Effect of manganese on the secretion of manganese-peroxidase by the basidiomycete Ceriporiopsis subvermispora

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1. Introduction

Ceriporiopsis subvermispora is a white-rot basidiomycete that preferentially degrades lignin over polysaccharides when growing on wood (Akhtar et al., 1992; Ferraz et al., 2003; Costa et al., 2005; Fackler et al., 2007). Its ligninolytic machinery is composed mainly of two kinds of enzymes: a manganese-dependent peroxidase (MnP) and a copper-containing enzyme known as laccase. The former is a heme-center-containing glycoprotein that catalyzes the H2O2-dependent oxidation of Mn3+ to Mn4+ (Glenn and Gold, 1985; Paszczynski et al., 1985). Mn3+ is then complexed by dicarboxylic organic acids for the subsequent oxidation of a wide variety of phenolic compounds deriving from lignin (Wariishi et al., 1988, 1989). Laccase, on the other hand, is a phenol oxidase member of the multicomponent oxidase family of proteins that includes mammalian ceruloplasmin, plant ascorbate oxidases and fungal Fet3 ferroxidases, among other proteins (Solomon et al., 1996; Hoegger et al., 2006). Laccase is proficient in the one-electron oxidation of a variety of phenolic and non-phenolic compounds, with the concomitant reduction of O2 to H2O (Solomon et al., 1996; Baldrian, 2006). In C. subvermispora, MnP and laccase are encoded by three genes, respectively, with their respective alleles (Lobos et al., 1994, 1998; Karahanian et al., 1998; Tello et al., 2000, 2001; Larrondo et al., 2001, 2003).

Manganese is an abundant and essential trace element among transition metals. It has been recognized to be essential for numerous physiological functions that include the metabolism of carbohydrates, lipids and proteins besides serving as cofactor for several enzymes (Keen et al., 1984, 2000; Crowley et al., 2000; Cullotta et al., 2005). On the other hand, studies with ligninolytic fungi such as Phanerochaete chrysosporium (Brown et al., 1991), Dichotomus squamens (Perié and Gold, 1991), Trametes versicolor (Johansson et al., 2002) and Pleurotus ostreatus (Cohen et al., 2002) have demonstrated that there is a correlation between transcript levels and MnP production. This suggests a transcriptional role for Mn2+, which would likely imply a putative transcription factor that has not yet been identified. In this regard, a novel hypothetical promoter sequence found in the mnp1 gene from P. chrysosporium has been reported, supporting the idea of a metal-mediated transcriptional regulation of MnP-encoding genes (Ma et al., 2004). In addition to this effect at the transcriptional level, our laboratory has suggested that at least in C. subvermispora, manganese may also have a role at a post-transcriptional level. This conclusion is based on experiments conducted at different Mn2+ concentrations, in which an inverse correlation between mnp-transcript levels and MnP extracellular activity is observed (Manubens et al., 2003).

In this work we provide evidence confirming the aforementioned hypothesis. Using absolute RT-qPCR quantification, high levels of mnp transcripts were observed in the absence of Mn2+,
although almost no MnP was detected in the extracellular medium. Nevertheless, under these conditions, the enzyme was identified in intracellular vesicles and compartments. Upon addition of Mn²⁺, the enzyme was secreted to the culture medium even in the presence of cycloheximide, indicating that in C. subvermispora manganese is involved in the post-translational control of MnP production, by affecting secretion.

2. Materials and methods

2.1. Strain and culture conditions

C. subvermispora strain FP-105752 was obtained from the Center for Mycology Research, Forest Products Laboratory, Madison, Wisconsin, USA. The fungus was maintained on slants of potato dextrose agar (Difco). Stationary cultures grown with 5 μM MnSO₄ were used for the preparation of inocula. Liquid cultures of C. subvermispora were grown for 10 days at 30 °C with orbital shaking (180 r.p.m.) in 250 ml Erlenmeyer flasks containing 100 ml of salts medium with 0, 20, 80, 160 and 320 μM MnSO₄ among other salts (Rüttimann et al., 1992). Mycelia were frozen in liquid nitrogen and kept at −80 °C, no longer than 2 weeks, until RNA extraction.

