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The Effect of Heavy Metal-Induced Oxidative Stress on the Enzymes in White Rot Fungus *Phanerochaete chrysosporium*

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Abstract Prevalence of heavy metals in the living environment causes chemical stress and reactive oxygen species (ROS) formation in *Phanerochaete chrysosporium* (*P. chrysosporium*). However, the mechanisms involved in ROS defense are still under investigation. In the present study, we evaluated the effect of lead- and cadmium-induced oxidative stress on the activities of catalase (CAT), peroxidase (POD), lignin peroxidase (LiP), and manganese peroxidase (MnP). A time-dependent change in all enzyme activities was observed following exposure to 50 μM cadmium and 25 μM lead. The lowest values were recorded at 4 h after exposure. Both cadmium and lead inhibited CAT and POD. The cytochrome P450 (CYP450) levels increased under 50–100 μM cadmium or lead exposure and decreased when heavy metal concentration was under 50 μM; this suggested that ROS is not the only factor that alters the CYP450 levels. The cadmium removal rate in the sample containing 900 μM taxifolin (inhibitor of CYP450) and 100 μM cadmium was reduced to 12.34 %, 9.73 % lower than that of 100 μM cadmium-induced sample, indicating CYP450 may play an indirect but key role in the process of clearance of heavy metals. The pH of the substrate solution decreased steadily during the incubation process.

Keywords *Phanerochaete chrysosporium* · Heavy metal · Reactive oxygen species · Antioxidant defense system · Cytochrome P450

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Introduction

Excessive release of heavy metals from metalliferous ore mining, industrial smelting, shale-gas extraction, and fossil fuel combustion is one of the major concerns in the last few decades [1]. Heavy metals such as copper, cadmium, lead, and arsenic are harmful to many organisms due to their high bioaccumulation and potential mutagenic, carcinogenic, and teratogenic properties [1, 2]. Microbial remediation has become remarkably attractive as an eco-friendly technique for the removal of both heavy metals and organic pollutants [3]. In order to expand the scope of this technology, it is important to understand the microbial defense mechanisms that protect against metal- or heavy metal-induced toxicity at the cellular level.

Heavy metal ions exert their toxicity by multiple mechanisms. Many metals are known to induce oxidative stress by scavenging thiols (glutathione and cysteine) that act as important non-enzymatic antioxidants. They may also cause physiological stress due to the generation of chemical stressors that in turn induce the production of reactive oxygen species (ROS) [4] such as H_2O_2, $O_2\cdot$, $\cdot OH$, etc. These species cause oxidative cellular damage in organisms. The regulation of the antioxidant defense system that equilibrates oxidative and antioxidative reactions is essential for determining the fate of the organism. As antioxidant systems represent the cellular defense mechanism and the potential targets of heavy metals, their study is important in clarifying the effect of metal exposure.

The cellular antioxidant defense system is composed of a range of non-enzymatic and enzymatic antioxidants. Previous studies have demonstrated the important roles played by superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in the defense against oxidation [5]. However, the availability of excess free radicals may decrease the level of antioxidants. Moreover, enzymatic antioxidants may be susceptible to heavy metal-mediated inactivation via metal interaction with sulfhydryl or other functional groups or via the replacement of cofactors essential to enzyme function.

Understanding the role played by enzymes such as CAT, peroxidase (POD), manganese peroxidase (MnP), lignin peroxidase (LiP), and cytochrome P450 (CYP450) in the anaerobic degradation of organic pollutants and adsorption of heavy metals is necessary in order to understand the microbiology, biochemistry, and molecular biology of the biodegradation process. Microbiological responses to chemical stresses have been studied at a molecular level [6]. However, hitherto, to the best of our knowledge, a complete and thorough explanation of the mechanism is unavailable.

Phanerochaete chrysosporium (*P. chrysosporium*), as the most extensively studied white rot fungus, due to its efficient lignin degradation and its potential ability to remove heavy metals [1, 3], was chosen for the study. Until now, little is known about the physiological effects of heavy metals on white rot fungi. The present study was designed to determine the response/functions of microbial antioxidant enzymes following exposure to heavy metals. We investigated the effects of lead and cadmium on enzymatic responses (particularly the CAT, POD, MnP, and LiP [7]) of *P. chrysosporium*, in order to examine the specific changes in the cell and the oxidative defense mechanisms of *P. chrysosporium*. Further, we conducted experiments to determine the effect of the *P. chrysosporium* growth environment on the heavy metal-induced modulation of CYP levels. Other factors that affect enzyme activities or levels, including heavy metal concentration, time of exposure, and pH, were also studied.

