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Short communication

The furofuran-ring selectivity, hydrogen peroxide-production and low K_m value are the three elements for highly effective detoxification of aflatoxin oxidase

Yuan-zhen Wu [a], Fu-Pu Lu [a], Cui-Ping Tan [a], Dong-Sheng Yao [b], Mao-Qing Chu [a], Chun-Fang Xie [a], Da-Ling Liu [a,*]

[a] Department of Biotechnology, Jinan University, Guangzhou City 510632, Guangdong Province, China
[b] National Engineering Research Center of Genetic Medicine, Jinan University, Guangzhou City 510632, Guangdong Province, China

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ABSTRACT

AFO (aflatoxin oxidase), an enzyme from *Armillariella tabescens* previously named aflatoxin detoxifizyme, exhibits oxidative detoxification activity toward aflatoxin B_1 and sterigmatocystin. Bioinformatics reveals that AFO is a newly discovered oxidase because AFO does not share any significant similarities with any known oxidase. It is critically important to understand how AFO acts on aflatoxin B_1. In this study, in addition to aflatoxin B_1 (AFB_1) and sterigmatocystin (ST), five other chemicals that have furan or pyran structures were investigated. The results indicated that in addition to AFB_1 and ST, AFO is also able to act on versicolorin A, 3,4-dihydro-2H-pyran and furan. These results suggested that 8,9-unsaturated carbon—carbon bond of aflatoxin B_1 is the potential reactive site for AFO. Further findings indicated that the action of AFO is oxygen-dependent and hydrogen peroxide-producing. The simultaneously produced-hydrogen peroxide possibly plays the essential role in detoxification of AFO. In addition, the extremely low K_m value of 0.33 μmol/l for AFO-AFB_1 and 0.11 μmol/l for AFO-ST signifies that AFO is highly selective for AFB_1 as well as ST.

1. Introduction

Aflatoxins are a group of carcinogenic mycotoxins that have attracted considerable attention due to their carcinogenicity, immunosuppressive ability, hepatocyte toxicity, and wide presence as contamination in food and feedstuffs (Tirado et al., 2010; Wagacha and Muthomi, 2008; Wild and Turner, 2002). The toxic structural component of aflatoxin B_1 is considered to be the bisfuran ring or the 8,9-unsaturated carbon—carbon bond (Fig. 1). There have been several reports about the degradation of aflatoxins by microbes or microbial enzymes (Alberts et al., 2006; Cserháti et al., 2013; Farzaneh et al., 2012; Guan et al., 2008; Smiley and Draughon, 2000; Teniola et al., 2005). AFO (aflatoxin oxidase), which was isolated from the mushroom of *Armillariella tabescens*, exhibits aflatoxin B_1 detoxification and was named aflatoxin detoxifizyme (ADTZ) in our previous reports (Liu et al., 1998, 2001). It has been found that after AFO (or ADTZ) treatment, the typical IR adsorption peaks of acetal structure (that is, the furofuran ring) of AFB_1 disappeared (Liu et al., 1998). After about 10 years, the findings on the direct electrochemistry of AFO via intramolecular electron transfer had been reported (Chen et al., 2010). In that study, the transfer of one electron to the electrode by a molecule of AFO was discovered. However, little is known about the action of AFO. AFO (coded by AY941095, NCBI gene bank) has been fully sequenced and cloned in an engineered yeast (Cao et al., 2011). The bioinformatics analysis indicates that the amino acid sequence of AFO shares 42% similarity with a known hydrolase (i.e., dipeptidyl peptidase III, BLAST, EC 3.4.14.4). However, both the gene and the protein sequence of AFO do not exhibit any significant similarities with any known oxidase. Therefore, a more in-depth investigation of AFO is required. In this paper, the features of AFO have been investigated, including the enzyme's substrate-selectivity. Electrochemistry is a convenient tool in oxidase study. The AFO-based electrode was constructed by immobilizing AFO onto a single-walled carbon nanotube (SWCNT)-modified gold electrode. Various potential substrates (Fig. 1), such as aflatoxin B_1 (AFB_1), sterigmatocystin (ST), versicolorin A (Ver A), 3,4-dihydro-2H-pyran (DHP), furan (FR), benzofuran (BF) and 8-methoxypsoralen (8-MOP), were investigated.