2.2. RNA extraction and quantitative Real-Time RT-PCR (RT-qPCR)

Frozen mycelia were processed for mRNA extraction from total RNA using Dynabeads (Invitrogen), accordingly with the manufacturer’s directions. Thereafter, this mRNA preparation was used for cDNA synthesis using the MMLV reverse transcriptase (Invitrogen). The complete procedure and control experiments intended for RT-qPCR have been described previously (Canessa et al., 2008). Additionally, mRNA quantification was determined using RiboGreen dye (Invitrogen) according to the manufacturer’s instructions. Quantification of mnp transcript levels was performed in real time using the Brilliant SYBR Green QPCR Master Reagent kit and the Mx3000P detection system (Stratagene). Primer sequences and predicted Tₘ values, as well as amplicon length, are shown in Table 1. Transcript levels are expressed as copies of the housekeeping gene (mnp gapdh−¹). All experiments were performed in three biological and technical replicates. Detailed RT-qPCR methodology (Bustin et al., 2009) is available as Supplementary information with the online version of this paper.

2.3. Enzymatic assay

MnP activity was measured at 30 °C in a Shimadzu UV–VIS model 160 as described previously (Paszkynski et al., 1985). Reaction mixtures (1 ml) contained 100 mM sodium tartrate pH 5.0, 100 μM MnSO₄, 100 μM H₂O₂ and 0.4 mM 2,6-DMP (2,6-dimethoxyphenol). One unit of MnP activity was defined as the amount of enzyme required to oxidize 1 mmol of 2,6-DMP min⁻¹. For MnP activity normalization (Mn²⁺ affects fungal growth), triplicate flasks for each culture condition were harvested and filtered to determine mycelial dry weight as described previously (Manubens et al., 2007). Consequently, MnP activity is expressed as U ml⁻¹ g⁻¹. Since both MnP and laccase can oxidize 2,6-DMP, laccase activity was subtracted by control experiments performed in the absence of H₂O₂ and manganese.

2.4. Anti-MnP antibody synthesis

A female rabbit was initially injected subcutaneously with 200 mg of a synthetic peptide comprising conserved amino acids among the three MnPs described in C. subvermispora (NH₂-KTTGPQDLQTC-OOH). This peptide was chemically coupled to the KLH peptide carrier (keyhole limpet hemocyanin, Calbiochem) in complete Freund’s adjuvant. Thereafter, three 200 mg aliquots of the synthetic peptide-KHL in incomplete Freund’s adjuvant were subsequently injected at 3 weeks intervals. Antibody titers were monitored by ELISA assays (data not shown).

2.5. Western blot

Supernatants from manganese-supplemented cultures were concentrated 25-fold by ultrafiltration using a 10 kDa cut-off membrane. Proteins (10 μg per lane) and prestained molecular mass marker (Fermentas) were first separated by SDS–PAGE using standard procedures and then transferred to a nitrocellulose membrane for 3 h at room temperature and 150 mA, in a BioRad chamber containing transfer buffer (25 mM Tris–HCl pH 8.4, 192 mM glycine and 20% (v/v) methanol). The nitrocellulose membrane was incubated with gentle agitation for 30 min at room temperature in PBS solution containing 3% (w/v) bovine serum albumin (PBS–BSA) and thereafter immersed in anti-MnP serum diluted 1000-fold in PBS–BSA for 30 min. The nitrocellulose membrane was washed three times for 5 min with 10 ml PBS solution. Thereafter, the membrane was incubated for 30 min in a solution containing an anti-IgG antibody conjugated to alkaline phosphatase (BioRad; 1:2000 dilution in PBS–BSA). The nitrocellulose membrane was washed as before and developed by soaking it for 5–15 min in alkaline phosphatase buffer (100 mM Tris–HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂) using the Alkaline Phosphatase Conjugate Substrate Kit (BioRad) containing nitroblue tetrazolium (NBT) and 5-bromo–4-chloro–3-indoly phosphate (BCIP) as substrates, according to manufacturer’s instructions.