Materials and Methods

Strains

P. chrysosporium (strain BKMF-1767) was obtained from the China Center for Type Culture Collection (Wuhan, China). Stock cultures were maintained on potato dextrose agar (PDA) slants at 4 °C. The spores on the agar surface were gently scraped and blended in ultrapure water to obtain a spore suspension, which was used to inoculate Kirk's liquid culture medium [8] in 500-mL conical flasks. The spore concentration was measured and adjusted to 2.0×10^6 spores/mL.

Sample Preparation

P. chrysosporium pellets were grown in a shaker incubator (37 °C, 150 rpm) for 3 days and then exposed to the heavy metals. Pellets were harvested following a 6-h exposure to lead/cadmium. The wet weight of each sample was maintained at 7.5 g. Buffer solution of 5 mL was added to the sample, and the sample was then disrupted using an ultrasonicator/ultrasonic homogenizer (Xianou Instruments Manufacture Co., XO-1000.1, Nanjing, China), the technical parameters for which were as follows: a total time of 3.5 min with 3 s of sonication cycles spaced at 8-s intervals, set at 50 % power. The temperature was maintained at less than 25 °C with an ice bath. The homogenized suspension was then centrifuged at 4 °C to obtain cell-free extract of the homogenized tissue.

Preparation of Stock Solutions

Stock solutions of cadmium (2000 mg/L) and lead (3000 mg/L) were prepared separately by dissolving appropriate quantities of analytical grade $Cd(NO_3)_2$ and $Pb(NO_3)_2$, respectively, in ultrapure water; the stock solution contains 2 % HNO_3. The exact concentrations of the stock solutions were determined by flame atomic adsorption spectrometry (Perkin Elmer, AA700, USA). The working solutions were obtained by diluting the stock solutions to the required concentration.

Determination of Manganese Peroxidase (MnP) Activity

The MnP activity was measured using the method described by Rogalski et al. [9]. The enzyme was incubated with a 50-mM ammonium tartrate buffer (pH 4.5) containing 200 μM $MnSO_4$ and 200 μM H_2O_2. The above assays were repeated four times, and the mean and standard deviations for enzyme activity were calculated. One unit of MnP activity was expressed as the rate of oxidation of Mn^{2+} to Mn^{3+} (per minute) at the wavelength of 290 nm. Spectrophotometric measurements were performed with a UV–visible light spectrophotometer (UV–2550, Shimadzu Company, Tokyo, Japan).

Determination of Lignin Peroxidase (LiP) Activity

The LiP activity in the extracellular medium was assayed by monitoring the rate of hydrogen peroxide-mediated oxidation of Azure-B at 25 °C at 650 nm [10]. The enzyme was incubated with 0.5 mL Azure-B (10 mM), 100 mM tartaric acid buffer solution (100 mM, pH 3.0), and 0.5 mL enzyme solution. The reaction was stared with 0.1 mL H_2O_2 (10 mM). One unit of LiP

activity was expressed as optical density change per minute per milligram of protein in 1 mL of solution at 650 nm.

Determination of Catalase (CAT) Activity

The CAT activity was determined according to the method described by former researchers [11, 12]. The 3-mL reaction mixture contained 0.1 mL of extract of homogenized tissue, 1 mL H_2O_2 (0.3 %), and 1.9 mL potassium phosphate buffer (50 mM, pH 7.0). The enzymatic decomposition of H_2O_2 was followed by monitoring the decrease in absorbance at 240 nm. One unit of enzyme activity was defined as the optical density decrement per minute per milligram of protein in 1 mL of solution at 240 nm.

Determination of Peroxidase (POD) Activity

The 3-mL reaction system used to determine peroxidase activity contained 1 mL H_2O_2 (0.3 %), 1 mL guaiacol (0.2 %), and 0.9 mL phosphate buffer (50 mM, pH 7.0). The extract of homogenized tissue (0.1 mL) was added to start the reaction. One unit of enzyme activity was defined as the optical density increment per minute per milligram of protein in 1 mL of solution at 470 nm [13].

