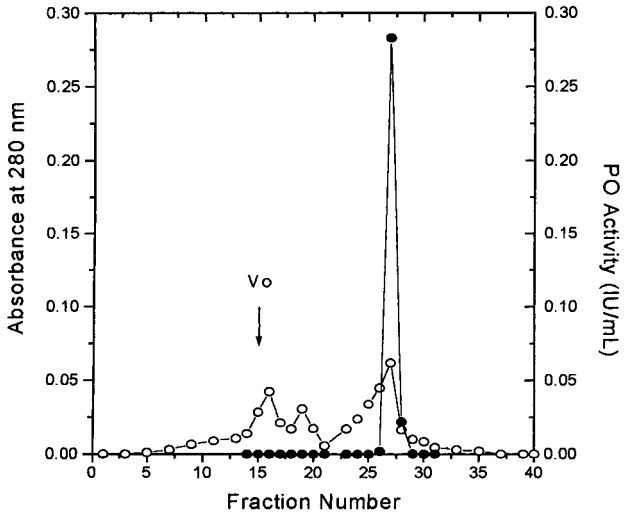

FIG. 1. Gel filtration profile on Sephadex G-10 of *T. aurantiacus* extracts prior to ultrafiltration (1 kDa cutoff membrane). The samples were applied on Sephadex G-10 column (1.5 × 50 cm), equilibrated, and eluted with distilled and deionized water (pH 6.2). Fractions of 2.5 mL were collected at 0.21 mL min^{-1}, and the absorbance at 280 nm (○) and PO activity as o-dianisidine oxidation (●) were determined.

of enzymes have high molecular mass. During the isolation procedure, other techniques such as dialysis and ammonium sulfate precipitation were applied unsuccessfully. After dialysis of the fungal extracts, the PO activity was lost and more than 90% of the activity was detected in the outside solution (dialysate) and the PO activity was not precipitated by salt treatment. All these results were unexpected and consequently reoriented our isolation scheme.

After different tests were carried out with the aim of isolate the component with PO activity, two simple procedures that yielded similar results (only one peak of high PO activity) were choose. A procedure using a UF through 1 kDa cutoff membrane of the extracts previously treated with PVP was carried out. Next, the filtrate (< 1 kDa) was lyophilized and put on a Sephadex G-10 column (Figure 1). The gel filtration profile of the filtrate, containing high PO activity, shows three peaks of A_{280} and only one peak of PO activity determined by o-dianisidine oxidation. The fractions (No 27–29) comprising peak of PO activity were pooled, lyophilized and re-applied on the same Sephadex G-10 column. Only one peak of A_{280} and PO activity was obtained, which was collected and lyophilized for posterior analysis.

The other procedure, the extracts, previously treated with PVP, was lyophilized, ressuspended in water and directly applied on a Sephadex G-10 column. Under these conditions, three peaks of A_{280} and two peaks of PO activity were detected (Figure 2). The major peak of PO activity appeared in the low molecular mass fractions (No 26–31), and one minor peak in the void volume (Vo) fractions (No 15–17). The major peak was pooled, lyophilized and again applied on the same Sephadex G-10 column. The gel filtration profile of the major peak of PO activity yielded only one peak with activity.

The characterization studies presented in this paper were carried out with the LMMC with PO activity isolate and partially purified from *T. aurantiacus* cultures through the first procedure here described, which include an UF by 1 kDa cutoff membrane. The results exclude the possibility that a ligninolytic enzyme is responsible by PO activity present in the *T. aurantiacus* extracts and suggests the presence of a low molecular mass component (LMMC) with phenoloxidase-like catalytic activity.

