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Enzymatic Sensor for Sterigmatocystin Detection and Feasibility Investigation of Predicting Aflatoxin B_1 Contamination by Indicator

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1. Introduction

1.1 Enzymatic sensory detection of sterigmatocystin

The development of fast and sensitive sensor for mycotoxins' detection has drawn a great attention in resent years (Prieto-Simom, B. et al., 2007). However, to construct anti-interference biosensor for the practical samples is still challenge.

Sterigmatocystin, a biogenic precursor of aflatoxin B_1, has been classified as group 2B by the International Agency for Research on Cancer (IARC). Its chemical structure consists of a xanthone nucleus attached to bisfuran and it bears a close structural similar to aflatoxin B_1 (Fig. 1) (Versilovskis et al., 2008). The toxicity of sterigmatocystin is primarily confined to the liver and kidney and closely correlated to the occurrence of hepatocellular carcinoma, gastric carcinoma and esophagus carcinoma (Purchase & van der Watt, 1970). Contamination of cereals with *Aspergillus* fungi refers to harmfulness, due to the potential of sterigmatocystin production by these fungi. Sterigmatocystin is similar to aflatoxin B_1 both in the carcinogenicity and fluorescence excitability. While the fluorescence of sterigmatocystin is not so strong as aflatoxin B_1 and the sterigmatocystin-antibody not commercially available, the detection of sterigmatocystin is harder or/and cost more. Several methods for the detection of sterigmatocystin have been established, including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), liquid

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chromatography with mass spectrometry (LC-MS), gas chromatography with mass spectrometry (GC-MS), high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Versilovskis et al., 2007; Turner et al., 2009;). Although accurate and sensitive, most of the chromatographic methods are often considered laborious and time intensive, requiring expensive equipments and extended cleanup steps. Therefore, developing a rapid and sensitive method for sterigmatocystin detection is urgently needed. Due to the advantages of enzymatic recognition which offer the response signal with diplex recognitions: the selective binding coupled with the catalytic action of the enzyme toward its substrate, the false results might occur less compared with immuno-sensor or ELISA (enzyme-linked immunosorbent assay) methods which has been concerned the false results (Lim et al., 2007; Massart et al., 2008; DeForge et al., 2010).

Aflatoxin-oxidase (AFO), confirmed to possess oxidation activity toward sterigmatocystin, was utilized as bio-recognition element to constructing the enzymatic biosensor for sterigmatocystin detection. Our previously reported AFO biosensors for fast detection of sterigmatocystin have indicated their potential practicability (Yao et al., 2006; Chen et al., 2010). However, to develop anti-interference enzymatic biosensor for the practical food samples is an arduous target. Recently, we have developed a Prussian blue-base AFO biosensor which revealed effective anti-interferent quality (detailed investigations are going to be published else where). Prussian blue, a prototype of mixed-valence transition metal hexacyanoferrates, has been extensively used as an electrontransfer mediator in amperometric biosensors due to its excellent electrocatalysis toward the reduction of hydrogen peroxide (Karyakin et al., 1994; Ricci & Palleschi, 2005; Zhao et al., 2005; Ricci et al., 2007; Liu et al., 2009). Because of its selective catalysis of hydrogen peroxide in the presence of oxygen and other interferents, Prussian blue is regarded as "artificial peroxidase" (Itaya et al., 1984; Karyakin et al., 1998, 1999, 2000; Karyakin & Karyakina, 1999). The extremely low applied potential of 0.0 V and effectively perselective barrier effect of the Prussian blue - chitosan composite were supposed to be a major attribution towards the interferents from real samples. Here reports the procedure of the biosensor (chitosan – AFO - Single wall carbon nanotubes / Prussian blue – chitosan / L-Cysteine / Au) construction and the results for the sensor's practical use.

