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Alteration of culture fluid proteins by cadmium induction in *Phanerochaete chrysosporium*

Guiqiu Chen[1,2], Ying Zhou[1,2], Guangming Zeng[1,2]*, Hongyu Liu[1,2], Ming Yan[1,2], Anwei Chen[1,2], Song Guan[1,2], Cui Shang[1,2], Huanke Li[1,2] and Jianmin He[1,2]

[1] College of Environmental Science and Engineering, Hunan University, Changsha, P. R. China
[2] Key Laboratory of Environmental Biology and Pollution Control, Hunan University, Ministry of Education, Changsha, P. R. China

Microorganisms need to resist the hazards posed by heavy metals during the process of metal adsorption. *Phanerochaete chrysosporium* is a fungus that is efficiently used for heavy-metal biosorption in wastewater treatment. Extraction and analysis of proteins induced by heavy metals can help to understand the regulatory mechanisms of *P. chrysosporium* in wastewater treatment. In this study, *P. chrysosporium* was exposed to 50 μM cadmium nitrate. A maximum cadmium adsorption capability of 77.1 mg/g dry biomass was found after 65 h, which was accompanied by a relatively higher protein concentration. After separation of the culture fluid proteins by two-dimensional difference gel electrophoresis (2D-DIGE), three differentially expressed proteins were detected from 17 spots. By using 2D-DIGE, followed by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS), glutathione *S*-transferase, glyceraldehyde-3-phosphate dehydrogenase, and malate dehydrogenase were identified. All three enzymes play important roles in the oxidative stress caused by cadmium.

Keywords: Cadmium / 2D-DIGE / MALDI-TOF/TOF MS / *Phanerochaete chrysosporium* / Wastewater

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Introduction

Heavy-metal pollution is a major environmental problem because of the persistence and bioaccumulation in the food chain [1]. The presence of heavy metals in natural aquatic ecosystems and industrial wastewater poses a major inorganic contamination problem. Cadmium (Cd) is an extremely toxic metal commonly found in industrial workplaces and represents a sanitary and ecological threat because of its negative effects on the environment [2, 3]. Industrial processes, e.g., electroplating, pigments, alloy preparation, and mineral processing, are the main sources of Cd release into the environment [4, 5].

With the development of bioremediation using microorganisms, conventional treatment methods for Cd-contaminated wastewater are considered expensive and inefficient, especially when the metal concentrations are low [6, 7]. *Phanerochaete chrysosporium* is a white-rot fungus that has been efficient in complete lignin mineralization and degradation of various xenobiotic compounds, as well as in the biosorption of heavy-metal ions from solutions [8–10]. Furthermore, *P. chrysosporium* showed a high accumulation of Cd(II) compared to other white-rot fungi [11]. The Cd(II) metal removal capacity of living *P. chrysosporium* was the highest when the metal concentration was 47.2 mg L^{-1} [12].

Toxic metals cause a series of stress responses, such as protein oxidation, lipid peroxidation, and nucleic acid oxidation, during the adsorption process [13]. Özcan et al. [14] have studied the soluble protein fraction of *P. chrysosporium* and its changes in response to Cu(II) and Cd(II). Some cellular proteins such as isocitrate dehydrogenase, short-chain acyl-CoA dehydrogenase, and UDP-glucose pyrophosphorylase are involved in lead detoxification in *P. chrysosporium* [15]. Vigneshwaran et al. [16]

Correspondence: G. Chen, College of Environmental Science and Engineering, Hunan University, Changsha 410082, P. R. China
E-mail: gqchen@hnu.edu.cn
Phone: +86 731 88822829
Fax: +86 731 88823701

*Additional correspondence: G. Zeng **E-mail:** zgming@hnu.edu.cn

demonstrated that microbe proteins related to biological functions, such as metabolism, proliferation, transcription, and signal transduction, and proteins in the extracellular culture fluid of *P. chrysosporium* were closely associated with the crystallization of heavy metals. Because the relationship between culture fluid proteins of *P. chrysosporium* and heavy metals is not well understood, further studies on the regulatory mechanisms of *P. chrysosporium* in heavy-metal poisoning and the interactions between them should be carried out.