Kinetics Properties

A summary of the kinetic properties of partially purified LMMC with PO activity is presented in the Table 1. Previous results showed that crude extracts of *T. aurantiacus* oxidizes a wide range of phenolic and nonphenolic typical phenoloxidase substrates at pH 3.0 (2). The component with PO activity was able to oxidizes the same substrates as the crude extract. However, the oxidation rates attained with LMMC exhibited high values. The substrates efficiently oxidized by LMMC include ABTS, 2,6-dimethoxyphenol, o-dianisidine and 5-hydroxyindol, whereas syringaldazine and guaiacol were slightly oxidized (Table 1). Furthermore, veratryl alcohol and tyrosine were not oxidized under our assay conditions, which corroborate results obtained with

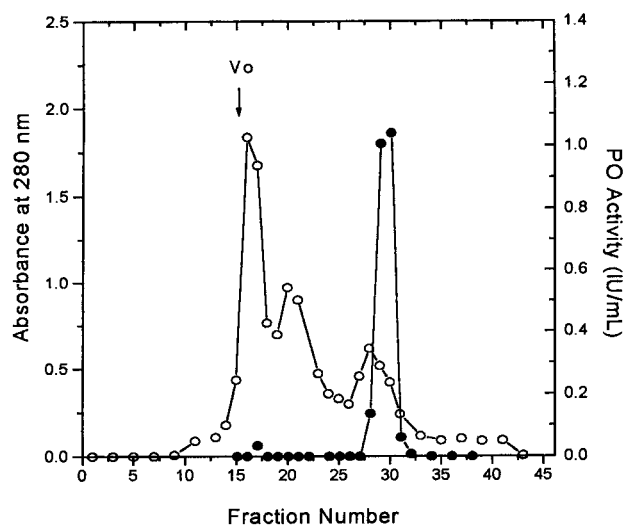

FIG. 2. Gel filtration profile on Sephadex G-10 of *T. aurantiacus* extracts previously treated with PVP and lyophilized. The samples were applied on Sephadex G-10 column (1.5 × 50cm), equilibrated and eluted with 25 mmol L^{-1} Tris-HCl buffer (pH 7.5). Fractions of 2.5 mL were collected at 0.21 mL min^{-1}, and the absorbance at 280 nm (○) and PO activity as o-dianisidine oxidation (●) were determined.

TABLE 1

Kinetic Properties of the LMMC Partially Purified from *T. aurantiacus*

Property	Low-molecular mass component
pH optimum	2.8
Temperature optimum	80°C
Km o-dianisidine	28.5 μmol L^{-1}
ABTS	8.7 μmol L^{-1}
Substrates oxidized	o-Dianisidine (100)[a]
	ABTS (561.3)
	2,6-DMP (170)
	5-Hydroxindol (58.1)
	Syringaldazine (2.9)
	Guaiacol (2.0)

[a] Values in parentheses correspond to the relative PO activity to 1 mmol L^{-1} o-dianisidine oxidation at pH 2.8 in 50 mmol L^{-1} citrate-phosphate buffer.

crude extracts of *T. aurantiacus* (2). LMMC oxidized all substrates in the absence of H_2O_2 in the same manner as observed with crude extracts. However, in contrast to that observed with the crude extract, the addition of 1 mM H_2O_2 to the reaction mixture containing LMMC increased the o-dianisidine oxidation rate by 1.5 times.

The LMMC appeared to be active in a narrow and acidic pH range (2.6–3.0), with an optimum at 2.8 for o-dianisidine oxidation. Over pH 6.0, small or no PO activity was observed. For the other substrates tested, the same range of optimum pH was obtained (data not shown). When the stability of the component was studied after pre-incubation in citrate-phosphate buffer at 25°C, a very different behaviour was observed. While LMMC showed maximal PO activity in an acidic pH range, their stability at these pH values was very low (Figure 3). The LMMC presented maximum stability between pH 5 and 6. At pH 2.6 and 2.8, the component was completely inactivated after 3 and 7 h of incubation, respectively.