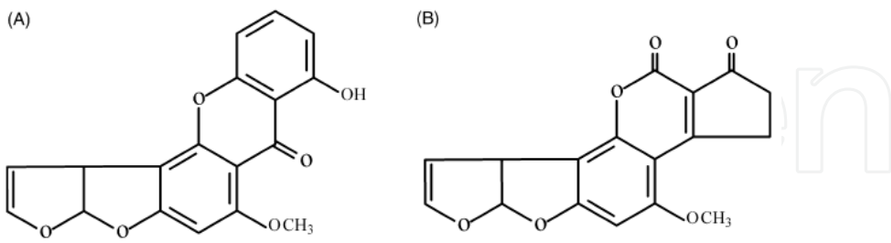


Fig. 1. Chemical structures of sterigmatocystin (A) and aflatoxin B_1 (B).

1.2 Predictive detection of aflatoxins

The prompt and fast method is valuable for food safety and feed. However, the early awareness may be more informative for both consumers and producers. Versicolorin A is the first compound having the toxic bisfuran structure in biosynthesis of aflatoxin B_1. The possibility and feasibility to predict the contamination of aflatoxin B_1 using versicolorin A as the indicator have been reported in the present chapter, also.

Aflatoxins are secondary metabolites produced by filamentous fungi *Aspergillus*, particularly *flavus* and *parasiticus,* which are ubiquitous and can grow extensively in crops and their products. The carcinogenic and immuno-suppresant toxicity of aflatoxins is a serious health risk both to human beings and animals. Among the aflatoxins variants, aflatoxin B_1 is the most toxic and is strictly controlled under food and feed safety regulations in many countries. As is known, mycotoxins may occur at any stage of crops' growth, harvest, storage, transport and marketing. The "fast detection" is still not fast enough to assure life safety and diminish the economic loss since the detection is "after-event" (detectable after the contamination occurred). Development of pre-alert or early-awareness methods has aroused general interests, especially in a time of constant climate changes and food and feed shortage. There is an extensive demand to develop methods for the early identification of emerging hazards to food safety (Concina et al., 2009; Kleter & Marvin, 2009; Marvin & Kleter, 2009).

Biosynthesis of aflatoxins is a complex process (Fig. 2) (Shier et al., 2005), with more than 20 genes involved. Yu (Woloshuk et al., 1994; Yu et al., 1995) revealed that most of these genes were located on the aflR gene (aflatoxin biosynthetic pathway regulatory gene), and that their physical order and distance is highly correlated to the aflatoxin biosynthetic pathway. This gene cluster has been further investigated and expanded (Yu et al., 2004).

