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Oxidation of zearalenone by extracellular enzymes from *Acinetobacter sp.* SM04 into smaller estrogenic products

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Abstract Zearalenone is a mycotoxin mainly produced by *Fusarium* mold, which has been associated with hyperestrogenism and other reproductive disorders in pigs, sheep, and other farm animals. Since zearalenone engendered economic losses to farm animal production, its detoxification in contaminated grains or by-products would be advantageous. In this study, enzymes from the *Acinetobacter sp*. SM04 extracellular extracts of liquid cultures were isolated by Sephadex G-100 column, and an active fraction capable of efficiently degrading zearalenone was obtained. Zearalenone could be oxidized into smaller estrogenic products by the active fraction, and two intermediate products, ZEN-1([M-H]$^-$ at m/z 489) and ZEN-2([M-H]$^-$ at m/z 405), were found. Zearalenone degradation activity of the active fraction was significantly inhibited by low oxygen gas content, protease, SDS, and EDTA treatment. Further, enzymes in the active fraction were analysed by SDS–PAGE and MALDI-TOF/TOF/MS, and three proteins were found that matched the database for *Acinetobacter* genus with great homology. They were identified as peroxiredoxin, a possible cytochrome and a putative fimbrial protein precursor.

**Keywords** Detoxification · Zearalenone · *Acinetobacter* sp · Peroxiredoxin

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Introduction

Zearalenone is a nonsteroidal oestrogenic mycotoxin biosynthesized through a polyketide pathway by a variety of *Fusarium* fungi (Zinedinea et al. 2007). Its chemical structure is presented in Fig. 1. Many of the toxigenic species of *Fusarium* are major pathogens of cereal plants in many countries, and a high level of zearalenone often accumulates in *Fusarium*-infected grains and derived cereal products (D'Mello et al. 1999; Placinta et al. 1999; Lu and Chen 2004), which causes hyperestrogenism and related toxicoses of farm animals and humans. Hyperestrogenic syndromes can be characterized by uterine enlargement, swelling of the vulva, prolapse of the vagina or rectum, prolonged or interrupted estrus, pseudopregnancy, and reduced litter size (Doll et al. 2003; Zinedinea et al. 2007). Because of the economic losses engendered by ZEA and its impact on human and animal health (Felicia et al. 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 zearalenone by microorganisms is an attractive approach for efficiently detoxifying zearalenone.

It was found previously (Yu et al. 2010) that zearalenone can be degraded into smaller estrogenic metabolites by proteins in extracellular extracts of *Acinetobacter* sp. SM04. In this report, enzymes in the extracellular extracts were isolated by Sephadex G-100 column, and zearalenone degradation by the active fractions was characterized. Further, enzymes in the active fractions involved on
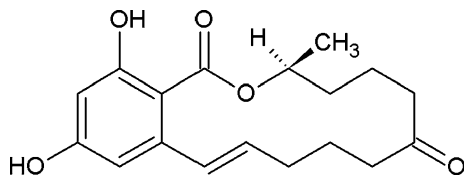
Fig. 1 The chemical structure of zearalenone

zearalenone degradation were also identified by SDS–PAGE and MALDI-TOF/TOF/MS analysis.

Materials and methods

Chemicals

Zearalenone was purchased from Sigma (St. Louis, USA), and dissolved in methanol (10 mg ml^{-1}). Methanol and ethyl acetate were of chromatographic pure grade and water was purified by a Milli-Q academic water system. Sephadex G-100 was purchased from Amersham Pharmacia Biotech (China) Ltd. Other chemicals used were all of analytical grade.