Determination of Cytochrome P450 Contents

The lysate containing 1 mM dithiothreitol (DTT) was centrifuged at 10,000×*g* for 10 min in a high-speed refrigerated centrifuge (Hitachi, Himac CR22G, Japan). The supernatant was collected and centrifuged at 20,000×*g* for 20 min to collect the mitochondria-containing light fractions, and the supernatant was then centrifuged at 114,000×*g* for 90 min in a micro ultracentrifuge (Hitachi, Himac CS150GX, Japan) to precipitate the microsomal fraction. The sediment was dissolved in 50 mM Tris–HCl buffer (pH 8.0) and stored at −80 °C (Thermo scientific, Forma 700 Series, US) for analysis.

The carbon monoxide (CO) difference spectrum was recorded as described previously [14]. Initially, the protein concentration of the pooled microsomal fractions was determined using the Bradford method [15]. Subsequently, the sample was dispensed equally into two cuvettes, and the baseline was recorded using a spectrophotometer in the range of 400–500 nm (Shimadzu, UV–2550PC, Japan). One cuvette was gently gassed with CO, and the other one was gassed with N_2. Exactly 10 mg of sodium dithionite were added into each cuvette, and their difference spectra were recorded. The concentrations of P450 and P420 (an inactive form of P450) were calculated using the corresponding values for the extinction coefficients (91 mM^{-1} cm^{-1} for ε450–490 and 110 mM^{-1} cm^{-1} for ε420–490) [16]. The P450 and P420 content in microsomal fractions was expressed by nanomole per milligram of protein.

Analytical Procedures

The initial and residual concentrations of cadmium and lead were determined using an atomic absorption spectrophotometer. During the experiment, the pH was monitored with a FE20 laboratory pH meter (Mettler Toledo, FE20, Shanghai, China). The absorbance was recorded by a UV–visible spectrophotometer (Shimadzu Company, UV–2550, Tokyo, Japan), and the protein concentration was determined using a UV–visible spectrophotometer (INESA Instrument, UV754N, Shanghai, China) by recording the absorbance at 595 nm, following the Bradford [15] reaction with Coomassie brilliant blue G250.

Results

The Enzyme Responses to Low Concentrations of Cadmium and Lead

Following exposure to 50 μM of cadmium (Fig. 1a), in the first 8 h, the change in activities of the extracellular enzymes (LiP and MnP) could be divided into two stages with each stage lasting for about 4 h. In the first stage, the activities of both LiP and MnP decreased, whereas in the second stage, they rose rapidly and surpassed the starting activities recorded immediately on addition of cadmium. The lowest value of cadmium-induced MnP activity (49.44 U) was recorded upon exposure for 4 h. Thus, in the first stage, MnP activity decreased slowly from the original value of 73.15 to 49.44 U at 4 h and then peaked at 111.26 U after 8 h of exposure. Similarly, LiP activity increased to 65.30 U after 8 h of exposure, which was 43.08 U higher than that of the original value (Fig. 1a). The lowest value (12.47 U) was recorded after a 4-h exposure. A similar trend was also observed following treatment with 25 μM lead (Fig. 1b).

Remarkably, the alteration in the activities of the intracellular enzymes (CAT and POD) upon cadmium exposure (Fig. 1a) was similar to that of the extracellular enzymes, as described above. However, CAT and POD are less active than that of LiP and MnP. In the first 2 h following cadmium exposure, activities of both CAT and POD decreased maximally, falling to 34.44 and 3.35 U, respectively. Furthermore, as the POD activity was particularly low, with highest recorded value being 8.83 U, it is plausible that cadmium may have little influence on POD.