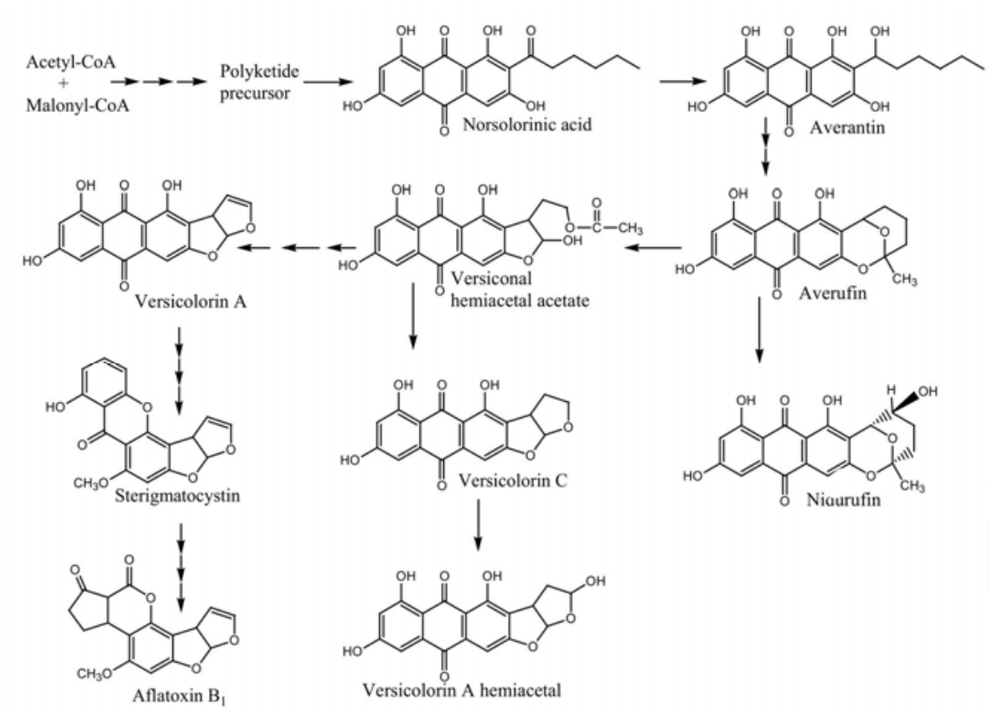


Fig. 2. Presumed biosynthetic pathway of aflatoxin by Shier et al., 2005

It has been proposed that aflatoxicosis is caused by the oxidation of the bisfuran group on aflatoxin B_1 and its variants to yield the ultimate carcinogen aflatoxin B_1-exo-8,9-epoxide in the liver (Jones & Stone, 1998; Smela et al., 2002). Versicolorin A, a precursor of aflatoxin B_1 in the biosynthetic pathway of aflatoxins (Ehrlich et al., 2003; Woloshuk et al., 1994; Yu et al., 1995, 2004), is a member of this toxic group of bisfurans along with its succeeding

metabolites sterigmatocystin and aflatoxin B_1. Versicolorin A, the metabolic precursor of aflatoxin B_1, was first separated by Lee (Lee et al., 1975) in a mutant strain of *Aspergillus parasiticus* named *Aspergillus versicolor*. Its molecular formula is $C_{18}H_{10}O_7$ with a molecular weight of 338. Its physical and chemical properties have been fully characterized (Lee et al., 1975; Shier et al., 2005). Some paper have reported the positive mutagenicity of Versicolorin A, and Versicolorin A has shown less mutagenic than aflatoxin B_1 (about 1.5% or 5% toxic of aflatoxin B_1) in Ames test. However, incomprehensive reports of the toxicity of Versicoloring A had been published (Wong et al., 1977; Dunn et al., 1982; Mori et al., 1985), thus we looked for the minimum dose of Versicoloring A mutagenicity using Ames tests with four tester strains and a human peripheral lymphocytes test.

As mentioned above, in the aflatoxin B_1 biosynthesis procedures versicolorin A is a key precursor and far away from the end product of aflatoxin B_1 with a lower toxicity. Versicolorin A might be a candidate indicator for pre-alert of aflatoxin B_1 pollution. This study expands report of versicolorin A and aflatoxin B_1 levels in pure cultures of *A. flavus* and *A. parasiticus* on different culture media, *A. parasiticus* inoculated white rice, and local (Guangdong province, China) commercial feed samples. To evaluate whether versicolorin A is feasible to pre-alert aflatoxin B_1 pollution, 34 feed samples (corn dregs) previously considered safe (aflatoxin $B_1 \leq 25$ μg/kg, China regulation [GB13078-2001]) but with a high level of versicolorin A (≥ 50 μg/kg) were chosen. The storage tests were performed. The final aflatoxin B_1 was determined and the relationships between original versicolorin A and the final aflatoxin B_1 have been analyzed.

2. Materials and methods

2.1 Enzymatic sensory detection of sterigmatocystin

2.1.1 Chemicals

Sterigmatocystin and L-Cysteine were obtained from Sigma-Aldrich Co. (St. Louis. USA). Single wall carbon nanotubes (SWCNTs) (95% purity) were purchased from Shenzhen Nanotech Port Co. (Shenzhen, China). Chitosan (CS) (95% deacetylation,) and other chemicals were of analytical grade without further purification. Phosphate buffer solution (PBS, 0.05 M) consisting of K_2HPO_4, KH_2PO_4 and 0.1 M KCl was employed as supporting electrolyte. The double-distilled water was used throughout. The preparation of aflatoxin-oxidase (AFO) followed a similar procedure according to the literature (Liu et al., 2001), and with corresponding specific enzyme activity of 320 U/mg (1 U was equal to the amount of enzyme that can decrease 1 nmol of sterigmatocystin per minute). Measurements were performed using CHI660C electrochemical workstation (CH Instrument, USA). The electrochemical system consists of gold working electrode, a platinum wire as the auxiliary electrode, and an Ag/AgCl (saturated with KCl) electrode as the reference electrode. All experiments were conducted at room temperature in a 10 ml electrochemical cell with respect to Ag/AgCl. The amplitude of the applied sine wave potential was 5 mV, with a formal potential 0.24 V. The current-time curves were recorded at 0.0 V under stirring.