Preparing of strain SM04 extracellular extracts of liquid cultures

Acinetobacter sp. SM04, isolated from agricultural soil (Yu et al. 2010) was inoculated into M1 medium and incubated at 30°C on a rotary shaker (200 rev min^{-1}) to exponential anaphase. The culture supernatants were pooled via centrifugation (12000g for 5 min) at 4°C, passed through 0.22 μm filter, and named as strain SM04 extracellular extracts of liquid cultures. M1 medium contained the following per liter: 15 g of sodium acetate, 1.0 g of yeast extracts, 3 g of NH$_4$NO$_3$, 1 g of KCl, 0.5 g of MgSO$_4$, 1.0 g of K$_2$HPO$_4$, 0.1 g of CaCl$_2$, and 10 ml of trace element (2 g l^{-1} FeSO$_4$·7H$_2$O, 0.4 g l^{-1} MnSO$_4$·4H$_2$O, 0.4 g l^{-1} CuSO$_4$·5H$_2$O, 0.4 g l^{-1} CoCl$_6$·6H$_2$O and 0.5 g l^{-1} ZnCl$_2$).

Sephadex G-100 column isolation of enzymes from strain SM04 extracellular extracts

The strain SM04 extracellular extracts were concentrated 8 times by rotary vacuum evaporator at 50°C. The concentrated extracellular extracts (10 ml) were then loaded onto a Sephadex G-100 column (2.0 × 25 cm) pre-equilibrated with 20 mM Tris–HCl (pH 9.0). The column was eluted by the same buffer at a flow rate of 2 ml min^{-1}, and fractions of 5 ml were collected and screened for zearalenone degradation activity.

Effects of low oxygen gas content, protease, SDS, and EDTA on zearalenone degradation activity

Low oxygen gas content

The active fractions from Sephadex G-100 column isolation were treated to remove dissolved oxygen gas by blowing with helium gas for 10 min, and promptly put into anaerobic incubator (Shanghai XinMiao Corporation, China) to perform the test of zearalenone degradation activity. All operations involved to the test of zearalenone degradation activity were done in the anaerobic incubator. The anaerobic incubator, which was filled with 95% CO$_2$ and 5% H$_2$ gases, was kept at 30°C. In control, the degasified active fractions were performed the test of zearalenone degradation activity under normal atmosphere.

Protease and SDS treatment

The active fractions from Sephadex G-100 column isolation were separately treated with 1 mg ml^{-1} proteinase K (sigma, USA), 1% (m/v) SDS (Sodium dodecyl sulfate), and 1 mg ml^{-1} proteinase K plus 1% SDS for 1 h at 55°C, and then were used to perform the test of zearalenone degradation activity. The active fractions exposed at 55°C for 1 h were included as control.

EDTA treatment

Before performing the test of zearalenone degradation activity, 0.1 M EDTA was added to the active fractions from Sephadex G-100 column isolation. The active fractions not treated with EDTA were included as control.

Test of zearalenone degradation activity

Samples (0.5 ml) was supplemented with zearalenone to give a final concentration of 20 μg ml^{-1}, and incubated at 30°C for 6 h. Following incubation, methanol (0.5 ml) was added to stop the reaction, and then the zearalenone concentration in the mixtures was quantified by using a Waters analytical HPLC system. In HPLC analysis, a Waters XTerra^R MS C18 column (4.6 × 150 mm, 5 μm) was kept at 30°C. The mobile phase, which consisted of methanol–water 65:35 (v/v), was used at a flow rate of 1 ml min^{-1} and the injection volume was 30 μl. The results were monitored at 274 nm. Concentrations of zearalenone were determined based on retention time and peak area compared to zearalenone standards dissolved in methanol. Zearalenone degradation rate (%) = [20 − remained ZEN concentration (μg ml^{-1})]/20 × 100. Results represent the averages of three independent experiments.

Product analysis of zearalenone oxidation by extracellular enzymes

The active fractions (50 ml) from Sephadex G-100 column isolation, which were supplemented with zearalenone (50 µg ml^{-1}), were put in conical flasks (250 ml) and incubated at 30°C on a rotary shaker (150 rev min^{-1}). After 6 h incubation, the mixtures were extracted twice with equal volume of ethyl acetate, acidified to pH 2.0 with 5 M HCl, and then extracted twice with an equal volume of ethyl acetate again. The ethyl acetate phases of base and acidic extracts were respectively evaporated under rotary vacuum evaporator at 45°C. The residue was dissolved in 1 ml methanol for HPLC analysis and further purification. The active fractions without zearalenone were included as control.