2.6. Effect of Mn²⁺ on MnP secretion

First, the effectiveness of cycloheximide (CHX) as inhibitor of protein synthesis and the protocol previously established for that purpose (Pakula et al., 2003) was determined as follows: cultures were grown for 10 days in the absence of manganese and then supplemented with CHX (250 μg/ml). Ten minutes later, 35S-methionine (0.5 mCi, New England Nuclear) was added to the cultures during an additional period of 50 min. Thereafter, cultures were supplemented with MnSO₄ (194 μM final concentration) (the standard Mn²⁺ concentration; see Rüttimann et al. (1992)) and further incubated during 3 or 6 h. Secreted proteins were concentrated with 20% TCA and radioactive incorporation was measured in a scintillation counter (Beckman LS 6500). Values were normalized with the sample’s protein concentration (cpm mg⁻¹). In addition, MnP specific activity was determined in samples taken from culture supernatants. A similar procedure was also carried out from intracellular proteins purified from mycelia extracts as previously described (Aguilar et al., 1999, data not shown).

Table 1

<table>
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<tr>
<th>Primer name</th>
<th>Sequence (5’–3’)</th>
<th>Use</th>
<th>Tₘ (°C)</th>
<th>Amplicon (bp)</th>
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2.7. Immunodetection of intracellular MnP, mycelia samples from 10-day old cultures were harvested and fixed immediately for 3 h at 4 °C in 3% (v/v) paraformaldehyde containing 0.5% (v/v) glutaraldehyde, in 0.1 M phosphate buffer, pH 7.2. Samples were then washed three times during 15 min. in the same buffer. After this procedure, samples were further washed in distilled water and then dehydrated using ethanol in increasing concentrations (50%, 70%, 95% and 100%) during 20 min. each time. Thereafter, samples were preincluded in epon:acetone (1:1) for 4 h (Medcast) and finally included in LR-White resin. Samples were then polymerized at 50 °C for 18 h and sectioned with a Sorval MT2-B ultramicrotome and collected on nickel grids. Samples were then blocked in 1% PBS–BSA and incubated with anti-MnP serum (1:1000) in 0.1% PBS buffer. After incubation with the antisera, sections were washed three times in a solution containing 0.1% PBS–BSA and 0.05% Triton X-100, followed by incubation with protein A (1:100) coupled to colloidal gold (20 nm). Thereafter, samples were washed three times with the same solution mentioned above and finally, in distilled water. Grids were observed without post-staining in a Philips Tecnai 12 transmission electron microscope operated at 80 kV.

3. Results

3.1. Effect of Mn2+ on mnp transcript levels

C. subvermispora was grown in the presence of different Mn2+ concentrations (see Section 2) and mnp transcripts were analyzed by RT-qPCR in order to establish their absolute abundance. Fig. 1 shows that high levels of mnp2 transcripts were obtained at all Mn2+ concentrations tested. mnp1 showed a remarkable low expression, reaching levels that are 100- to 350-fold lower than those of mnp2. In turn, mnp3 transcripts were almost negligible. On the average, 3.7 mnp3 transcripts per gapdh were identified. Interestingly, mnp2 was the only gene whose transcript levels were induced by Mn2+, reaching a peak at 20 μM of this metal. In contrast, mnp1 transcript levels were down-regulated with increasing manganese concentrations. In summary, this data reveals that most of the mnp transcripts in liquid cultures of C. subvermispora derive from the mnp2 gene.

3.2. Effect of Mn2+ on extracellular MnP production

Levels of extracellular MnP activity were measured in culture supernatants after 10 days of growth using the same Mn2+ concentrations employed above. Very low MnP activity was detected in cultures grown in the absence of Mn2+ (Fig. 2A), in spite of the presence of significant mnp1 and mnp2 transcript levels (Fig. 1). The highest MnP activity was observed at 20 μM Mn2+ (Fig. 2A). We also analyzed extracellular MnP by Western blot hybridization (Fig. 2B). This assay showed a positive correlation between MnP enzymatic activity and protein levels, implying that neither there is a correlation between MnP protein levels and mnp transcript levels. This situation is particularly evident in cultures lacking Mn2+.