In our study, we extracted culture fluid proteins of *P. chrysosporium* induced by Cd(II) and analyzed the differentially expressed proteins, in order to study the functional regulation of *P. chrysosporium* under heavy-metal stress. The protein concentrations and the adsorption of Cd(II) were investigated to determine the optimum protein extraction time. Further proteomics analyses were carried out to identify proteins by two-dimensional difference gel electrophoresis (2D-DIGE), followed by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS). These studies will help to understand the functional regulation of *P. chrysosporium* under Cd(II) stress in wastewater.

Materials and methods

Culture conditions

Spores of *P. chrysosporium* (CCTCC AF 96007; The China Center for Type Culture Collection, Wuhan) were separated from potato dextrose agar slant surfaces by scraping and homogenized in sterile distilled water. The fungal spore concentration was adjusted to 2.0×10^6 colony-forming units/ml using a turbidimeter (WGZ-200; Shanghai, China). The suspension was then transferred into 250 ml Erlenmeyer flasks each containing 100 ml Kirk's medium [17]. The mixture was incubated at 150 rpm in a shaker at 35 °C until it showed logarithmic growth. Then, $Cd(NO_3)_2 \cdot 4H_2O$ solution was added to the culture medium to a final concentration of 50 μM, and the cultures were further cultivated for 4 h (treatment). A control without Cd(II) was included in each set of Cd(II) treatment experiments.

Adsorption study

Control and treatment cultures were incubated for different times (50, 55, 60, 65, and 70 h). The cultures were then centrifuged for 10 min at 10,000 rpm and the supernatants (1 ml each) were used to determine the heavy-metal concentrations. The pellets were dried in a freezer–dryer. The amount of adsorbed metal ions per unit of dry biomass was calculated using Eq. (1):

$$Q = \frac{(C_0 - C)V}{M} \quad (1)$$

where Q is the amount of metal ions adsorbed on the biomass (mg/g), C_0 is the initial metal concentration, C is the final metal concentration after biosorption (mg L^{-1}), V is the volume of the medium (L), and M is the amount of dry biomass (g).

Protein extract preparation

After cultivation, the extracellular culture fluids were collected by centrifugation at 10,000 rpm for 30 min at 4 °C. A Bradford assay [18] was performed to preliminarily quantify the protein concentration for extraction. Trichloroacetic acid/acetone extraction was applied to the supernatant according to Fragner et al. [19], with some modifications. Briefly, 100% trichloroacetic acid was added at one-tenth of the volume of extracellular culture fluid obtained. The solution was left overnight at 4 °C, centrifuged at 10,000 rpm for 15 min, and the supernatant was decanted. This precipitation step was repeated once. The protein precipitate collected was suspended in 5 ml 80% cold acetone (containing 0.07% β-mercaptoethanol), incubated at −20°C for 5 h (vortexed every 30 min) and then re-centrifuged and subjected to an additional acetone rinse. After discarding the supernatant, the final precipitate was desalted, quantitatively analyzed with a 2D Quant kit (GE Healthcare) and then subjected to lysis.

2D-DIGE

For 2D-DIGE, 50 μg each of control, treatment, and internal standard protein sample were labeled with cyanine dyes, i.e., Cy3, Cy5, and Cy2, according to the instructions of the CyDye DIGE™ kit (GE Healthcare). The internal standard used was a mixture of equal amounts of protein from control and treatment samples. Each cultivation, extraction, or labeling experiment was performed in triplicate in order to obtain statistically significant data. Before first-dimension isoelectric focusing (IEF), a labeling mixture was combined with rehydration buffer (containing 8 M urea, 2 M thiourea, 4% w/v CHAPS, 20 mM Tris, 20 mM dithiothreitol (DTT), 0.5% v/v immobilized pH gradient buffer, and bromophenol blue) to a final volume of 450 μl. IEF was performed in 24-cm linear immobilized pH gradient strips (pH range 3–10; GE Healthcare) and carried out as follows. After rehydration of the samples at 50 V for 12 h, the voltage was linearly increased from 50 to 500, 500 to 1000, and 1000 to 10,000 V, each for 1 h, maintained at