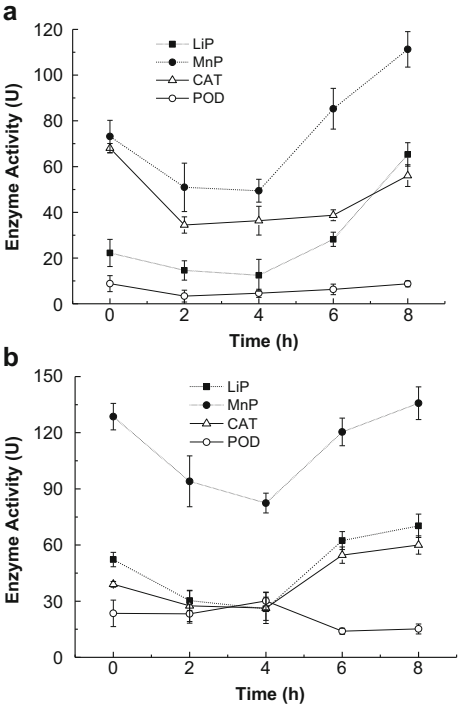

Fig. 1 Change in enzyme activities with time on exposure to different heavy metals. *P. chrysosporium* was treated with 50 μM cadmium (**a**) and 25 μM lead (**b**)

The trend of the lead-induced change in *P. chrysosporium* CAT and POD activities (Fig. 1b) was similar to that induced by cadmium. Notably, however, as the CAT activity considerably increased between 4 and 8 h, it may be more sensitive to lead. After 8 h of incubation with lead, CAT activity increased to 60.01 U, which was much higher than that of the initial value (39.02 U).

Effect of the Heavy Metal Concentration on Intracellular Enzyme Activity

The role of oxidative stress in the modulation of the intracellular environment was evaluated by examining the effect of treatment with various concentrations (5–150 μM) of the heavy metals. In general, both the enzymes displayed a drop in activity with increasing metal concentration. The POD activity was much lower than that of CAT (Fig. 2a, b), especially upon lead exposure. Comparatively, on exposure to 100 μM of cadmium, POD activity

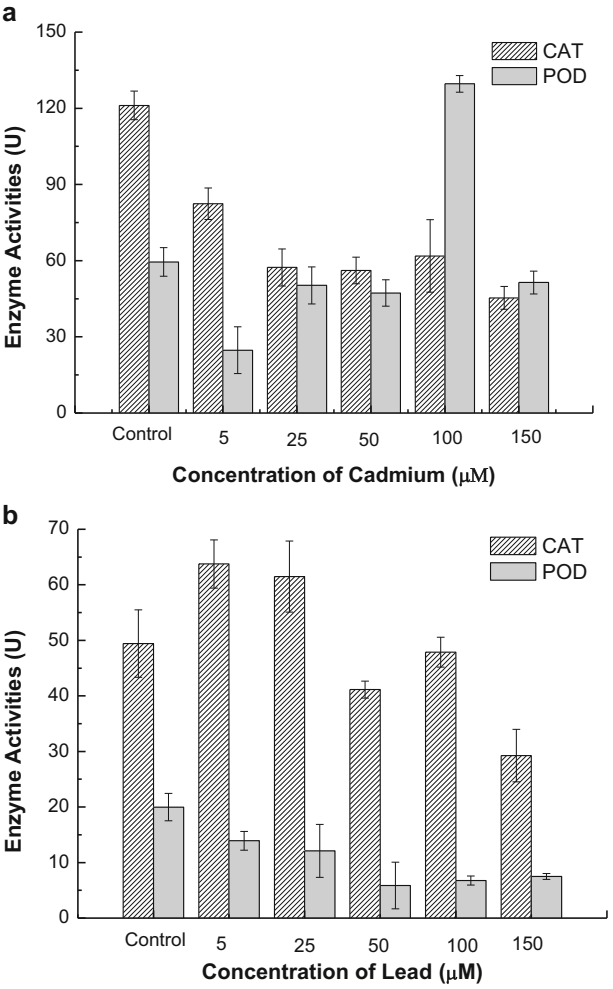

Fig. 2 Alterations in the *P. chrysosporium* CAT and POD activities following treatment with various concentrations of cadmium (**a**) and lead (**b**), determined following a 6-h exposure

increased sharply, whereas cadmium stress strongly depressed the *P. chrysosporium* CAT activity (Fig. 2a). Notably, the change in CAT activity was not obvious with 25 μM or more cadmium induction. Interestingly, the differences were very small when exposed to high concentrations of heavy metals and remarkably large for the lower concentrations. CAT activity was induced by lead, with the peak value (63.75 U) being recorded following exposure to 5 μM lead (Fig. 2b). However, when the lead concentration was increased to 50 μM, the CAT activity was distinctly reduced. In contrast, POD activity was suppressed by all the concentrations of lead, with an increase in concentration resulting in a reduction in POD activity when the concentration was less than 50 μM.