The optimum temperature for o-dianisidine oxidation by LMMC was reached at 80°C (working limit of the spectrophotometer). During 5 h of incubation at 50°C, the component remained completely stable, while at 80°C about 85% of residual PO activity was maintained (Figure 4). The LMMC showed a high thermostability, maintaining more than 50% residual PO activity after 4 h of incubation at 100°C.

Physicochemical Properties

To determine the nature of the LMMC of *T. aurantiacus* with catalytic activity, several physicochemical properties of the fraction partially purified by Sephadex G-10 were studied.

During the purification procedure, component with PO activity presented a molecular mass lower than 1,000 Da, since the active component passed through 1 kDa cutoff membrane. In fact, the estimated molecular mass by Sephadex G-10 showed that the partially purified component with PO activity has a relative molecular mass of 530 Da (Table 2). Attempts to determined the molecular mass of LMMC by mass spectrometry were unsuccessful because the molecular ion was not detected and only several low-molecular mass peaks were observed.

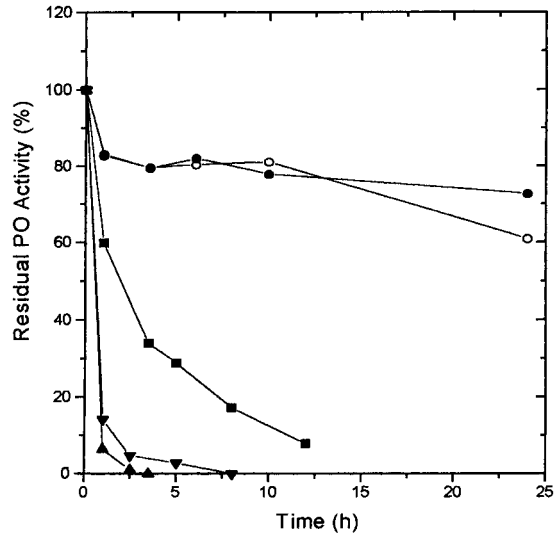

FIG. 3. Residual activity of LMMC partially purified from *T. aurantiacus* after preincubation at different pH values. One hundred percent activity refers to 0.75 U/mL, using 1mM o-dianisidine in 50 mmol L^{-1} citrate-phosphate buffer at pH 2.6 (△), 2.8 (▽), 3.5 (■), 5.0 (○) and 6.0 (●).

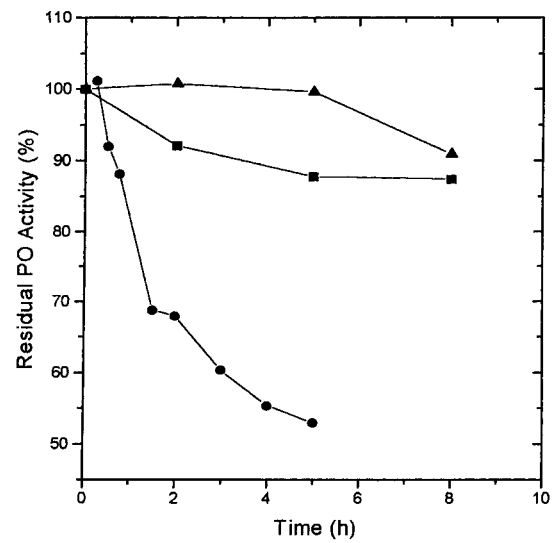

FIG. 4. Residual activity of LMMC partially purified from *T. aurantiacus* after preincubation at different temperatures. One hundred percent activity refers to 0.75 U mL^{-1}, using 1mM o-dianisidine in 50 mmol L^{-1} citrate-phosphate buffer (pH 2.8) at temperatures of 50 (△), 80 (■), and 100°C (●).

TABLE 2

Physico-Chemical Properties of the LMMC with PO Activity Partially Purified from *T. aurantiacus*

Property	Low-molecular mass component (LMMC)
Molecular mass	530 Da (Sephadex G-10)
Protein reaction	Bradford (negative), Lowry (positive)
Amino acid residues	Not detected
Structure	Hydroxamate derivative (IR, ^{1}H- and ^{13}C-NMR)
Metal	Fe, Ca, Mg (X Ray-Fluorescense, PGS)
CAS assay	Negative

Note. PGS: plane grating spectroscopy.