Product purification of zearalenone oxidation was done using a Waters preparative HPLC system. Samples (50 µl) were isolated on a Agilent Zorbax 300SB-C18 column (9.4 × 250 mm, 5 µm) with a linear gradient of methanol/water solution[10–70% (v/v) in 30 min] contained 0.1% (v/v) formic acid. Flow was kept at 4 ml min^{-1}, and results were monitored by PAD (photodiode array detector) between 210 and 400 nm. Product fractions were collected according to their retention time, concentrated 3 times by rotary vacuum evaporator at 50°C, and then were extracted three times with ethyl acetate. Combined extracts were evaporated in a rotary vacuum evaporator at 45°C, and the residue was dissolved in methanol or methanol-D4 to be analysed by mass spectroscopy (MS) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy analysis.

Mass spectrometric detection was performed with an ion-trap mass spectrometer equipped with an ESI interface (Bruker Corporation, Germany).^{1}H NMR spectra and ^{13}C NMR spectra were recorded on a AVANCE Digital 400 M Hz NMR Spectrometer (Bruker Corporation, Germany). The chemical shifts were reported in parts per million relative to tetramethylsilane, an internal standard.

The MTT (tetrazolium salt) MCF-7cell proliferation assay

The active fractions (0.5 ml) from Sephadex G-100 column isolation were supplemented with zearalenone (10 µg), and incubated at 30°C. After 0 and 6 h incubation, methanol (0.5 ml) was added to stop the reaction, and then the mixtures were used to analyse the loss of estrogenic effect of zearalenone by the MTT MCF-7 cell (estrogen receptor positive, human breast cancer cell) proliferation assay (Shen et al. 2003; Yuksel and Bullerman 2005a; Yuksel and Bullerman 2005b). The active fractions without zearalenone were included as control (incubated for 6 h). Each well in a 96-well plate was inoculated with MCF-7 single-cell suspension (100 µl, at a density of 5×10^4 cell/ml). After attachment of cells to the wells, the seeding medium was replaced with fresh 180 µl of phenol red-free DMEM/F-12 containing 10% charcoal-stripped serum. Samples were diluted 100 times using 1% ethanol water solution, and the diluted samples (20 µl) were also added to the 96-well plates and incubated at 37°C in a humidified atmosphere of 5% CO_2. To the blank was only added 20 µl of phenol free DMEM/F-12 medium containing 1% ethanol. Every sample was repeatedly added to five wells. After 4 days incubation, the MTT solution (25 µl, 5 mg ml^{-1}) was added to each well of the 96-well plates and incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2. Thereafter, the medium was discarded. 100 µl of dimethyl sulfoxide was added to each well, and the 96-well plates were placed on an orbital shaker at 37°C for 20 min. The absorbance was measured at 490 nm using a microplate reader. The % increase of MCF-7 cell proliferation was calculated using the formula: % increase of cell proliferation = $[(A_{490}$ test/A_{490} blank$) - 1] \times 100$, where A_{490} test = absorbance of test sample, A_{490}blank = absorbance of blank sample.

SDS–PAGE and MALDI-TOF-TOF/MS analysis of extracellular enzymes

The proteins in the active fractions from Sephadex G-100 column isolation were collected by TCA (Trichloroacetic acid) plus DOC (Na deoxycholate) protein precipitation method (Jiang et al. 2004), and separated with 15% polyacrylamide gel discontinuous denaturing SDS–PAGE (Laemmli 1970). Visualization of bands was performed by Coomassie Brilliant Blue R-250 staining. Proteins in the gel were excised for further identification with MALDI-TOF-TOF/MS (ABI 4800 plus MALDI-TOF/TOF MS, Applied Biosystems, USA).

