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Comparative study of *in vitro* prooxidative properties and genotoxicity induced by aflatoxin B1 and its laccase-mediated detoxification products

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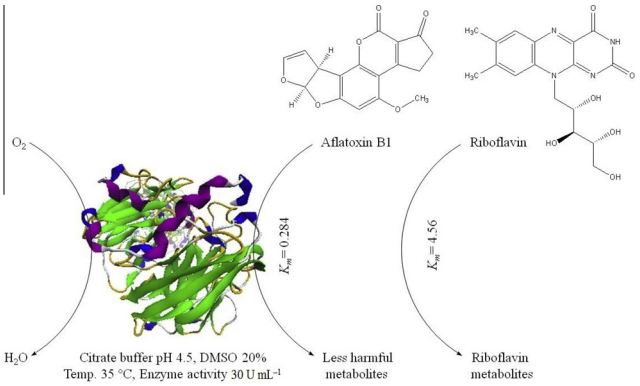
HIGHLIGHTS

- Enzymatic detoxification of aflatoxin B1 by laccase was studied.
- Maximum removal observed at pH 4.5 and 35 °C.
- Enzymatic derivatives showed less prooxidative activities.
- Metabolized and non-metabolized forms of products did not indicated mutagenicity.
- Laccase affinity for aflatoxin B1 is significantly more than riboflavin.

GRAPHICAL ABSTRACT



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ABSTRACT

In this paper, the enzymatic detoxification of aflatoxin B1 (AFB1) by laccase was studied, and the prooxidant properties and mutagenicity of the detoxification products were compared with those of AFB1. The optimal enzymatic reaction occurred in 0.1 M of citrate buffer containing 20% DMSO at 35 °C, a pH of 4.5, and a laccase activity of 30 U mL^{-1}. After 2 d, sixty-seven percent of the toxic substrate was removed. The prooxidative properties of the detoxified products (27% versus 86%) and the mutagenicity were significantly decreased in comparison with the parent toxin. Unlike AFB1, which promoted metabolism-dependent genetic mutations by base-pair substitution, the detoxified products did not induce genotoxicity. Comparison of the K_m values for AFB1 and riboflavin, a valuable food nutrient, indicated that laccase showed greater affinity for the toxin than for riboflavin.

1. Introduction

With worldwide increases in population, the need for nutrient-rich food is rising. Contamination of foods by toxins, bacteria, viruses, parasites, allergens, and prions may lead to serious

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diseases; unhealthy foodstuffs are implicated in approximately one-third of cancer cases (Oroojalian et al., 2010). Controlled storage conditions, improved packaging, and strict hygiene regulations for food production, preservation, and distribution are essential to diminish such problems (Galati et al., 2002). Aflatoxin B1 (AFB1), often formed by *Aspergillus flavus*, is a prevalent food pollutant, found typically in tropical countries. It imposes great costs on the world's economy and health (Algül and Kara, 2014). Thus it is important to eliminate AFB1 from food resources and prevent production of the toxin. Due to lack of infrastructure, poor and third-world countries are the major victims of AFB1. The established carcinogenesis, teratogenesis, and severe multi-organ toxicity associated with AFB1 have made it a substantial challenge for scientists (Partanen et al., 2010; Niknejad et al., 2012). AFB1's molecular toxicity is primarily due to DNA alkylation and subsequent mutagenesis and AFB1's prooxidant activities, which result in the generation of oxygen radicals (Eaton and Gallagher, 1994; Payabvash et al., 2006; Nili-ahmadabadi et al., 2011).

AFB1 is highly resistant to traditional detoxification protocols, such as heat, solvents, and radiation, which have consequences for food quality and safety (Sabzevari et al., 2006). Biological procedures using microbial or enzymatic tools that possess great specificity with minimal consequences are the appropriate choices for the treatment of contaminated foodstuffs; they also offer ease of application, affordability, and environmentally-friendly behavior. The use of biological procedures requires optimized conditions, such as pH and temperature, for maximum efficiency (Forootanfar et al., 2011; Aghaie-Khouzani et al., 2012).

Numerous enzymes can degrade toxins (Karlovsky, 2011); among these, laccase, a copper-based oxidase, can be used in the synthesis or degradation of organic compounds (Rogalski et al., 1990; Ostadhadi-Dehkordi et al., 2012; Mirzadeh et al., 2014; Mogharabi and Faramarzi, 2014; Heidary et al., 2014). Recent studies demonstrated that laccase can convert several environmental pollutants and toxins, such as phenols, oxidizing alkenes, carbazoles, fluorene, dibenzothiophene, and polycyclic aromatic hydrocarbons, to less or non-poisonous products (Bressler et al., 2000; D'Acunzo et al., 2002; Mayer and Staples, 2002; Punnapayak et al., 2009).

