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Cloning, expression of a peroxiredoxin gene from *Acinetobacter* sp. SM04 and characterization of its recombinant protein for zearalenone detoxification

Yuanshan Yu[a,b], Hui Wu[b,*], Yuqian Tang[b], Liping Qiu[c]

[a] College of Light Industry and Food, South China University of Technology, Guangzhou 510641, China
[b] Sericulture and Agro-food Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou 510610, China
[c] Guangdong Food and Drug Vocational College, Guangzhou 510520, China

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ABSTRACT

Zearalenone (ZEN) is a *Fusarium* mycotoxin, which has been associated with hyperestrogenism and other reproductive disorders in farm animals. ZEN-contaminated grains as well as its by-products had engendered numerous economic losses to farm animals' production, so the detoxification of ZEN-contaminated grains and its by-products would be necessary and beneficial. In this study, a peroxiredoxin (Prx) gene from *Acinetobacter* sp. SM04 was cloned, and over-expressed in *Escherichia coli* BL21 (DE3). The Prx gene of *Acinetobacter* sp. SM04 encodes a protein of 187 amino acids residues and NCBI BLAST program analysis of deduced amino acids shows high identity with 2-Cys Prx family. Interestingly, recombinant Prx show efficient ability to degrade ZEN using H_2O_2. Results of MCF-7 cell proliferation assay also found ZEN were oxidized into little estrogenic metabolites by purified recombinant Prx plus H_2O_2. Further, model experiments on decontamination of ZEN-contaminated corn using recombinant Prx were performed, and results found nearly 90% of ZEN was degraded when crushed ZEN-contaminated corn samples (nearly 1000 μg ZEN per kg grain) were treated with purified recombinant Prx plus 0.09% (m/m) H_2O_2 for 6 h at 30 °C. In addition, the optimum pH and temperature of purified recombinant Prx for ZEN degradation were 9.0 and 70 °C respectively.

1. Introduction

Zearalenone (ZEN) is a nonsteroidal oestrogenic mycotoxin which causes hyperestrogenism and related toxicoses to farm animals and humans. Hyperestrogenic syndromes can be uterine enlargement, swelling of the vulva, prolapse of the vagina or rectum, prolonged or interrupted estrus, pseudopregnancy and reduced litter size (Doll et al. 2004; Zinedinea et al. 2007). This mycotoxin is produced by some *Fusarium* fungi (D'Mello et al. 1999; Zinedinea et al. 2007). Many of the toxigenic species of *Fusarium* are major pathogens of cereal plants in many countries, and a high level of ZEN often accumulates in *Fusarium*-infected grains and derived cereal products (D'Mello et al. 1999; Placinta et al. 1999). Because of the economic losses engendered by ZEN and its impact on human and animal health (Wu and Munkvold 2008), many strategies for detoxifying contaminated foods and feeds had been described in the literature, including adsorption (Shawna et al. 1998; Kabak et al. 2006), biodegradation (Kamimura 1986; Megharaj et al. 1997; Mortensen et al. 2006; Molnar et al. 2004; Takahashi-Ando et al. 2005), extrusion cooking (Yuksel and Bullerman 2005b) and ozonation (Mckenzie et al. 1997). Among them, transformation or degradation of ZEN by microorganisms is an attractive approach for efficient detoxifying ZEN.

Based on the previous findings (Yu et al. 2011) that ZEN can be degraded into little estrogenic metabolites by enzymes in the extracellular extracts of *Acinetobacter* sp. SM04 liquid cultures in M1 medium, and these enzymes involved on ZEN degradation were isolated from *Acinetobacter* sp. SM04 extracellular extracts of liquid cultures. Results of SDS-PAGE and MALDI-TOF/TOF/MS analysis found a protein from these enzymes involved on ZEN degradation, which was identified as peroxiredoxin (Prx), matched the NCBI database for *Acinetobacter* genus with great homology.