Poultry are the most sensitive species to the toxic effects of aflatoxin B_1 (AFB_1), and younger poultry are more sensitive to this mycotoxin (Giambrone et al., 1985; Klein et al., 2002). AFB_1 toxicity is the metabolic activation by cytochromes P450 (CYPs) to

Abbreviations: AFB_1, aflatoxin B_1; AFO, aflatoxin oxidase; BF, benzofuran; CV, cyclic voltammetry; DHP, 3,4-dihydro-2H-pyran; DPV, differential pulse voltammetry; EDC, 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide; FR, furan; HRP, horseradish peroxidase; ITC, isothermal titration calorimetry; 8-MOP, 8-methoxy-psoralen; NHS, N-hydroxysuccinimide; PBS, phosphate buffer solution; ST, sterigmatocystin; SWCNT, single-walled carbon nanotube; Ver A, versicolorin A.

* Corresponding author. Department of Biotechnology, Jinan University, Guangzhou City 510632, Guangdong Province, China. Tel.:+86 020 85228422.
E-mail address: tldl@jnu.edu.cn (D.-L. Liu).

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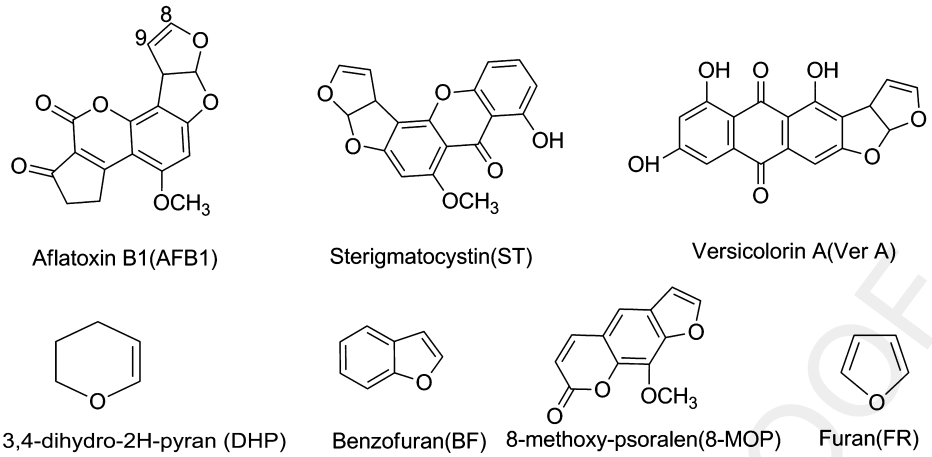

Fig. 1. Structures of the various chemicals under investigation.

AFB_1-8,9-epoxide (AFBO) (Guengerich et al., 1998). In this study, we report that AFO reacts with AFB_1 at a much lower K_m value when compared with young turkey's hepatic microsomal activation.

2. Materials and methods

2.1. Reagents

AFO was obtained from the engineered *Pichia pastoris* GS115 PSA and purified according to previous procedures (Li et al., 2011). The concentration of AFO used in the study was 0.2 mg/ml with an activity of 300 U (1 U was equal to the amount of AFO that was able to decrease one unit of fluorescence intensity at 450 nm of AFB_1 (Ex 365 nm)). VerA was purified from *Aspergillus versicolor* ATCC 36537 (Trail et al., 1995). The SWCNTs (95% purity) were obtained from Shenzhen Nanotech Port Co., China. EDC (1-ethyl-3-(3-dimethylamine propyl) carbodiimide), NHS (N-hydroxysuccinimide), AFB_1, ST, DHP, FR, BF and 8-MOP were obtained from Sigma-Aldrich, USA. All other reagents were of analytical reagent grade.