Heavy Metal Removal Rates

Both lead (25 μM) and cadmium (50 μM) in the solution were removed rapidly from the solution during the first 6 h. However, this process subsequently slowed down and reached equilibrium after 8 h of exposure (Fig. 3b). The removal rate for lead was found to be 43.32 %, as compared to 49.35 % for 50 μM cadmium. Previous work demonstrated a maximum removal rate of 55 % for a 25-μM lead solution [17].

The mechanisms by which *P. chrysosporium* tolerate heavy metals are numerous and varied, including extracellular metal sequestration and precipitation [1]. The microbial cell wall, which consists of polysaccharides, proteins, and lipids, offers a range of functional

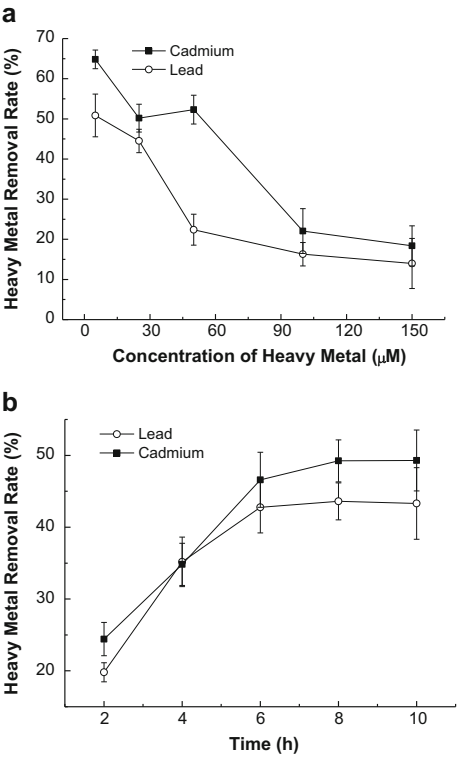

Fig. 3 The effect of the heavy metal concentration on the removal rate (**a**). Cells were exposed to different concentrations of the heavy metals for 6 h prior to analysis. Determination of heavy metal removal rates for *P. chrysosporium* treated with 25 μM lead and 50 μM cadmium at varied time intervals (**b**)

groups capable of binding to heavy metals. Scanning electron micrographs have revealed that *P. chrysosporium* can synthesize extracellular cadmium crystals with the diameters ranging from about 100 to 2 or 3 nm [18]. Lead uptake by dead or living *P. chrysosporium* cells occurs in two stages: rapid surface binding and slow intracellular diffusion [19]. Our study suggests that the first stage (surface binding of the metal ion) lasts for about 6 h (Fig. 3a). As the fixed cell biomass offers a finite number of surface-binding sites, the initial process (surface adsorption) would be expected to show saturation kinetics with increasing metal ion concentration [20]. The surface-bound metal ion is then transported into the cytoplasm. The second stage may last for several days or even longer.

pH Change in the Heavy Metal-Treated Samples

The pH of the substrate decreased with an increase in heavy metal concentration (Fig. 4). This decrease was relatively rapid in the 9- to 33-h period. A previous study has reported that cadmium in the medium may enter the *P. chrysosporium* cells during the uptake process and boost the production of organic acids such as oxalic acid [21], further increasing H^+ efflux and consequently lowering pH. Moreover, the turbidity of the substrate solution after 33 h of incubation was greater than that after 57 h, indicating reduced extracellular secretion of *P. chrysosporium* during the intervening 24-h period. In contrast, following incubation with lead, the change in pH with time was rather unremarkable as compared to that with cadmium during the first 33 h. Acidulation of the cytoplasm may prevent growth by reducing active transport [22] or by interfering with signal transduction [23].