2.1.2 Preparation of sterigmatocystin biosensor

Gold electrodes (2 mm in diameter, CH Instruments Inc.) were cleaned following the reported protocol (Zhang et al., 2007) and then rinsed with water. After flowing dry with nitrogen, electrodes were immediately immersed into 0.02 M L-Cysteine solutions for 6 h at 4 ^{o}C to form self-assembly monolayer modified electrode. Extensively washed with water to

remove the unbound L-Cysteine (Cys), the self-assembly monolayer modified electrodes were denoted as Cys/Au. A 0.2 wt.% CS solution was prepared by dissolving chitosan (CS) powder in 1% (V/V) acetic acid solution with magnetic stirring for about 2 h followed with filter removal of the undissolved particles and adjusting the pH to 5.5 with condense NaOH. The prepared and CS solution was then stored in 4 ^{o}C. The Prussian blue-chitosan (PB-CS) hybrid film was deposited onto the Cys/Au modified electrode according to the following four steps:

1. Preparation of the film: A PB-CS solution consisting of 2.5 mM $K_3[Fe(CN)_6]$, 2.5 mM $FeCl_3$, 0.1 M KCl, 0.1 M HCl, and 0.01% CS was deoxygenated by purging high-purity nitrogen for 10 min. PB-CS was then electrodeposited onto Cys/Au by applying a constant potential of 400 mV (*vs.* Ag/AgCl) for 40 s.
2. Activation of the film: The PB-CS layer was then further activated in an electrolyte solution containing 0.1 M KCl and 0.1 M HCl, which was used for film growth by successive cyclic scanning from -50 mV to 350 mV for 30 cycles at 50 mV/s.
3. Drying of the film: After carefully rinsed with doubly distilled water, the modified electrode was then baked at 100 ^{o}C for 1 h since it was reported in the literature (Ricci et al., 2003) that a more stable and active layer of Prussian blue (PB) could be obtained with 1 h baking at 100^{o}C.
4. Conditioning of the film: A potential of -50 mV was applied for 600 s in 0.05 M PBS consisting of K_2HPO_4, KH_2PO_4 and 0.1 M KCl (pH 6.5). And then a 20 cycles of scan from -50 mV to 350 mV at 50 mV/s was followed.

After the four steps procedure, the electrode, constructed with PB-CS electrically depositing onto Cys/Au modified electrode, was referred to as PB-CS/Cys/Au electrode. For the enzyme biosensor, the modification was carried out by dropping 10 μl of an aqueous suspension containing 0.5 mg/ml carboxylated single wall carbon nanotubes (SWCNTs), 2.5 mg/ml aflatoxin-oxidase (AFO), and 0.2 wt.% CS on the PB-CS/Cys/Au electrode. Before used, SWCNTs were carboxylated in a 3:1 (V/V) mixture of concentrated H_2SO_4/HNO_3 with sonication at 60 ^{o}C according to the literature (Zhang et al., 2008). The AFO-modified electrode (referred to as CS-AFO-SWCNTs/PB-CS/Cys/Au) was then dried at 4 ^{o}C in a refrigerator for 24 h. The enzyme electrodes must be washed thoroughly with PBS before experiments and store at 4 ^{o}C when not in use.

2.1.3 Rice samples preparation

Non-infected rice sample (purchased from the local market) was first grounded in a household blender. Aliquots (0.5 g) of the rice powder were then spiked with sterigmatocystin at different concentrations and mixed in a vortex mixer. After adding 4 ml of extraction solvent (80% methanol), the sample was fully mixed by shaking for 30 min, and then, centrifuged at 6000 g for 10 min at 4 ^{o}C. The supernatant was carefully removed and diluted with PBS (1:10, V/V) for further analysis.