The protein determination in the LMMC was carried out by two methods. When the LMMC was analyzed by Lowry method, a development of the typical color (accompanied by increase in absorbance at 660 nm) reaction was observed. However, the LMMC did not react with Coomassie brilliant blue dye (Bradford method) as observed by absence of color development when compared with a control sample. Thus, since the LMMC showed significant absorption at 280 nm and reacted positively in the Lowry method, but it fails to reacted in the Bradford method, the protein content was measured by an amino acid analyzer. For this, the LMMC was submitted to acid-catalyzed hydrolysis (HCl 6N/ 104°C, 24 h) and then analyzed by ion-exchange chromatography. After hydrolysis, the LMMC completely lost the PO activity and the chromatogram of hydrolysate showed total absence of amino acids. However, under acid conditions the amino acid tryptophan is destroyed and consequently, we can not assert that this amino acid is not present in the LMMC.

The absorption spectra obtained for partially purified LMMC dissolved in *dd* water (pH 7.6) showed three absorption bands: one at 360 nm, one at around 280–290 nm and the third and most intense at about 230 nm. Variation of pH to 12 or 1.5 did not shifted the absorption bands, however, an slight increase on the intensity could be observed.

In order to elucidate some structural properties of the LMMC, IR and NMR spectroscopies studies were carried out. The IR spectrum showed two characteristic bands that corresponding to carbonyl stretching. One band at 1,550 cm^{-1} was attributed to the carbonyl group of carboxylic acid and the other at 1,632 cm^{-1} to the carbonyl group of amide. The ^{1}H and ^{13}C-NMR spectra of LMMC showed few signals. The main signals at 3.4 and 8.35 ppm in the ^{1}H-NMR spectrum were attributed to -CH_2O and -CH_2N, respectively. ^{13}C-NMR spectrum showed two signals at 59 and 60 ppm which were attributed to -CH_2O and -CH_2N. The distortionless enhancement by polarization transfer (DEPT) experiment revealed the presence only of *CH2* and *C0* type carbons (quaternary) in LMMC, eliminating the possibility of -CH and -CH_3 groups presence in its structure.

For detection of metal ions in the structure of LMMC, several techniques were used. The combination of X-Ray-Fluorescence and plane grating spectroscopy revealed the presence of iron, calcium and magnesium in the compound. The concentrations of calcium and magnesium were higher than that of iron as estimated by Inductively Coupled Plasma (ICP) and atomic absorption (data not showed). All these metals probably are derived from wheat bran and from the Czapek medium, which contains high calcium and magnesium and only traces of iron (0.36 μM). Nevertheless, the quantification of metal ions needs to be repeated to corroborate these results. Among the metal ions detected in the LMMC, only the iron could be participating in the oxidation reactions such as o-dianisidine and ABTS oxidations (Table 1).

These results demonstrate that LMMC with high PO activity purified from *T. aurantiacus* cultures is an extracellular compound of low-molecular mass and non-proteic nature. No amino acid were found in its structure, but under our analysis conditions, the presence of tryptophan in the LMMC could no be eliminated. However, by IR and NMR spectra showed the absence of aromatic groups in the compound and based on data in the literature (16,17) one can suggest a hydroxamic acid derivative structure. Atkin and Neilands (17) attributed the IR band at 1.594 cm^{-1} to carbonyl group of hydroxamic acid (C=O stretching) of the rhodotorulic acid siderophore. However, the authors emphasize that this band is in a region below of those normally observed for acetohydroxamic acid and desferri-ferrichrome (1,630–1,660 cm^{-1}). Thus, partially purified LMMC have similar characteristics as siderophores which are iron-binding compounds of low-molecular mass produced and excreted by many fungi and bacteria.