2.2. Apparatus and instrumentation

A bioanalytical system BAS100 (BAS Co., USA) with a three-electrode system was employed as we reported previously (Chen et al., 2010). The determinations were performed at 25 °C in a supporting electrolyte of a 0.1 mol/l Na_2SO_4 solution buffered with 0.01 mol/l PBS (phosphate buffer solution) (pH 7.0). Prior to modification, the bare gold electrode and SWCNTs were treated following our previous procedures (Chen et al., 2010). The AFO-SWCNT/Au electrode was fabricated layer-by-layer by using EDC-NHS as the cross-linker. Ten microliters of the active SWCNTs (0.5 mg/ml) were dropped onto the cleaned gold electrode surface and dried to form a film at 50 °C. After a PBS (PH 7.4, 0.01 mol/l) washing procedure, the SWCNT-modified electrode was immersed in 50 ml of an EDC-NHS (1:1 v/v) solution with a concentration of 100 g/l (resolved in 0.01 mol/l pH 7.4 PBS) for 30 min. Then, 10 ml of the enzyme solution was dropped onto the NHS-EDC/SWCNT-modified gold surface. The electrode was placed upright in a humid airtight environment at 4 °C for more than 24 hours. To detect the hydrogen peroxide, a horseradish peroxidase (HRP)-modified electrode was fabricated following the same procedure except that the AFO solution was replaced with 0.2 mg/ml HRP. The electrodes were suspended in a salt solution (0.1 mol/l Na_2SO_4 buffered with 0.01 mol/l PBS, pH 6.5) during storage.

2.3. Electrochemical investigation of AFO

During the electrode fabrication, the properties of the electrodes were analyzed in a step-by-step manner using a 2 mmol/l $K_4Fe(CN)_6$ solution containing 0.1 mol/l KCl. The characteristics of the response of the AFO-modified electrode to various substrates at various concentrations were investigated with cyclic voltammetry (CV) and differential pulse voltammetry (DPV). H_2O_2 was detected using an HRP-modified electrode. AFO and its substrates were added to a base solution that contains 1 mmol/l hydroquinone with a 0.01 mol/l PBS solution (pH 6.6).

2.4. Enzymatic kinetics investigation of AFO

Kinetic constants play an important role in understanding the characteristics of an enzyme. Here, the AFO used is prepared with higher than 90% purity. The determinations were performed using the isothermal titration calorimetry (ITC) (Auto-ITC MicroCal, Unicam 500, Thermo Spectronic) technique. Thermograms were measured with the addition of AFB_1 or ST (72 separate 5 µl injections from a 10 µmol/l AFB_1 or ST solution with 0.3% Triton X-100 added) to a 10 nmol/l AFO solution in a 0.02 mol/l phosphate-citrate buffer of pH 5.8 with 0.3% Triton X-100 at 30 °C.

3. Results and discussion

3.1. AFO-modified electrode response to the various substrates at various concentrations

The substrate selectivity of AFO, AFB_1, ST, Ver A, 8-MOP, DHP, BF and FR (shown in Fig. 1) were investigated. The DPV responses of the AFO-SWCNT-modified electrode were recorded. As shown in Fig. 2, the AFO-based electrode demonstrates valid signals that gradually increase in strength with increasing concentrations of AFB_1 (Fig. 2a), ST (Fig. 2b), Ver A (Fig. 2c), DHP (Fig. 2d) and FR (Fig. 2e). The peaks for AFB_1 (Fig. 2a), ST (Fig. 2b), Ver A (Fig. 2c), DHP (Fig. 2d) and FR (Fig. 2e) are located at 550, 600, 560, 580 and –200 mV, respectively, and at –600 mV. In addition, the AFO electrode does not exhibit a significant response to increasing concentrations of BF and 8-MOP (Figs. 2f and g). In BF and 8-MOP the benzene structure strongly stabilizes these chemicals via the conjugation of pi bonds, which leads to the difficulty in the oxidation of benzofuran and methoxy benzofuran by AFO. In contrast, being free from benzene ring, DHP and FR are small molecules, and easily enter and adjust to the correct position in the active center of the enzyme. The above results suggest that AFO is selective to furan or pyran structure. This finding is consistent with what we reported previously that the furofuran-ring of AFB_1 was transformed by AFO (or ADTZ) (Liu et al., 1998).

3.2. The impact of oxygen on the catalysis of AFO

Previous experimental results have confirmed the oxidase activity of AFO (Chen et al., 2010). However, there are various types of redox enzymes. To determine the influence of oxygen is essential for understanding the active mode of AFO. This study was performed by bubbling oxygen and nitrogen into the reaction mixtures, separately. As shown in Fig. 3, the oxygen bubbling enhanced the redox-response signals of the AFO electrode for AFB_1, ST, Ver A, DHP, and FR. The results suggest that the action of AFO is oxygen dependent.

3.3. Confirming the production of hydrogen peroxide

AFO is an oxidase without a hydrogen donor cofactor such as NADH, NADPH or FADH. Therefore, the substrate should act as the hydrogen donor during the AFO reaction. However, AFO reacts with

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