



Hexavalent chromium induced oxidative stress and apoptosis in *Pycnoporus sanguineus*[☆]



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ABSTRACT

White rot fungi have been proved to be a promising option for the removal of heavy metals, understanding their toxic response to heavy metals is conducive to developing and popularizing fungi-based remediation technologies so as to lessen the hazard of heavy metals. In this study, Cr(VI)-induced oxidative stress and apoptosis in *Pycnoporus sanguineus*, a species of white rot fungi were investigated. The results suggested that high level of Cr(VI) promoted the formation of ROS, including H₂O₂, O₂^{•−} and •OH. With the increment of Cr(VI) concentration, the SOD and CAT activity along with GSH content increased within the first 24 h, but decreased afterward, accompanied with a significant enhancement of MDA content. Cr(VI)-induced oxidative damage further caused and aggravated apoptosis in *P. sanguineus*, especially at Cr(VI) concentrations above 20 mg/L. Cr(VI)-induced apoptosis was involved with mitochondrial dysfunction including mitochondrial depolarization, the enhancement of mitochondrial permeability and release of cytochrome c. The early and late apoptosis hallmarks, such as metacaspase activation, phosphatidylserine (PS) externalization, DNA fragmentation and the nuclear condensation and fragmentation were observed. Moreover, we also found disturbances of ion homeostasis, which was featured by K⁺ effluxes and overload of cytoplasmic and mitochondrial Ca²⁺. Based on these results, we suggest that Cr(VI) induced oxidative stress and apoptosis in white rot fungi, *P. sanguineus*.

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1. Introduction

Hexavalent chromium has drawn considerable attention across the globe as one of the most ubiquitous and toxic heavy metals. The industrial activities identified as the main source contributing to the massive discharge of Cr(VI) were involved in electroplating, dyes and pigments, leather tanning, metallurgy and wood treatment (Gu et al., 2015). The alarming amount of emissions containing Cr(VI) gave rise to severe pollution of waters and soils and posed a great threat to human beings since Cr(VI) has been recognized as one of the Group 1 carcinogens by IARC owing to its confirmed carcinogenic effect (Banerjee et al., 2017; Wu et al., 2016a). What is more noteworthy is that the high water solubility

and mobility of Cr(VI) together with its interaction with crucial component of the cellular machinery after readily penetrating through cell membranes have enhanced the toxicity and hazards of this heavy metal to ecological systems (Garavaglia et al., 2010). Plenty of studies have demonstrated that Cr(VI) can exert cytotoxicity, genotoxicity and mutagenic effects to plants, aquatic species and animals, primarily manifested as mitochondrial impairment, chromosomal aberrations, ultrastructural alterations as well as interference of cell proliferation (Eleftheriou et al., 2015; Kováčik et al., 2015).

Considering the widespread presence and severe risk of Cr(VI), it is imperative to explore economical and effective approaches to remove it from the contaminated environment. Biological treatment, an emerging environmentally-friendly technology regarding utilizing microorganisms to eliminate and transform hazardous substances in environment, has been proposed to be a promising alternative to the traditional physico-chemical methods for Cr(VI) elimination (Gutiérrez-Corona et al., 2016; Joutey et al., 2014). However, the toxicity of the objects disposed has serious

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implications for the colonization and bioactivity of microorganism, and thus the removal capacity by microorganisms changed considerably with the types of contaminants and nature of microbial cell (Chen et al., 2014). As is evidenced by the fact that Cr(VI) could result in enzymes inactivation, plasma membrane disruption and protein denaturation, accordingly disturbing the normal metabolism and inhibiting the growth of microorganism (Kamaludeen et al., 2003; Poljsak et al., 2010), these poisonous effects of Cr(VI) are certain to produce negative consequences on its bioremoval and make it increasingly difficult for bioremediation of Cr(VI)-polluted site. Therefore, it is of great importance to further investigate the toxic response of microorganism to heavy metal stress when intending to acquire more useful information about the tolerance of microorganism and maximize its application potential in bioremediation of environments contaminated with heavy metals.

During the past several years, mounting interest has been paid to evaluating the bioremediation potential of white rot fungi, a kind of fungi with high metabolic versatility, extensive adaptability to environment, and desired ability to eliminate heavy metals (Chen et al., 2011; Hanif and Bhatti, 2015). *Phanerochaete chrysosporium*, a typical strain of white rot fungi, was found to remove more than 95% of Cr(VI) with initial concentration of 10 mg/L after 100 h (Murugavelh and Mohanty, 2012). *Coriolus versicolor* has also been shown to transform Cr(VI) through sequestration together with binding of Cr(VI) to the groups present in the fungal cell wall followed by reduction to Cr(III) (Sanghi et al., 2009). In addition, the potential of the *Ganoderma lucidum*, *Agaricus bitorquis* and *Pleurotus sajor-caju* as the bioremediation agent for the treatment of industrial effluents containing Cr(VI) was also reported (Hanif and Bhatti, 2015). In our previous study, *Pycnoporus sanguineus* has also been proven to possess the ability to remove Cr(VI) via bioreduction (data shown in Supplementary Information). Although there are numerous researches verifying that some species of white rot fungi were available for the bioremoval of Cr(VI), most of these were primarily concerned with the removal mechanisms and the factors related to the removal efficiency, scarce study is underway to explore the toxic responses of white rot fungi subject to Cr(VI) stress.