10,000 V for 9 h (90,000 V h) and finally reduced to 500 V for 10 h. After IEF, the strips were gently shaken for 15 min in equilibration buffer I (50 mM Tris–HCl pH 8.8, 6 M urea, 30% v/v glycerol, 1% SDS, 0.2% w/v DTT, and bromophenol blue) and equilibration buffer II (same as buffer I except that DTT was replaced with 0.5% w/v indoleacetic acid). Second-dimension separation was carried out in polyacrylamide gels of 12.5% T (acrylamide concentration) at 12 W for 30 min and then at 48 W until completion.

2D-DIGE image analysis

Gels were scanned with a Typhoon TRIO+ fluorescence scanner (GE Healthcare), using the DeCyder™ 2D software for image analysis. Differential in-gel analysis for the three gels was carried out first, including point detection, background removal, and gel normalization, in order to obtain quantitative information on each gel's protein sample with different fluorescent markers. Later, biological variation analysis – including gel matching and calculating the molecular weights and isoelectric points (IP) – was performed in order to obtain coordinates for the spots and the standardization and statistical analysis of protein point values. Statistical analysis was performed using Student's *t*-test using the SPSS 12.0 software, and differences with a $|\text{Ratio}| \geq 2$ and $p \leq 0.05$ were recognized as statistically significant. Group comparisons were carried out using the anti-subject method after gel matching. Then, the spots of differentially expressed proteins could be selected by using the software.

Protein identification by mass spectrometry

Another gel was stained with Coomassie Brilliant Blue G. After matching with 2D-DIGE images, protein spots were excised, washed twice with distilled water and destained with 25 mM NH_4HCO_3 in 50% acetonitrile (ACN) for 30 min at 37 °C. The solution was discarded and ACN was added. After 10 min, the ACN was discarded and the spots were lyophilized for 20 min, after which 10 mM DTT in 25 mM NH_4HCO_3 was added. The supernatant was discarded and 55 mM indoleacetic acid in 25 mM NH_4HCO_3 was added for 30 min. Each spot was washed with 25 mM NH_4HCO_3, dehydrated, lyophilized, and digested overnight with trypsin. The peptides were extracted with 20 μl 67% ACN and 2.5% trifluoroacetic acid and incubated for 30 min at 37 °C. Peptide solutions were concentrated and pointed to the target, crystallized with an equal volume of saturated α-cyano-4-hydroxycinnamic acid and dried at room temperature for MALDI-TOF/TOF MS. Proteins were identified by searching these peptide molecular-weight values against the National Center of Biotechnology Information non-redundant (NCBInr) protein database, by using the online version of Mascot (www.matrixscience.com). The searching process took into consideration the modification of cysteine by carbamidomethylation and oxidation of methionine as well as maximally one cleavage site missing.

Results

Concentration of proteins and adsorption of cadmium

The changes of the protein concentration in the culture fluid and the adsorption of Cd(II) after 50–70 h are shown in Fig. 1. The concentration of proteins in the extracellular culture fluid under Cd(II) stress showed an upward trend in this period (Fig. 1A). The protein concentration was in the range of 8.94–10.02 mg L^{-1} during the incubation period of 60–70 h, which was higher than that of the 50–60-h period and suitable for extraction. The removal rate of Cd (Fig. 1B) gradually increased