3.3. Immunolocalization of intracellular MnP in C. subvermispora

These results strongly suggest that manganese could have a post-transcriptional role in the production of extracellular MnP. With this hypothesis in mind, the presence of intracellular MnP was determined in mycelium obtained from cultures grown at 0 and 160 μM Mn2+. The immunogold micrographs depicted in Fig. 3 show significant quantities of intracellular MnP protein in both culture conditions, indicating that the enzyme is synthesized in the absence of the metal. Interestingly, either in the presence or in the absence of metal, the immunomarker was predominantly located in the peripheral part of the hyphae, near the cell wall and inside membrane vesicles. Similar results were obtained using 20 or 320 μM Mn2+ (data not shown).

3.4. Effect of Mn2+ on the secretion of MnP

Since the MnP protein was detected in intracellular compartments in cultures lacking manganese, we decided to analyze...
whether the addition of Mn$^{2+}$ to the medium would promote secretion of MnP to the extracellular medium. To assure that any MnP detected in the liquid medium had been synthesized prior to the addition of the metal, we designed a protocol involving the protein synthesis inhibitor cycloheximide (CHX). First, we confirmed that CHX does indeed inhibit protein synthesis in the culture conditions used. Before the addition of Mn$^{2+}$ (see Section 2), no significant variations in the protein’s fraction radioactive incorporation were observed in cultures supplemented or not with CHX ($0.145 \pm 0.021 \times 10^{-6}$ cpm/mg). In the absence of CHX, a 4.9- and 5.7-fold increment in the radioactive incorporation were observed after 3 and 6 h of supplementation with manganese, respectively. In contrast, in the presence of CHX, a 2.7-fold increment was observed after 3 h of the manganese pulse that remains constant after 6 h with the metal ($0.395 \pm 0.007 \times 10^{-6}$ cpm/mg). An analogous result was obtained from intracellular protein fractions (data not shown). Since no increment in the radioactive incorporation was observed, between 3 and 6 h after the treatment with CHX, the protein synthesis was blocked in the analyzed period of time. Consequently, after 6 or 10 h in the presence of Mn$^{2+}$ and CHX, the effect of the metal on the secretion of MnP was determined as indicated in Methods. As shown in Fig. 4, a pulse of MnSO$_4$ in 10-day-old cultures lacking this metal led to the appearance of MnP activity in the culture fluid. Interestingly, the enzymatic activity was somewhat higher in the cultures that did not contain CHX (see Section 4).

4. Discussion

In this work we first corroborated the effect of Mn$^{2+}$ on mnp transcript levels in C. subvermispora. Although preliminary results on this subject had been previously reported by our group (Manubens et al., 2003), the new data established that transcripts of mnp2 are the most abundant among all MnP-encoding genes in this fungus, at least under the culture conditions tested. While previous data had already suggested that, the use of Real-Time RT-qPCR allow us to absolutely and independently quantify the expression of the different mnp genes. We also confirmed that there is no correlation between transcript levels and extracellular MnP activity (Manubens et al., 2003), a phenomenon most clearly observed in
the absence of manganese. This observation led us to postulate a post-transcriptional role of manganese in the production of extracellular MnP activity in Ceriporiopsis subvermispora (Manubens et al., 2003). In the present work, we have also observed this lack of correlation by Western blotting, ruling out the presence of inactive extracellular MnP in the absence of manganese.