2.1.4 Safety conditions

Sterigmatocystin is a very potent carcinogen, so great care should be taken to avoid personal exposure. It is necessary to wear lab dresses, gloves, and mask when doing experiments. All laboratory glassware and consumables contaminated with sterigmatocystin were soaked overnight in a 5% sodium hypochlorite solution containing 5% acetone. The decontamination solution was allowed a minimum of 30 min before disposal.

2.2 Feasibility investigation on predictive detection of aflatoxin B_1

2.2.1 Preparation of pure versicolorin A

Aspergillus versicolor ATCC 36537 (Lee et al., 1975) (purchased from ATCC) was regenerated on slants under 24 ºC in darkness according to the product manual. After 5d activation in liquid growth medium (malt extract 20g, glucose 20g, peptone 1g, distilled water 1L) twice, it was cultured in YES medium (sucrose 200g, yeast extract 20g, distilled water 1L) at 24 ºC in darkness without agitation for 7-10d for versicolorin A production. The mycelial mass was extracted with acetone until colorless and the combined extracts were filtered, dried with anhydrous sodium sulfate and evaporated to dryness at 50 ºC in a rotary evaporator. For each 1L culture, 10ml petroleum ether and 250ml 30% acetone-water was added to re-dissolve the residue and transferred to a separatory funnel, followed by partitioning with 100ml hexane thrice. Finally, the hexane partition was pooled and evaporated at 50 ºC until dryness and the residue was dissolved in 20ml methanol and stored at 4 ºC in darkness.

Crude versicolorin A was further purified by preparative HPLC (Billington & Hsieh, 1989) using 95: 5 methanol: water at a constant flow of 10ml/min on a 50×250mm 10μm Agilent Prep-C_{18} column mounted on Agilent 1100 series installed with a DAD detector. Versicolorin A was eluted at 18.432min detected by absorbance at 214nm and 290nm. Pure versicolorin A was lyophilized and stored at -20 ºC in darkness. Pure versicolorin A powder was re-dissolved in methanol and verified by LC-MS when in use.

2.2.2 Mutagenicity tests

Ames tests with *Salmonella typhimurium* TA97, TA98, TA100 and TA102 tester strains and the human peripheral lymphocytes test were carried out with pure Versicoloring A in the Guangzhou Disease Prevention and Control Center, Guangzhou, China. In the Ames tests, we used the positive controls of 50μg/plate Dexon in the TA97 and TA98 tests, 1.5μg/plate NaN3 in the TA100 test, and 0.5μg/plate Mitomycin C in the TA102 test in the absence of S9 mix; for S9+ tests, 10μg/plate 2-aminofluorene served as positive control in the TA97, TA98 and TA100 tests, and 60μg/plate Chrysazin in the TA102 test. The experiment group consisted of Versicoloring A at variable concentrations of, 20.0, 10.0, 5.0 and 2.5 μg/plate. A blank control and a negative control of DMSO were also included. Experiments were repeated twice in triplicate. The TA98 test was repeated twice in triplicate with Versicoloring A concentrations of 0.8, 0.6 and 0.4 μg/plate.

In the human peripheral lymphocytes test, Versicoloring A concentrations of 1.6, 0.8, 0.4 0.2 and 0.1 μg/mL were used with peripheral lymphocytes of 8 healthy patients in parallel. A blank control, a negative control of DMSO and a positive control of 40μg/mL Mitomycin C were also included.

2.2.3 Detection of versicolorin A and aflatoxin B_1 production time course in pure medium cultures

Pure cultures of *A. flavus* and *A. parasiticus* on different culture media were studied. The three media used were: CAO (sucrose 30g, $MgSO_4$ 0.5g, $FeSO_4$ 0.01g, K_2HPO_4 1g, $NaNO_3$ 3g, KCl 0.5g, distilled water 1L), YES (as described above) and PG (peptone 100g, glucose 10g, distilled water 1L). 1ml 1.0 x 10^6 CFU/ml *A. flavus* or *A. parasiticus* spore suspension fluid was inoculated in 100ml liquid medium and cultured at 28 ºC without agitation in darkness. Toxins were extracted according to a protocol previously described by Bennett (Lee et al., 1975, 1976; Bennett et al., 1976). TLC developed by toluene: ethyl acetate: glacial acetic acid at a ratio of 50:30:4 (V/V/V) on a 12×12cm silica plate was used for detecting metabolites in crude extract with reference to reported R_f values (Shier et al., 2005).