Peroxiredoxins are thio-redoxin peroxidases that catalyze the reduction of hydrogen peroxide, organic hydroperoxides and peroxynitrite to less reactive products (Zachary et al. 2003). The characteristic feature of all Prx is the peroxidatic Cys residue (Cys–S_PH) which is oxidized by the peroxide substrate to Cys sulfenic acid (Cys–S_POH) during the reaction cycle. Prx are classified either as 1-Cys or 2-Cys depending on whether they contain one or two conserved Cys residues (Zachary et al. 2003). As a new and expanding family of thio-redoxin peroxidase, Prx have received considerable attention. Its functions are diverse,

* Corresponding author at: South China University of Technology, Wushan RD, TianHe District, Guangzhou 510641, China. Tel.: +86 13922782528; fax: +86 20 222 36189.
E-mail address: 499072725@qq.com (H. Wu).

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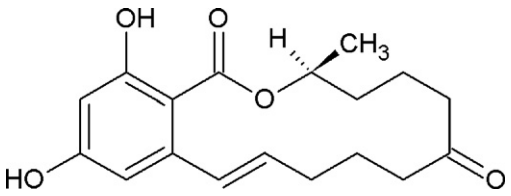

Fig. 1. The chemical structure of ZEN.

ranging from antioxidant enzymes to regulators of signal transduction. This diversity is reflected in slight evolutionary modifications in sequence and structure, built around a common peroxidatic active site. The literature within the Prx field is currently focused on their more recently identified roles as regulators of redox-sensitive signaling (Jacobson et al. 1989; Poole and Ellis 1996; Bryk et al. 2000; Peshenko and Shichi 2001; Hofmann et al. 2002).

No studies on degradation of lignin, dyes and aromatic pollutants using peroxidase activity of Prx have been reported so far, but many typical hemoperoxidase, including horseradish peroxidase, soybean peroxidase, lignin (LiP) peroxidase and manganese (MnP) peroxidase, had been widely applied in clinical analysis, decolorization in paper and textile industries, and decontaminated of aromatic pollutants. ZEN is somewhat structurally related to the above-mentioned aromatic pollutants (Fig. 1). However, not many works have been reported on the degradation of ZEN by these peroxidases (Peter 2000; Ogawaa et al. 2004; Zinedinea et al. 2007). To further conform whether Prx identified on the previous findings (Yu et al. 2011) was involved to ZEN degradation, a gene of Prx from *Acinetobacter* sp. SM04 was cloned and expressed in *E. coli* BL2 (DE3). Meanwhile, the activity of recombinant Prx to degrade ZEN was also assessed.

2. Materials and methods

2.1. Chemicals

ZEN was purchased from Sigma–Aldrich (St. Louis, USA), and dissolved in methanol as standard stock solution (1 mg ml^{-1}). Methanol was of chromatographic pure grade and water was purified by a Milli-Q Academic Water system. Sephadex G-75 and phenyl-Superpose HR were purchased from Amersham Pharmacia Biotech (China) Ltd. Other chemicals used were all of analytical grade.

2.2. Cloning of Prx gene from Acinetobacter sp. SM04

A pair of degenerate primers (5′-GTWGCTTCGCCTTCTTTCCATTT and 5′-AATCCAAATCGTWGARHTNAAYGC) was used to clone Prx gene fragment. Degenerate primers were designed on basis of the sequences of protein identified by previous studies (Table 1). Genomic DNA (PCR template) was purified from *Acinetobacter* sp. SM04 by the Bacterial DNAout kit (TIANDZ, China). The PCR products were cloned into pMD^{TM} 18-T vector (Takara, Japan) and sequenced by Invitrogen Corporation (Guangzhou), and then specific primers from the PCR products (sectional sequences of Prx gene obtained) were designed for amplifying its flanking nucleotide sequence information using Tail-PCR (Liu and Chen 2007). The nucleotide and deduced amino acid sequence of Prx ORF were analyzed using NCBI BLAST search program and Expasy search program (http://au.expasy.org/tools/).