In a similar vein, cultures of P. chrysosporium grown in a low concentration of manganese and in temperature-stress conditions show high levels of mnp transcripts with no MnP in the extracellular medium (Brown et al., 1993). Likewise, in low-glucose cultures of P. chrysosporium, an analogous result has also been obtained for a lignin peroxidase (LiP) gene. In this case, high expression levels were observed, without a concomitant appearance of LiP activity in the culture supernatants (Broda et al., 1995). In Pleurotus eryngii, highest levels of a versatile peroxidase mRNA were obtained without added Mn2+ (Ruiz-Dueñas et al., 1999). More recently, the lack of correlation between mnp transcripts levels and MnP activity in cultures of the white-rot fungus Phlebia sp. strain MG-60 also led to the proposition of a post-transcriptional role of Mn2+ in the MnP production (Kamei et al., 2008). In contrast, other ligninolytic fungi exhibit a linear correlation between MnP-encoding transcripts and extracellular MnP activity at various concentrations of Mn2+ (Brown et al., 1991; Perié and Gold, 1991; Cohen et al., 2002; Johansson et al., 2002). On the other hand, in C. subvermispora we have also observed a linear correlation between laccase transcripts and its corresponding enzyme activity at different Mn2+ concentrations (Manubens et al., 2007).

Not much is known about the regulation of gene expression by manganese at a post-transcriptional level. Nevertheless, one of the few examples includes Saccharomyces cerevisiae, where manganese affects the production of the Smt1 and Smt2 Nramp manganese transporters (Cullotta et al., 2005). In manganese-deficient conditions and in agreement with their physiological function, transcripts levels of both transporters increase. However, when cultures are supplemented with the metal, the levels of their corresponding encoded proteins are remarkably diminished at the plasma membrane and post-Golgi vesicles, respectively. In this post-transcriptional regulatory mechanism, both proteins are targeted to the vacuole by the endoplasmic reticulum protein Bsd2p, where they are degraded by proteases. The mechanisms that control all these processes are still a matter of debate (Liu and Cullotta, 1999; Van Ho et al., 2002; Cullotta et al., 2005).

To date, there is little information regarding a possible role of manganese in protein secretion. Notably, it has been reported that the metal is able to stimulate the release of hypothalamic luteinizing hormone in rats through a cGMP/protein kinase G pathway, but to our knowledge no information is available for other species (Lee et al., 2007; Prestifilippo et al., 2007, 2008). In P. chrysosporium, production of extracellular MnP involves autolysis of post-Golgi vesicles by hydrolases (Jimenez-Tobon et al., 2003). Since we observed that in cultures lacking Mn2+ MnP localizes in the fungal hyphae, mainly within the cell wall and post-Golgi vesicles, it is conceivable that in C. subvermispora manganese may influence the protein secretion.

Although immuno localization experiments were not intended for protein quantification, no significant differences in MnP were observed within intracellular compartments in cultures grown in the absence or presence of manganese. A pulse of Mn2+ in the presence of CHX confirmed that the enzyme was present inside the cell and that the mechanism of MnP secretion is most probably independent of protein synthesis. On the other hand, the higher titers of MnP in the cultures lacking CHX most likely are due to de novo enzyme synthesis after the pulse of Mn2+.

Accumulation of intracellular MnP in the absence of Mn2+ is somewhat intriguing. In order to be sure that the antibody is actually detecting MnP, and that there is no cross-reaction with other proteins, the immune antisera was used to determine its specificity against heterologously expressed MnP from C. subvermispora using Aspergillus nidulans (Larrondo et al., 2001). Also, no cross-reaction was observed with the pre-immune antisera (data not shown). Since it is well known that the metal is essential for the MnP catalytic activity (Glenn and Gold, 1985; Paszczynski et al., 1985), one may think that secretion of the enzyme would be unnecessary. Then, what would be the purpose of producing intracellular MnP if it is not going to be utilized? One possibility is that it would allow a faster response to initiate ligninolysis in the case that the metal becomes available. On the other hand, we have observed that the extracellular laccase titers are highest in cultures with low Mn2+ (Manubens et al., 2007). Being laccase activity a source of reactive oxygen species (ROS), it is conceivable that membrane-bound MnP, utilizing low or trace levels of Mn2+, may serve as a defense mechanism for protection against these harmful compounds. It must be kept in mind that the ROS scavenging capacity of manganese-dependent superoxide dismutase would be limiting under these conditions. In the future, further work will be needed in order to establish an appropriate protocol to indentify the mechanisms that control the MnP secretion, thus understanding the underlying processes of this interesting phenomenon.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2010.04.003.

References


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