2.2.4 Detection of versicolorin A and aflatoxin B_1 production time course in contaminated white rice

Versicolorin A and aflatoxin B_1 was detected in pure cultures of *A. parasiticus* on white rice by thin layer chromatography (TLC) or high performance liquid chromatography (HPLC).
Commercial bulk white rice was purchased in a supermarket and exposed to UV prior to fungal contamination. 1ml of 1.0 x 10^6 CFU/ml *A. parasiticus* spore suspension fluid was inoculated on 20g rice. Culture conditions were indicated in the context or under the diagrams. Toxins were extracted and detected by TLC as described above. Otherwise, quantifications of crude samples were made with HPLC on 4.6×150mm 5μm Shimadzu ODS-C_{18} column mounted on Shimadzu 6AD series installed with a DAD and fluorescence detector. 10μl sample was loaded and eluted with solvent A (10mM ammonium acetate, 20μM sodium acetate in water) and solvent B (10mM ammonium acetate, 20μM sodium acetate in methanol) by a two-step gradient of 85%B for 10min and 100%B for 10min respectively at a constant flow of 0.3mL/min. Versicolorin A was eluted at 23.163min detected by absorbance at 222nm and 288nm; aflatoxin B_1 was eluted at 11.973min detected by fluorescence at an excitation wavelength of 365nm and an emission wavelength of 435nm.

2.2.5 Detection of versicolorin A and aflatoxin B_1 on commercial feed samples

A set of 100 animal feeds samples (corn dregs) were analyzed. Feed samples of 20g were crushed with blender. Aflatoxin B_1 and versicolorin A were extracted and determined by HPLC procedures as 2.2.4 described. Data analyzed by using the statistic soft ware of SPSS13.0.

2.2.6 Detection of the original versicolorin A and the after-storage aflatoxin B_1 for the samples which concern safe originally

Aflatoxin B_1 in 200 feeds samples were determined by ELISA (Aflatoxin Tube Kit, Beacon, USA) according to instructions in the product manual. Those which aflatoxin B_1 were not more than 25 μg/kg were screened. And followed by the determination of versicolorin A by HPLC method described in 2.2.4. Thirty-four samples with high levels of versicolorin A (≥ 50 μg/kg) of them were chose for the following storage tests.
The 34 chosen samples have divided into two groups. Seventeen samples of them were stored under darkness at 22 ± 2 °C with relative humidity 70 ± 2% for 10 days, and the rest were stored under darkness at 28 °C with 80% relative humidity for 4 days. After determinations of the final aflatoxin B_1 and versicolorin A content by HPLC methods, data of versicolorin A and aflatoxin B_1 before and after storage have been analyzed, and statistical soft ware of SPSS13.0 were used.

3. Results and discussions

3.1 Enzymatic sensory detection of sterigmatocystin

3.1.1 Analytical performance of the enzyme electrode for sterigmatocystin detection

Fig. 3 (A) shows the cyclic voltammograms of sterigmatocystin detected by CS–AFO–SWCNTs/PB–CS/Cys/Au electrode in 0.05 M PBS (pH 6.5) at a scan rate of 50 mV/s. With the addition of certain amount of sterigmatocystin, the cyclic voltammograms changed obviously with an increase in the cathodic peak current and a concomitant decrease in the

anodic peak current. The possible interferent usually appeared in drink and food samples were selected for interference studies to investigate the selectivity of the as-prepared biosensor. As shown in Fig. 3 (B), the biosensor shows no observable change of the response to 4 g/ml glucose, methanol, oleic acid, phenol, L-tryptophan, and ascorbic acid; in contrast, the biosensor exhibits very strong response to the successive addition of 20 ng/ml sterigmatocystin in the presence of the interfering substances.