The cell wall matrix of *P. chrysosporium* contains complex heteropolysaccharides that present amino, carboxyl, and sulfate groups [20]. At a low pH, the cell wall ligands are protonated and repel the approach of the metal cations. Upon increasing the pH, ligands such as amino, phosphate, and carboxyl groups become negatively charged, subsequently attracting

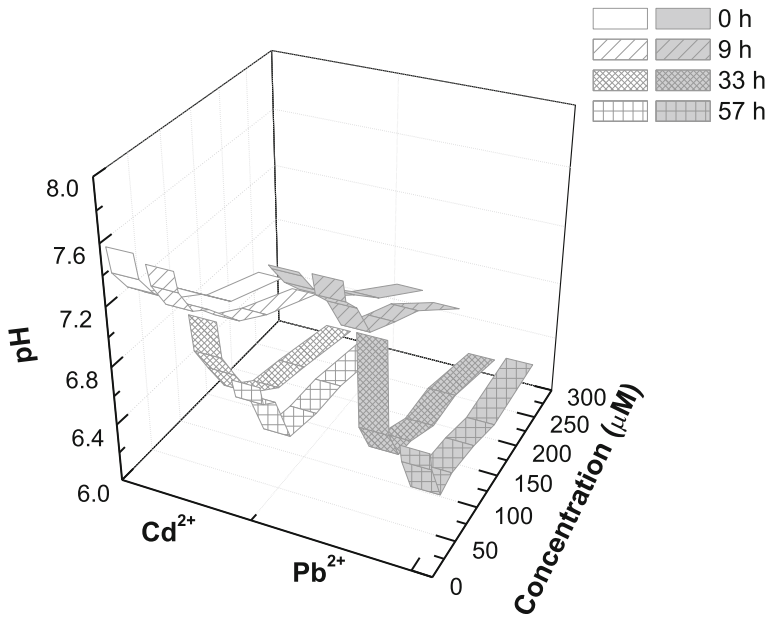

Fig. 4 pH changes at varying concentrations of lead and cadmium

metal ions [24]. The effect of pH may be further explained in relation to the competition effect between H_3O^+ and heavy metal ions. At low pH, H_3O^+ occupies the binding sites on the cell walls. As pH increases, the competing effect of H_3O^+ decreases, and positively charged metal ions take up the free binding sites, thus increasing the metal uptake capacity [25].

Effect of Heavy Metal Concentration on CYP Content

The potential function of CYP in the ROS defense system of *P. chrysosporium* was evaluated by measuring the CYP420 and CYP450 content following induction with lead and cadmium (Table 1). When the concentration of lead was below 50 μM, the CYP levels decreased with an increase in heavy metal concentration. Thus, on increasing heavy metal concentration to 50–100 μM, the CYP420 and CYP450 levels also increased substantially.

A similar trend was observed upon cadmium treatment, with both CYP420 and CYP450 decreasing with an increase in cadmium (Table 2). It is noteworthy that both CYP450 and CYP420 had an obvious increment under 50–100 μM cadmium inducement. Both CYP420 and CYP450 were not detected following 900 μM taxifolin treatment and 100 μM cadmium-induced *P. chrysosporium*. Moreover, the cadmium removal rate for the sample was only 12.34 %, which was 9.73 % lower than that of 100 μM cadmium-induced sample.

Discussion

Following a period of heavy metal exposure, *P. chrysosporium* immobilizes a portion of the heavy metals on the cell surface [18]. Some heavy metal ions are transported into the cytoplasm, inducing the generation of ROS, that affect the mitochondrial microsomes, metabolites, and intermediates, creating a strong oxidizing environment in vivo that may obstruct mRNA expression, replace the ligand in the active site in enzymes, and cause lipid peroxidation and DNA and membrane damage, leading to apoptosis [26, 27].