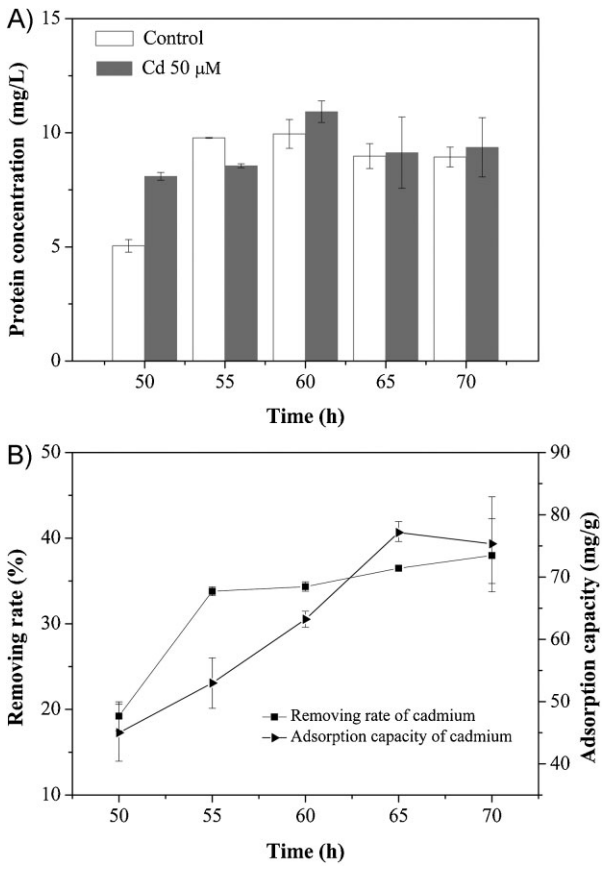

Figure 1. Changes in protein concentration (A) and adsorption of Cd(II) (B) of *P. chrysosporium* after Cd(II) stress at an initial Cd(II) concentration of 50 μM and pH 6.0.

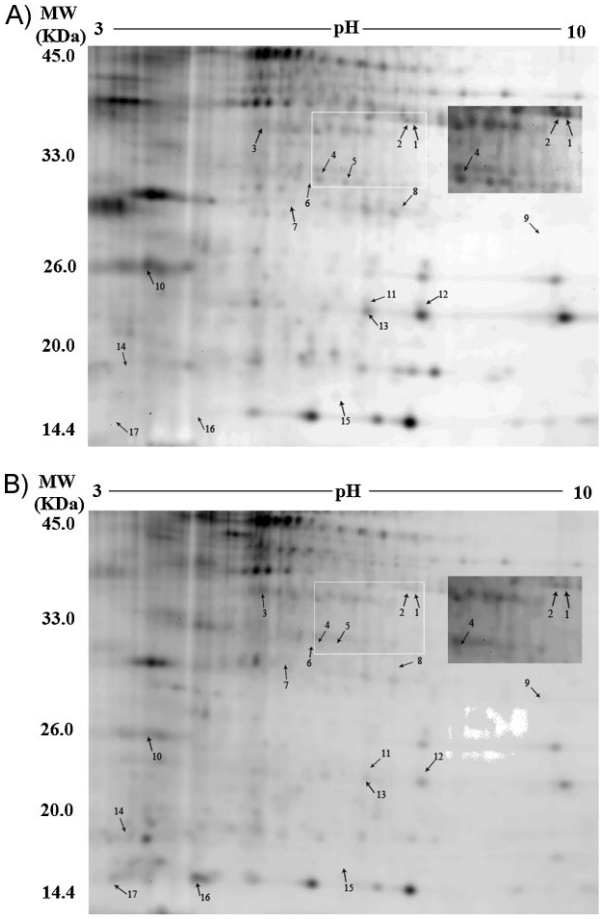

Figure 2. 2D-DIGE patterns of proteins in *P. chrysosporium* before (A) and after (B) Cd(II) stress. Fungal cultures were incubated for 65 h and then induced by Cd(II) at a concentration of 50 μM and pH 6.0. The 17 differentially expressed spots were labeled, numbered, and partially magnified in an overlay picture, to visualize the differences.

during the logarithmic growth phase, indicating that the mycelium had a strong ability to bind heavy metals. The maximum Cd adsorption capability of 77.1 mg g^{-1} occurred at 65 h, which correlated with a relatively high amount of protein. Thus, further protein extraction was carried out at this incubation time.