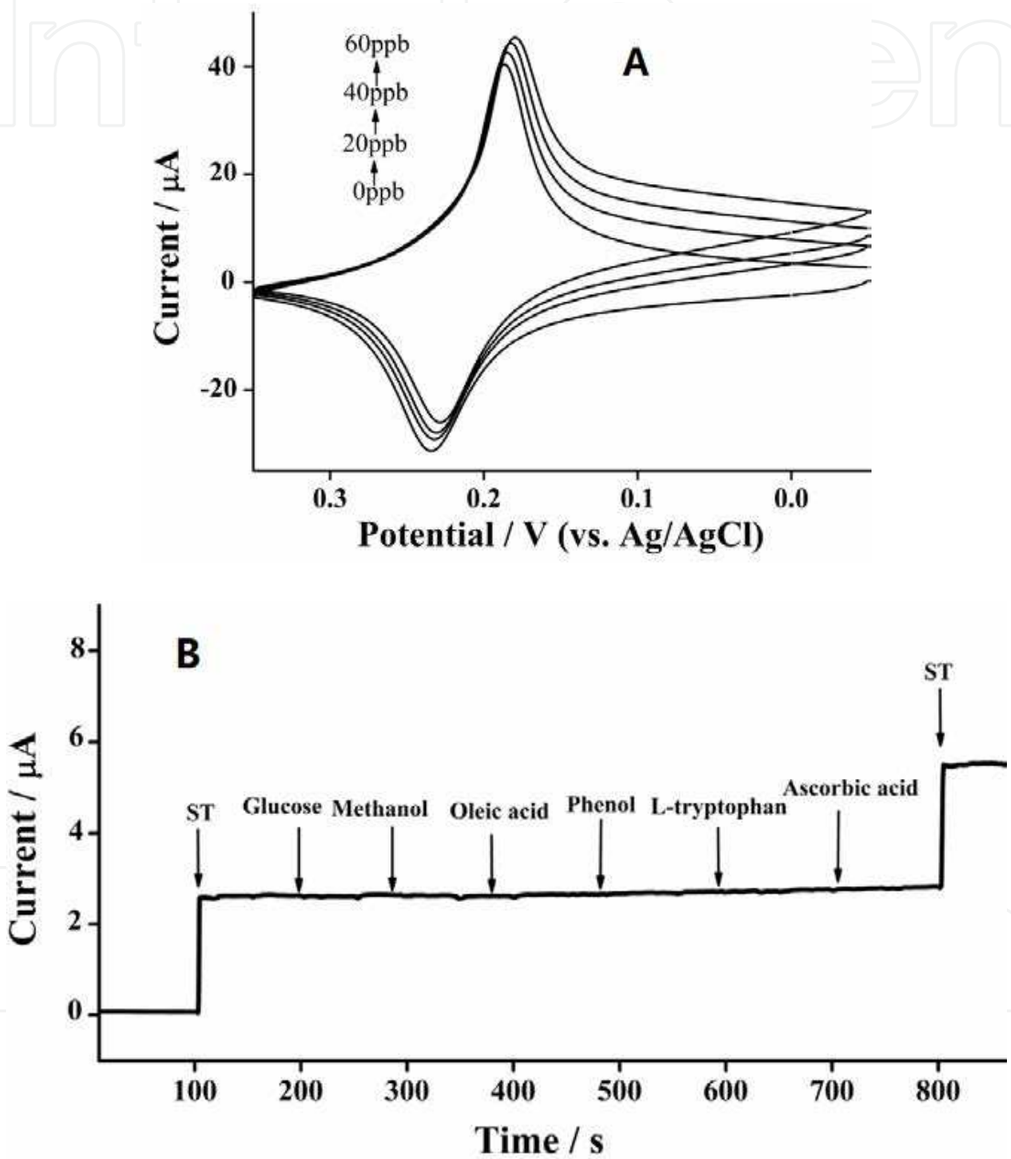


Fig. 3. (A) The cyclic voltammograms of CS-AFO-SWCNTs/PB-CS/Cys/Au electrode in 0.05 M PBS (pH 6.5) in the presence of different concentration of sterigmatocystin (ST). Scan rate: 50 mV/s. (B) Amperometric current-time curve illustrating the interferences free sensing of ST at the proposed biosensor in 0.05 M pH 6.5 PBS. ST (20 ng/ml) and the potential interfering substances (4 g/ml) were added at regular intervals as indicated by the arrows. Applied potential: 0.0 V.