In view of this, the objective of the present work was to investigate Cr(VI) toxicity to a species of white rot fungi, *Pycnoporus sanguineus*. We first assessed Cr(VI)-induced oxidative stress of *P. sanguineus*, with particular attention given to the variation of antioxidant enzyme activity, glutathione (GSH) and malondialdehyde (MDA) content and ROS production. And then, we further explored whether the oxidative stress caused apoptosis of *P. sanguineus*, through focusing on the specific markers of apoptosis, covering mitochondrial damage, metacaspase activation, DNA fragmentation, phosphatidylserine externalization and the disruption of the ion homeostasis.

2. Materials and methods

2.1. Fungal strains

The experimental strain *P. sanguineus* (CGMCC 5.00815) was obtained from China General Microbiological Culture Collection Center. After stock cultures were subcultured on potato dextrose agar plates at 30 °C for 5 days, spore suspension was prepared by scraping the spores from the agar surface, and then blending it with sterile normal saline. A 0.8-mL aliquot of spore suspension with approximate concentration of 2.0×10^6 CFU/mL was then incubated into 50 mL flask with a total volume of 19.2 mL of a liquid culture medium consisting of the following composition (in g/L of distilled water, pH 5.0): glucose 5.0, L-asparagine 0.2, KH_2PO_4 2.0,

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.002, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001, NaCl 0.005, $\text{AlK}(\text{SO}_4)_3$ 0.0002, $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ 0.0002, H_3BO_3 0.0002, thiamine hydrochloride 0.001.

After 3 days of incubation in an rotary shaker under 160 rpm at 30 °C, the mycelial pellets were formed and treated with Cr(VI). The initial Cr(VI) concentration in the medium was regulated at 5, 10, 20, 30 and 40 mg/L separately via the addition of various amount of potassium dichromate solutions (10 g/L). The mycelial pellets incubated without Cr(VI) were set as controls.

2.2. Assay of SOD, CAT activities and glutathione (GSH) content

The determination of SOD, CAT activities and glutathione content was conducted according to the instructions of total superoxide dismutase assay kit, catalase assay kit and GSH assay kit, respectively. All these kits were produced by Beyotime Institute of Biotechnology, China.

2.3. Analysis of malondialdehyde (MDA) content

The content of MDA was determined by utilizing thiobarbituric acid method (Jiang et al., 2015).

2.4. Measurements of reactive oxygen species (ROS) and superoxide anion ($\text{O}_2^{\cdot-}$), hydroxyl radicals ($\cdot\text{OH}$) and hydrogen peroxide (H_2O_2)

Intracellular ROS were assessed using specific dyes 2,7-dichlorodihydro-fluoresceindiacetate ($\text{H}_2\text{DCF-DA}$) (Kováčik et al., 2015). The pellets collected were washed twice with PBS (0.05M, pH 7.4) followed by staining in 20 μM working solution of $\text{H}_2\text{DCF-DA}$ in darkness for 40 min at room temperature. The stained pellets were washed with PBS again prior to observation using confocal laser scanning microscope (CLSM) (Pascal 2, Zeiss).

The estimation of the level of $\text{O}_2^{\cdot-}$ and $\cdot\text{OH}$ was conducted using the method based on the oxidation of hydroxylamine and deoxyribose separately (Gill et al., 2014) (See Supplementary Information). And the content of H_2O_2 was determined with a hydrogen peroxide detection kit provided by Nanjing Jiancheng Bioengineering Institute, China.

2.5. Assay of mitochondrial properties

To analyze the alterations of mitochondrial membrane potential, specific molecular probes 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1) was used. The mycelia pellets washed twice with PBS were then incubated with 2 $\mu\text{g/mL}$ JC-1 at 30 °C for 40 min in darkness. After incubation, the samples were rinsed with PBS three times, and the mean values of the JC-1 monomer fluorescence intensities (green fluorescence, 525 nm) and J aggregate fluorescence intensities (red fluorescence, 595 nm) were measured with CLSM (Yun and Lee, 2016).

The determination of membrane permeability of mitochondria was conducted following Chen et al. (2014). Mitochondrial extraction was performed with the method of successive differential centrifugation. The *P. sanguineus* pellets were homogenized in a ice-cold extraction buffer containing 0.4 M mannitol, 50 mM Tris-HCl, 10 mM EDTA, 0.1% (w/v) bovine serum albumin, and 0.05% (v/v) β -mercaptoethanol at pH 7.4. The biomass homogenate was centrifuged first at 800 g for 10 min to eliminate cell debris, and then the supernatant was further centrifuged at 10,000 g for 30 min at 4 °C. Finally, the remaining precipitate rinsed twice with the extraction medium mentioned above was used as the pure mitochondria. The prepared mitochondria were suspended in buffer A (200 mM mannitol, 70 mM sucrose, 5 mM HEPES, 5 mM sodium succinate, pH 7.2). The protein concentration of suspension

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