Siderophore Detection

Since the physicochemical studies of LMM-component suggested the presence of a compound with characteristics of siderophore, the detection of these compounds using typical assays was carried out. The universal CAS assay as described by Schwyn and Neilands (15) was used for detection of siderophore production by *T. aurantiacus* in solid and liquid media. When the fungus was inoculated on CAS-blue agar plates, the growth was very poor. However, a small orange halo appeared surrounding the fungal colony. In liquid cultures containing glucose (0.5%) or wheat bran (1.5%) as substrates, the fungus produced compounds which reacted positively with CAS after two or three days of incubation. Nevertheless, when the maximum PO activity was attained in the extracts (approx. 15 days), no CAS reaction could be detected (data not shown). The

fungal extracts reacted slowly with CAS reagent developing a reddish-orange coloration. However, the fractions with PO activity (N° reagent and even after the concentration step by lyophilization, the LMMC did not react with CAS.

DISCUSSION

We previously described the production of high levels of phenoloxidase activity by *T. aurantiacus* growing in liquid medium. The effect of several culture conditions on production of this activity was studied and the crude extracts containing activity were characterized (2). The activity present in the crude extracts showed characteristics of phenoloxidase laccase-type except for the high optimal temperature (70−80°C). On the other hand, extracts semi-purified from this fungus, with high PO activity, showed capability for kraft pulp bleaching and effluent mineralization (3). Thus, in order to elucidate the nature of the system (enzymatic or non-enzymatic) responsible for the catalytic activity of *T. aurantiacus,* we tried to isolate and characterize a component containing high PO activity from crude extracts of fungus growing in liquid medium.

The crude extracts were filtered and after treatment with PVP were concentrated by ultrafiltration (UF) using a 10 kDa cutoff membrane. Surprisingly, more than 90% PO activity passed through of these membrane and therefore the active filtrate (< 10 kDa) was submitted to a second UF using a 1 kDa cutoff membrane. Again, all PO activity was detected in the filtrate (< 1 kDa). These results were unexpected and eliminated the possibility that ligninolytic enzymes or other proteins of high molecular mass are responsible for PO activity of *T. aurantiacus.* The last filtrate (< 1 kDa) was then applied on Sephadex G-10 column and an only one peak with PO activity which eluted in a low-molecular mass region was obtained (Figure 1). Several applications with this column allowed us to obtain a sufficient quantity partially of purified LMMC.

However, when the extracts were applied directly on Sephadex G-10, without the prior UF step, two peaks of PO activity were obtained. One major peak that eluted in a low-molecular mass region and other small peak with activity that eluted in the Vo of the column (Figure 2) which could be related to the presence of typical ligninolytic enzymes. Several ligninolytic activities besides the PO activity describe here were previously detected in the *T. aurantiacus* cultures growing on *E. grandis* sawdust (1).

The PO activity of the partially purified LMMC showed similar kinetic properties as those of the crude extracts, except that these showed a activity which was not affected by addition of H_2O_2 or catalase in the reactional medium (2). The LMMC oxidized several laccase-typical substrates at pH 2.8 in the absence of H_2O_2, however, the addition of H_2O_2 to the reactional medium caused an increase of PO activity. The results of substrate specificity (Km) of LMMC with ABTS and o-dianisidine (Table 1) were comparable with those described in the literature for laccases from ligninolytic fungi (18,19). In addition, although the maximal o-dianisidine oxidation was observed at acid pH values (pH 2.8-3.2), the LMMC quickly lost its activity after pre-incubation at these pH values. The optimum pH (2.8) was within the range observed for the ligninolytic enzymes (lignin-peroxidase, Mn-peroxidase and laccase) isolated from many of wood-rot fungi. These results shows one to confirm that although the LMMC does not have an enzymatic nature, the kinetic properties displayed by it were very similar those of typical phenoloxidases, except for the high optimum temperature and elevated thermostability.