In the present work, the hypothesis that the enzymes CAT, POD, MnP, and LiP play a role in the protection of *P. chrysosporium* from heavy metal-induced oxidative stress was tested. Cadmium (50 μM) exhibited time-dependent effects on the activities of these antioxidant enzymes, by initially inhibiting their activities and then stimulating them. The change in the activities of these enzymes may be a part of the cellular stress response that protects against ROS. Addition of cadmium to the substrate triggered the generation of excessive ROS, thus activating the antioxidant enzymes. Subsequently, through transcriptional regulation, *P. chrysosporium* may generate more proteins including enzymes to eliminate the ROS both in vivo and in vitro (Fig. 5), which is in agreement with earlier results that ROS can induce expression of LiP and MnP at the transcriptional level [28, 29]. The antioxidant enzymes

Table 1 The effect of the lead concentration on CYP levels

Concentration of lead (μmol/L)	Concentration of P420 (nmol/mg protein)	Concentration of P450 (nmol/mg protein)
0	0.3645±0.018	0.2022±0.032
5	0.2938±0.023	0.0968±0.004
25	0.1775±0.009	0.0645±0.007
50	0.2115±0.027	0.2321±0.043
100	0.2278±0.031	0.3268±0.011

Table 2 The effect of cadmium concentration on CYP levels

Concentration of cadmium/μM	Concentration of P420 (nmol/mg protein)	Concentration of P450 (nmol/mg protein)
0	0.3759±0.042	0.3435±0.009
5	0.3729±0.033	0.2467±0.017
25	0.3272±0.009	0.2798±0.018
50	0.3349±0.013	0.3776±0.021
100	0.3797±0.027	0.4262±0.026
150	0.2714±0.015	0.2286±0.014

regained their activity once the ROS levels were decreased. Results from lead (25 μM)-treated samples (Fig. 1b) also supported this hypothesis.

A decrease in GSH levels following cadmium exposure has been reported previously [30], which further confirmed the cadmium-induced generation of ROS. Cadmium also decreases the availability of protein-bound sulfhydryl groups or other functional groups and may replace cofactors essential for enzyme functions, which renders these enzymatic antioxidants susceptible to metal-induced inactivation, leading to enhanced production of reactive oxygen species such as the superoxide ion, hydroxyl radicals, and hydrogen peroxide [31].

The feedback signals may also induce the production of ROS-scavenging enzymes, thus maintaining the redox equilibrium in vivo. Many other elements are involved in the feedback loop. Protein kinase C, as a Ca^{2+}-signaling factor, plays a role in the ROS-mediated signaling underlying LiP expression [32]. cAMP also acts as a signal for increasing the transcription of

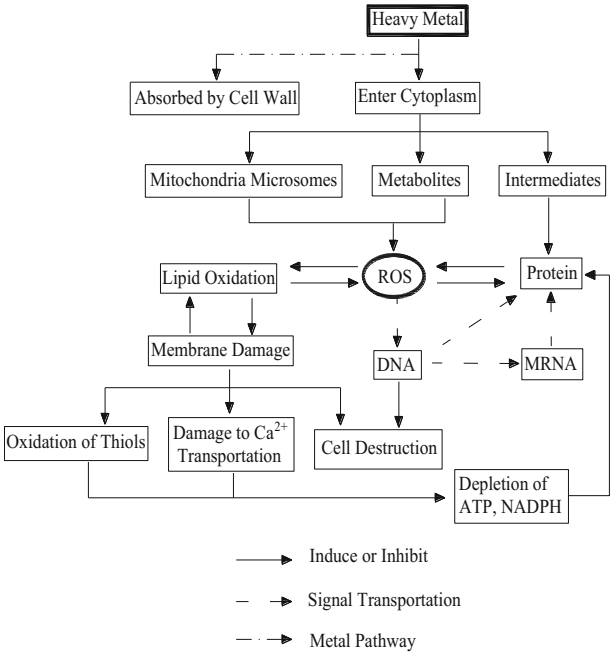

Fig. 5 Possible mechanisms involved in the metal-induced oxidative stress

LiP and MnP through the induction of calmodulin transcription [33]. These findings demonstrate the complexity of the antioxidant mechanisms in *P. chrysosporium* (Fig. 6).

In the present work, MnP was found to be more sensitive to lead or cadmium than LiP (Fig. 1a, b). Both MnP and LiP can catalyze the reduction of H_2O_2 to H_2O through a series of electron transfers [34–36]. The fact that CAT activity was stimulated by both lead- and cadmium-containing solutions highlights the importance of this enzyme as a major antioxidant defense in *P. chrysosporium*. As an intracellular enzyme, CAT shows high efficiency and specificity in the catalysis of H_2O_2. Some researchers have suggested that cadmium may lower the activity of antioxidant enzymes including catalase [37], this has been demonstrated in the case of *P. chrysosporium* during the rapid surface-binding stage (Fig. 1). The activity of POD remained appreciably lower compared to that of other enzymes under identical conditions. Both lead and cadmium had little influence on POD activity, suggesting that it is not the main ROS scavenger in the antioxidant system.