The aim of the present study was to evaluate the potential of laccase in AFB1 decontamination in order to be used as a safe food preservative. Accordingly, optimization of the enzymatic detoxification procedure, evaluation of enzyme selectivity, comparisons of genotoxicity and prooxidant potential of AFB1 and detoxified products were performed *in vitro*.

2. Materials and methods

2.1. Chemicals and instruments

Laccase from *Trametes versicolor* (>10 U mg^{-1}) and other chemicals were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). All organic solvents were obtained from Merck (Darmstadt, Germany). Aflaprep immunoaffinity columns were prepared from r-Biopharm AG (Darmstadt, Germany). Amicon ultracentrifugal filters were purchased from Merck Millipore (Billerica, Massachusetts, USA). *Salmonella typhimurium* (TA 98 and TA 100 strains) was obtained from the Persian Type Culture Collection (PTCC).

2.2. Laccase assay

The activity of laccase was determined using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the specific enzyme substrate. The reaction mixture comprised the enzyme (0.1 U mL^{-1}) and ABTS (5 mM) dissolved in citrate buffer (pH 4.5, 0.1 M). Absorbance changes were measured at 420 nm by an UV–visible spectrophotometer, UVD 2950 (Labomed, Los Angeles, CA, USA), after incubation for 10 min at 37 °C. The enzymatic activity was determined by the molar extinction coefficient of ABTS (ε_{420} = 36 000 M^{-1} cm^{-1}). One unit of laccase activity was equal to the quantity of the enzyme that oxidized 1 μM of ABTS per min (Alberts et al., 2009; Faramarzi and Forootanfar, 2011). The impact of 20% DMSO, as the co-solvent added to increase AFB1 solubility in the aqueous solution, was also assessed (data not shown).

2.3. Detection of AFB1 with HPLC–FLD

A high-pressure liquid chromatography machine, Smartline Series (Knauer, Berlin, Germany), equipped with a fluorescence detector (HPLC–FLD) was used for chromatographic analysis. A LCTech C18 liquid chromatography (LC) column (150 × 4.6 mm, 3 μM with a guard of 8 × 4.6 mm) was applied with a mobile phase containing water/acetonitrile/methanol (60:15:30 by volume; isocratic), a column temperature of 37 °C, a flow rate of 1 mL min^{-1}, and an injection volume of 20 μL. AFB1 detection was achieved using 375 and 430 nm as the wavelengths of excitation and emission, respectively. The limit of detection (LOD) and the limit of quantification (LOQ) of the system for AFB1 were 2.91 and 8.82 ng, respectively.

2.4. Optimum laccase activity for detoxification of AFB1

For optimization of the enzymatic detoxification of AFB1, different ranges of the variables affecting the enzymatic reaction were studied: substrate (AFB1) concentrations, enzyme activity, pH, and temperature. Based on a preliminary study, AFB1 concentrations of 10–100 μg mL^{-1}, a pH range of 2.5–7.0, and temperatures of 5–65 °C were applied, and the enzyme activity was assessed in 0–60 U mL^{-1}. All sample aliquots were prepared in 0.1 M of citrate buffer containing 20% DMSO. For maximum accuracy, all substrate concentrations, laccase activities, pHs, and temperature ranges were analyzed by a Biotek Synergy4 Plate-reader with a fluorescence spectrophotometer (Winooski, Vermont, USA) using 375 and 430 nm as the wavelengths of excitation and emission of AFB1. The activities of the enzyme at the optimal temperature and pH were used as the control (relative activity: 100%) and other data were expressed as percent of control.

2.5. Laccase-mediated catalysis of AFB1

AFB1 (80 μg mL^{-1}) and laccase (30 U mL^{-1}), both dissolved in a citrate buffer solution (pH 4.5, 0.1 M) containing 20% DMSO were incubated at 35 °C for 5 d. Changes of AFB1 concentration were measured using the HPLC–FLD. A blank sample without enzyme was also analyzed. For extraction of the applied enzyme, Amicon ultracentrifugal filters were used. The detoxified products and the remaining non-detoxified AFB1 were separated using Aflaprep immunoaffinity columns. Consequently, AFB1 as the parent toxin, the detoxified products, and remaining non-detoxified AFB1 (attached to immunoaffinity columns) formed three groups to compare their toxicities. The enzyme was removed to prevent its probable effects on the results. Remaining attached AFB1 was compared to intact AFB1.

2.6. Prooxidant potential of AFB1 and the laccase-mediated detoxified products

20 μL of each sample (from solutions of 50 μg mL^{-1}), including AFB1, the detoxified products, and the remaining non-detoxified