The proteins in the gel pieces were digested into dried tryptic peptide mixtures by the methods previously described (Yu et al. 2010), and were redissolved in 0.1% (w/v) TFA (Trifluoroacetic acid) water solution and subjected to MALDI-TOF-TOF/MS analysis. From the mass spectral data obtained, protein identification was automatically performed with ABI GPS Explorer software (V 3.6). This system ran a search for each protein against the NCBInr (National Center for Biotechnology nonredundant database) using the MASCOT (V2.1, Matrix Science, London, U.K) search engine.

Results

Sephadex G-100 column isolation of enzymes from strain SM04 extracellular extracts

In the Sephadex G-100 column isolation, 40 fractions were collected, and their activity to degrade zearalenone was tested. Reduction of zearalenone were observed in the between 16 and 22 fractions, and no significant reduction of zearalenone were found in the other fractions. Zearalenone degradation activity of different fractions was presented in Fig. 2. In addition, nearly 80 repetitions of Sephadex G-100 column isolation were performed, and all active fractions (between 17 and 21 fractions) were pooled for further experiments.

Effects of low oxygen gas content, protease, SDS, and EDTA on zearalenone degradation activity

Table 1 shows the effects of low oxygen gas content, protease, SDS, and EDTA on zearalenone degradation activity. After treated with protease K and SDS, the pooled active fractions retained low zearalenone degradation activity, which indicated that enzymes from the pooled active fractions are involved in the degradation of zearalenone. Oxygen gas was necessary to zearalenone degradation by the enzymes in the pooled active fractions, which indicated that some enzymes involved on zearalenone degradation may be oxidases. EDTA, as metal chelating agent, can destroy the zearalenone degradation activity, which indicated that some metal cations are required for the enzymes in the active fractions to degrade zearalenone.
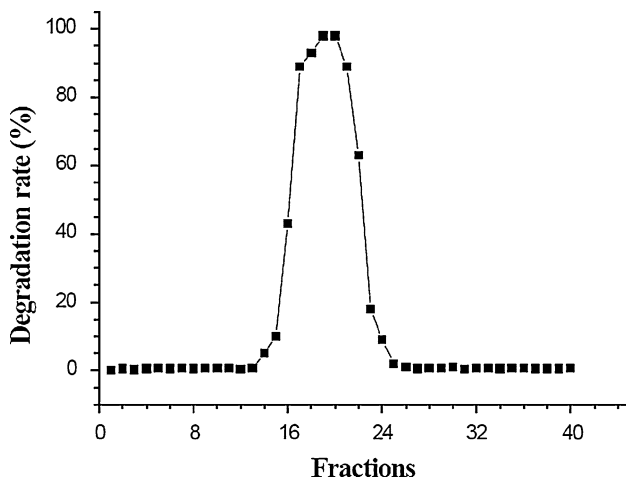
Fig. 2 Zearalenone degradation activity for different fraction from Sephadex G-100 column isolation

The MTT (tetrazolium salt) MCF-7 cell proliferation assay

A significant ($P < 0.001$) decrease of zearalenone proliferative effect on MCF-7 cells was observed when zearalenone was treated with the pooled active fractions for 6 h. Comparing with the blank, % increase of MCF-7 cell proliferation was nearly $10.2 \pm 1.4\%$ after zearalenone was treated with the pooled active fraction for 6 h, whereas 86 ± 0.6 increase of MCF-7 cell proliferation was observed when zearalenone was treated with the pooled active fraction for 0 h. In the control, only $0.2 \pm 0.8\%$ increase of MCF-7 cell proliferation was found. These results indicated that the pooled active fraction had no proliferative or anti-proliferative effect on MCF-7 cells, and zearalenone can be degraded into smaller estrogenic products by the pooled active fraction.

### Product analysis of zearalenone oxidation by extracellular enzymes

A total of 40 mg zearalenone were used for further product analysis of zearalenone oxidation by extracellular enzymes. After 6 h incubation, the reaction mixtures (100 μl) in each conical flask were taken to test the zearalenone degradation activity, and results showed that zearalenone degradation (%) was more than 86.7%. Meanwhile, products of zearalenone oxidation were extracted with ethyl acetate. Compared with the control, HPLC-PAD analysis of base extracts revealed no new products except remaining zearalenone, whereas two new products (named as ZEN-1 and ZEN-2) were found in the acidic extracts.