High concentrations of heavy metals generate high levels of free radicals in *P. chrysosporium*, thus creating a strong oxidizing environment in vivo. Higher levels of ROS may explain the decrease in CAT and POD activities with the increase of heavy metal concentration (Fig. 2). The contents of CYP450 and CYP420 decreased as the concentration of cadmium or lead increased from 5 to 50 μM, which further proved ROS could inhibit the generation or activity of antioxidant enzymes. However, when heavy metal concentration increased to 50 μM, a converse result was observed that CYP450 and CYP420 contents did not decrease but began to increase following the exposure of 50–100 μM heavy metal (Tables 1 and 2). These results implied there might exist other regulatory mechanisms which could eliminate the effect of ROS and stimulate the generation of CYP450 and CYP420 simultaneously (Fig. 6). The intermediate that plays a major role in the regulatory mechanism might be proved for its being activated upon the exposure of 50–100 μM cadmium or lead. It may also play a role in the removal of heavy metals and be involved in basic cellular defense mechanisms or the activation of other antioxidant enzymes.

Studies in the 1970s have established that lead was an inhibitor of a number of cytochrome P450-related oxidations [32]. However, to date, the effects of divalent metal ions on the activity of CYP450 enzymes have not been well understood [38]. The cadmium- and lead-induced CYP450 inhibition could be attributed to two main postulated mechanisms: the first being the combined effect of the metal on proteins. It has been reported that heavy metals inhibit δ-aminolevulinic acid dehydratase, an important enzyme in the heme synthesis pathway, via the binding of heavy metals such as lead to the thiol groups present at allosteric sites,

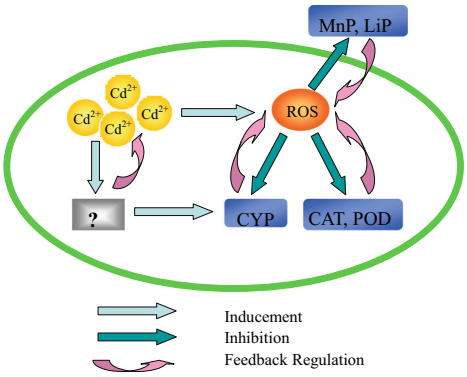

Fig. 6 Possible ROS removal mechanisms in vivo

provoking allosteric transitions to inactive forms of the enzyme, consequently leading to a decrease in heme synthesis [39]. Since heme is the prosthetic group of CYP450 [40], its reduced bioavailability may explain the decreased CYP450 protein levels. Secondly, the inhibition of CYP450 activity by lead, which potentially occurs through a lead-induced alteration in phospholipid conformation within biological membranes, may cause lipid peroxidation, as shown in previous studies [41]. This process may thereby affect lipid organization and electron transport among microsomal CYP450 components, indirectly leading to the inhibition of CYP450 activity.

Conclusions

LiP, MnP, and CAT activities were inhibited and then stimulated by heavy metal-induced ROS during the first 8 h of exposure to 50 μM cadmium and 25 μM lead. The lowest value of LiP and MnP activities was recorded upon exposure for 4 h. POD activity kept low during the exposure to heavy metals, suggesting it may contribute little to the elimination of ROS. LiP, MnP, and CAT activities decreased with the increase of heavy metal concentration. Cadmium of 150 μM or above and lead of 100 μM may cause irreversible inhibition to enzyme activities. CYP450 and CYP420 contents increased with cadmium and lead concentration only among 50–100 μM, suggesting the existence of an intermediate that can stimulate the production of CYP and play a role in the removal of heavy metals. The results obtained from this study contribute to a better understanding of changes in microbiological metabolism at sublethal concentrations of a chemical stressor. With the advent of new technologies, numerous questions regarding reactive oxygen species, their transport, tissue-damaging effects, and relative roles in the mechanistic sequences associated with toxicity and carcinogenicity may be addressed.

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