Several studies have established that low-molecular mass compounds from white- and brown-rot fungi may play a direct role in the wood decay process (4,5). Iron-binding compounds or Gt chelators with siderophore characteristics (molecular mass < 1000 Da) from *G. trabeum* were isolated through UF and ethyl acetate extraction and characterized as phenolate derivatives. The fraction extracted under those conditions showed high CAS reactivity. Gt chelators can mediated redox reactions, such as 2-keto-4-thiomethylbutyric acid (KTBA) oxidation through the systems Fe(II)/H_2O_2 or Fe (III)/H_2O_2 under air at pH values below neutrality. In the later system, the compound chelate Fe(III) and reduce it to Fe(II) to initiate the Fenton reaction (9,10,20). This iron reduction is favored at pH below 4.5 and at pH ≥ 7, the reduction is greatly limited (10).

Low-molecular mass substances (1,000−5,000 Da) were also isolated from *G. trabeum*, *P. chrysosporium* and other wood-rot fungi (8,11). These substances, purified by Sephadex G-50 and G-25, were characterized as glycopeptides containing carbohydrates and Fe (II). Even when these substances showed structural properties different from those of Gt chelators, the redox reaction mechanisms of both compounds were similar. Glycopeptides catalyzed one-electron oxidation reactions, such as KTBA oxidation and reduction of Fe(III) to Fe(II) (11) are dependent on H_2O_2. LMMC from *T. aurantiacus* showed some similar properties with Gt chelators and glycopeptides (low-molecular mass, iron presence). However, IR and NMR revealed the absence of aromatic groups in the structure of LMMC and indicated that it is composed of hydroxamic acid derivatives. At the same time, the absence of amino acids in the structure excluded the peptidic nature of component. Despite its nature, LMMC showed reactivity with Lowry protein assay which is based on the reaction between Folin reagent and specially when a a peptide bond in a tyrosine and tryptophan moieties are presents in a protein. This can be explained because the Folin reagent under tbe same conditions of Lowry

assay also is used for hydroxamate-type siderophores determination (21,22). Therefore, the positive reaction in Lowry assay provides further evidence of the presence of a polyhydroxamate derivative in LMMC. Furthermore, LMMC showed efficient capability to oxidize a range of substrates typical of phenoloxidases in the absence of H_2O_2 at pH 2.8 and elevated temperatures which was not demonstrate for those compounds. Preliminary assays with o-phenantroline revealed that *T. aurantiacus* LMMC in the same form as Gt chelators and glycopeptides are capable of reducing Fe(III) to Fe(II) (unpublished data).

CAS universal assay for siderophore detection indicated the production of these compounds by *T. aurantiacus* in solid and liquid media. However, partially purified LMMC did not react with CAS. Probably, the presence of metals in LMMC may be responsible for the lack of reactivity because the iron present in CAS reagent can be only remove by a non-complexed siderophore. Hence, the iron presence in LMMC may be responsible for both catalytic activity and CAS negative reaction.

More extensive studies are necessary for complete elucidation of structure of LMMC with PO activity. LMMC produced by *T. aurantiacus* was isolated by us in a complexed form and it probably interfered with most methodologies of characterization. It has been reported that the iron must be removed from the siderophore before any characterization studies principally through NMR, because the presence of Fe (III) in compound eliminate signals from the spectra (23,24). However, although the complete structure of LMMC remains to be elucidated, it is clear from our studies that the partially purified compound presents characteristics of hydroxamate-type siderophore complexed with iron and other metals. Very recently, we have published hydroxamate iron complex with phenoloxidase activity with the same characteristic of this metabolite (25). These results also suggested that the LMMC with high PO activity from *T. aurantiacus* must be related with the ability of fungus to degrade *E. grandis* wood, bleached kraft pulp and decontamined kraft effluent as previously described (1–3).

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