Detection of differentially expressed proteins by 2D-DIGE

After separation by 2D-DIGE, 17 protein spots from *P. chrysosporium*, with differential expression before and after Cd treatment, were detected through statistical analysis (*t*-test; $|\text{Ratio}| \geq 2$, $p \leq 0.05$). The 17 differentially expressed spots were labeled, numbered, and partially magnified in an overlay picture, to visualize the differences (Fig. 2). The tryptic peptides from the extraction were then analyzed by MALDI-TOF/TOF MS. Only four spots, from the 17 proteins analyzed, returned statistically significant scores. The peptide masses and predicted amino acid sequences of these four spots, obtained through homology searching of the fungi in NCBInr, are shown in Table 1.

Identification of the proteins

When subjected to 50 μM Cd(II), which is a low concentration for Cd adsorption of *P. chrysosporium*, only one up-regulated and three down-regulated protein spots were identified. Two spots were found to be the same protein, but they appeared at different positions in the gel due to differential modification. The three proteins could be assigned to corresponding protein accession numbers from the NCBI website and were identified as glutathione *S*-transferase (GST), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and malate dehydrogenase (MDH). GST was up-regulated 4.72-fold, whereas

Table 1. Peptide masses and predicted amino acid sequences of the four identified spots through homology searching of the fungi in NCBInr.

Protein spot	Mass (observed)	Mass (expected)	Mass (calculated)	Missed cleavages	Start/end	Database sequence
1	1006.3167	1005.3095	1005.6335	0	92–101	VVVIPAGVPR
	1205.3032	1204.2959	1204.6816	0	163–173	VFGVTTLDVVR
2	1006.4786	1005.4713	1005.6335	0	92–101	VVVIPAGVPR
	1364.4736	1363.4663	1363.6732	0	108–119	DDLFNTNASIVR
	1361.5361	1360.5288	1360.7827	1	162–170	RVFGVTTLD
4	1443.4342	1442.4269	1441.7525	0	31–43	NNIIPSSTGAAK
	1797.7443	1796.7370	1796.9744	1	31–47	NNIIPSSTGAAKAVGK
	1183.1980	1182.1907	1181.7132	1	44–55	AVGKVIPSLNGK
	827.3132	826.3059	826.4912	0	48–55	VIPSLNGK
9	825.4556	824.4484	824.3929	0	46–51	HPETWR
	1946.9205	1945.9132	1946.0163	1	150–165	LVTKGLNNGFYIHEWR
	827.4562	826.4489	826.4701	0	223–229	VWPNLAK

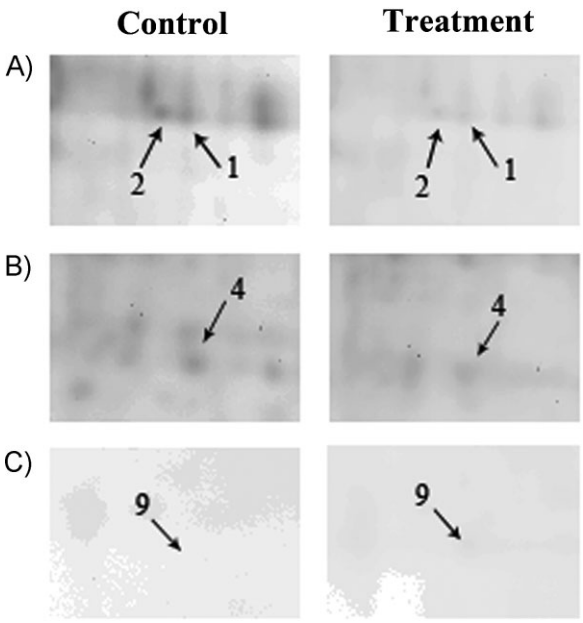

Figure 3. Comparison of the three identified proteins in response to 50 μM Cd(II): (A) MDH, (B) GAPDH, and (C) GST.