ZEN-1 and ZEN-2 were purified by the Waters Preparative HPLC system. The yield of isolated ZEN-1 and ZEN-2 was less than 1.0 mg, and the quantity of ZEN-1 was nearly half of ZEN-2. Therefore, the signal intensity of ^{1}H and ^{13}C NMR spectra was too low to determine the complete molecular structure of ZEN-1 and ZEN-2. ESI–MS (negative mode) analysis revealed $[M-H]^-$ of ZEN-1 at m/z 489, and $[M-H]^-$ of ZEN-2 at m/z 405, and UV–vis spectroscopy of ZEN-1 and ZEN-2 obtained from HPLC-PAD analysis showed ZEN-1 and ZEN-2 to have a high absorption at 300–330 nm (Fig. 3).

In addition, methanolic solutions of ZEN-1 and ZEN-2 (10 μl) (nearly 5% of total products obtained above) were respectively added to the active fraction (0.5 ml) again in order to test whether they could be further transformed. 20 mM Tris–HCl (pH 9.0) buffer was included as control. After 6 h incubation at 30°C, methanol (0.5 ml) was added to stop the reaction and ZEN-1 and ZEN-2 in the mixtures was purified by Waters analytical HPLC system (detailed methods not showed). Results showed only 10% of ZEN-

Table 1 Effects of low oxygen gas content, protease, SDS, and EDTA on zearalenone degradation activity of the pooled active fractions

Factors name	Treatment ways	ZEN degradation rate (%)
Low oxygen gas content	Treated	6.8 ± 0.4
	Control	93.5 ± 1.2
Protease and SDS	Protease	57.1 ± 0.8
	SDS	40.6 ± 0.7
	Protease plus SDS	32.5 ± 0.6
	Control	90.2 ± 0.9
EDTA	Treated	4.2 ± 0.5
	Control	99.2 ± 0.2
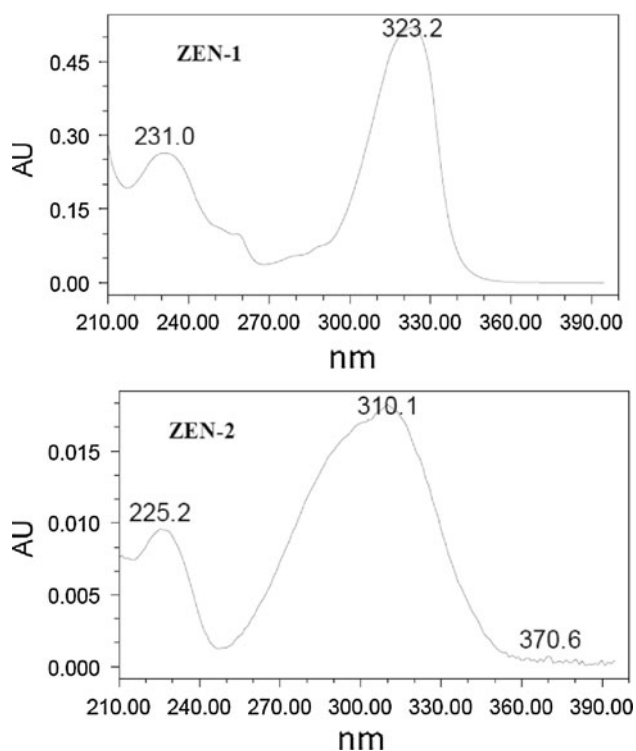
Fig. 3 UV-Vis spectroscopy of ZEN-1 and ZEN-2 obtained from HPLC-PAD analysis

1and 8% of ZEN-2 in the mixtures remained when compared with the control.