Table 1
Information of identified protein involved to ZEN degradation in the extracellular extracts of *Acinetobacter* sp. SM04 (Yu et al. 2011).

Protein name	Mass	Sequences of identified peptides
Peroxiredoxin	20874	K.IQIVELNAGGIGR.D K.NFDVLIESEGLADR.G

2.3. Cloning of Prx coding region sequence into the pET-31 b(+) expression vector

A pair of primers (5-CTGTATACATATGAGCTTGATTAATACTGAAGT and 5-TAGGCTGCTCGAGTTAGATTTTACCAACTAAGTCG) was used to amplify the Prx coding region sequence. Genomic DNA from *Acinetobacter* sp. SM04 was used as PCR template. The pET-31 b(+) vector and the PCR products above containing the Prx ORF were both digested with *Nde*I and *Xho*I, and were purified by agarose gel electrophoresis. The gel purified Prx ORF was ligated into the pET-31 b(+) vector using T_4 DNA ligase (Promega) and the ligation products were used to transform competent *E. coli* DH5α cells. Transformed cells were selected on LB agar containing Ampicillin (50 μg ml^{-1}), the presence of the Prx ORF was confirmed by colony PCR and sequencing, and then the resulting constructs were used to transform *E. coli* BL21 (DE3) cells.

2.4. Over-expression and purification of recombinant Prx

The recombinant Prx were over-expressed in *E. coli* BL21 (DE3) cells in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG). Briefly, *E. coli* BL21 (DE3) starter culture containing the Prx ORF in the correct orientation and reading frame was used to inoculate LB broth supplemented with Ampicillin (50 μg ml^{-1}) and the cells were cultured with shaking (250 rpm) at 37 °C until an OD_{600} value of 0.5 was reached. At this point, protein expression was induced by the addition of 0.5 mM IPTG and the cells were cultured for further 4 h before being harvested by centrifugation.

The resulting cell pellets were suspended in 5 times volume of 0.02 M Tris–HCl buffer (pH 9.0), and were disrupted at 4 °C with Ultrasonic Cell Disruptor (Sonics Materials, Inc., USA). The cell debris was removed by centrifugation at 15,000 × *g* for 5 min at 4 °C, and then the supernatant (crude extract) was used for further Prx enzymes purification.

In the further Prx enzyme purification, the supernatants (crude extracts) were supplemented with 2.0 mol l^{-1} $(NH_4)_2SO_4$, and then applied on phenyl Sepharose column (2.0 × 10 cm) equilibrated with Buffer A containing 2.0 mol l^{-1} $(NH_4)_2SO_4$ + 0.02 mol l^{-1} Tris–HCl (pH 9.0). After washing the column with the same buffer, the active fraction was eluted with decreasing concentrations of $(NH_4)_2SO_4$. Fractions containing ZEN degradation activity (10 ml) were then loaded onto a Sephadex G-75 column (2.0 cm × 25 cm) pre-equilibrated with 0.02 mol l^{-1} Tris–HCl (pH 9.0), and the column was eluted by the same buffer at a flow rate of 0.5 ml min^{-1}, and fractions of 5 ml were collected to analyze ZEN degradation activity. In addition, protein samples were run on 12.5% SDS-PAGE with a protein marker. Gels were stained using 0.05% Coomassie Blue R-250, followed by a standard destaining procedure. Protein concentrations were determined with a Bradford protein assay with bovine serum albumin as a standard.

2.5. Effect of H_2O_2 on ZEN degradation activity of recombinant Prx

The reaction mixture contained 50 μl of Prx enzyme solution, 400 μl of 0.05 mol l^{-1} Tris–HCl buffer (pH 9.0), 40 μl different concentration of H_2O_2 water solution, and 10 μl of ZEN standard stock solution (1 mg ml^{-1}). The reaction mixture was incubated