GAPDH and MDH were down-regulated 2.16-fold and 2.15/2.49-fold, respectively. Functions and other information about the differentially expressed proteins (Fig. 3) are listed in Table 2.

Discussion

Microorganisms need to resist the hazards posed by heavy metals during the process of metal adsorption. This self-protection regulation is closely associated with enzymes. GST is an antioxidant-scavenging/defense protein whose expression was increased in response to Cd in our study. Intracellularly, GST is commonly found to catalyze nucleophilic group ($HO^{\bullet}$, O_2^-, H_2O_2) and glutathione (γ-Glu-Cys-Gly) conjugations in order to protect nucleic acids and proteins against the by-products of oxidative stress [20, 21]. The impact of GST on heavy-metal detoxification was reported not only in fungi but also in plants, animals, and bacteria [22]. GST as binding protein may be a transporter of hydrophobic compounds [23]. Adamis et al. [24] reported that GST could catalyze the formation of glutathione-Cd conjugates in *Saccharomyces cerevisiae* involved in Cd detoxification. Following induction by Cd, the intracellular expression of this enzyme has been found to be up-regulated in *P. chrysosporium* [14]. Another previous study pointed out that this enzyme could be secreted extracellularly and may be associated with nutrient recycling [25], which was shown in the present study. The induction by Cd is likely to cause secretion of GST into the culture fluid of *P. chrysosporium*. The role of intracellular GST in Cd detoxification has been studied, but whether and how the secreted GST has effects on Cd detoxification requires further analysis.

Cd reduces the outward transport of intracellular dehydrogenases such as GAPDH and MDH. Thus, the concentration of these enzymes is decreased in the culture fluid. It is likely that Cd inhibits the growth of *P. chrysosporium* and influences the metabolic activity to some extent. However, both GAPDH and MDH were shown to be involved in the adaptive response to oxidative stress [26–28]. Intracellularly, GAPDH can act as reversible metabolic switch under oxidative stress. The temporary inactivation of GAPDH by oxidant treatment may re-route the metabolic flux from glycolysis to the pentose phosphate pathway, allowing the cell to generate more NADPH as an antioxidant cofactor [27]. MDH catalyzes the reversible conversion of malate and oxaloacetate. It provides protection against oxidative damage caused by Zn in *Escherichia coli* through the action of oxaloacetate [29]. The chelation mechanism of oxalic acid as an extracellular metabolite has a positive effect on the heavy-metal defense in white-rot fungi [30].

Although Cd(II) is not a redox-active metal ion, it can cause oxidative stress resulting in the change of antioxidant enzymes in wastewater. The adaptive response to oxidative stress by Cd could be adjusted by

Table 2. Information on the putative enzymes and the functions of the four identified spots.

Spot	Putative enzyme	Function	Accession no.	Treatment/ control ratio	Mass (kDa)	IP	Score
1/2	MDH	malate or carbohydrate metabolic process, oxidoreductase activity	GI:169865690	−2.03/−2.49	35.160/35.114	9.02	110/94
4	GAPDH	oxidoreductase activity, NAD or NADP as acceptor	GI:224042366	−2.16	15.143	8.18	78
9	Predicted GST	transferase activity	GI:380352846	4.72	31.237	9.13	75

The nominal mass and calculated IP values are also shown. A score value higher than 74 was considered as successful protein identification.

enzymes such as GST, GAPDH, and MDH, and the antioxidant regulatory mechanisms, especially the regulation of intracellular antioxidants, are probably the key to the response of *P. chrysosporium* against the damage caused by the heavy metal Cd. This research lays the foundation for a comprehensive understanding of the Cd detoxification mechanism in white-rot fungi. Further research combined with exposure time and metal concentration variation will enable better use of white-rot fungi in heavy-metal wastewater treatment.

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