SDS–PAGE and MALDI-TOF-TOF/MS analysis of extracellular enzymes

Enzymes in the active fractions were isolated by SDS–PAGE. Two bands of approximately 20 kDa and 11 kDa respectively appeared in gel and were excised for protein identification by MALDI-TOF/TOF/MS analysis. Three proteins were found from the active fractions, and they matched the database for *Acinetobacter* genus with great homology. They were identified as peroxiredoxin, a possible cytochrome and a putative fimbrial protein precursor (Table 2).

Discussion

In this study, an active fraction capable of efficiently degrading zearalenone was obtained after enzymes in the strain SM04 extracellular extracts were isolated by Sephadex G-100 column. Three proteins including peroxiredoxin, a possible cytochrome and a putative fimbrial protein precursor were identified by SDS–PAGE and MALDI-TOF/TOF/MS analysis. Putative fimbrial protein precursor, as a skelemin, should not be concerned in zearalenone degradation. The characteristic of peroxiredoxin and the possible cytochrome was accordant with our results, which indicated that metal-dependent oxidases were involved on zearalenone degradation. Peroxiredoxins (Prxs) have received considerable attention in recent years as a new and expanding family of thiol-specific antioxidant proteins, also termed the thio-redoxin peroxidases and alkyl-hydroperoxide-reductase-C22 proteins (Zachary et al. 2003). Prxs exert their protective antioxidant role in cells through their peroxidase activity (ROOH + 2e → R-OH + H_2O), whereby hydrogen peroxide, peroxynitrite and a wide range of organic hydroperoxides (ROOH) are reduced and detoxified (Jacobson et al. 1989; Poole and Ellis 1996; Bryk et al. 2000; Peshenko and Shichi 2001; Hofmann et al. 2002). Peroxidases can degrade dyes and a variety of aromatic pollutants (Peter 2000; Ogawaa et al. 2004). Cytochromes are hemoproteins and many cytochromes can catalyse the oxidation of organic substances (Alves et al. 1999; Erman and Vitello 2002). Therefore, we inferred that cytochrome may catalyse the oxidation of zearalenone involving molecular O_2 as the electron acceptor. In this reaction, oxygen is reduced to hydrogen peroxide, and then hydrogen peroxide continues to oxidize zearalenone and its products by the catalysis of peroxiredoxin.

Table 2 List of proteins in the active fraction from Sephadex G-100 column isolation, identified by MALDI-TOF/TOF/MS using Mascot server

Protein name	Mass	Sequences of indentified peptides	Accession no
Fimbrial Protein	22035	K.VATYFEPGATVDFNTGR.L	gi|169796199
Peroxiredoxin	20874	K.IQIVELNAGGIGR.D	gi|262279671
		K.NFDVLIESEGLADR.G	
Possible cytochrome	11724	K.NIATTIIFLVLIR.I	gi|226952653
		K.LPDTMSPMMK.T	

Zearalenone could be degraded into smaller estrogenic products by the enzymes in the pooled active fractions, which indicated they will be very useful for detoxification of zearalenone-contaminated feed. The acid condition was necessary to extract ZEN-1 and ZEN-2 by ethyl acetate, and there were low absorption at 230–270 nm and high absorption at 300–330 nm for UV–vis spectroscopy of ZEN-1 and ZEN-2, which indicated that the benzene ring of zearalenone may be cleaved and oxidized into the products containing carboxyl groups (Mckenzie et al. 1997; Megharaj et al. 1997). As two intermediate products, the yield of ZEN-1 and ZEN-2 was very low, so a higher amount of zearalenone is required to determine the complete molecular structure of ZEN-1 and ZEN-2 by NMR analysis. Considering the complexity of zearalenone degradation by peroxiredoxin and possible cytochrome (mainly via free radical mechanisms), the complete molecular structure of ZEN-1 and ZEN-2 may be not important to explain the mechanisms of ZEN degradation by enzymes in the active fractions (Peshenko and Shichi 2001; Ogawaa et al. 2004; Hofmann et al. 2002). Therefore, it is not necessary to prepare more ZEN-1 and ZEN-2. In the further study, purification of cytochrom and peroxiredoxin in the pooled active fractions should be performed, and their genes need to be cloned.

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