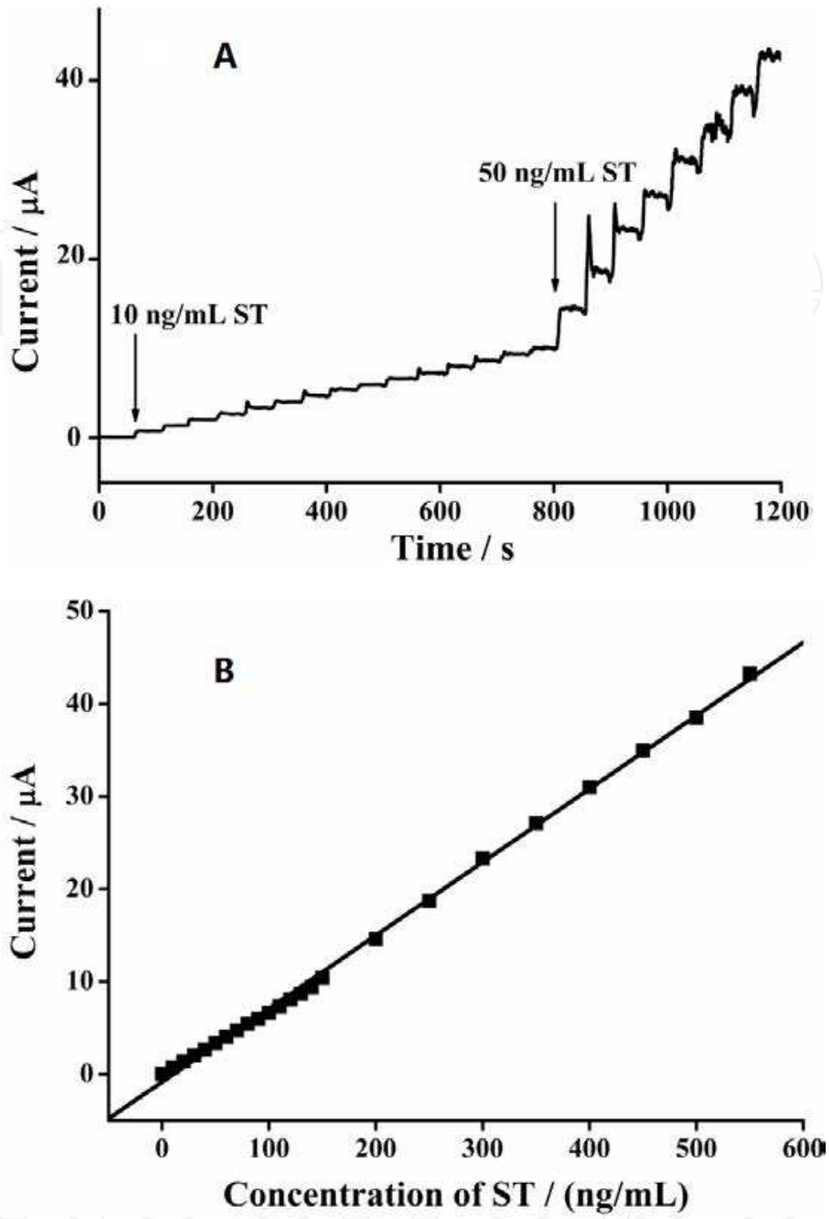


Fig. 4. (A)Typical amperometric current-time curve of CS-AFO-SWCNTs/PB-CS/Cys/Au electrode to successive addition different concentration of sterigmatocystin (ST) in 0.05 mol/L pH 6.5 PBS at 0.0V. (B) The corresponding calibration curve of the electrode.

Fig. 4 (A) shows the amperometric current-time responses of the biosensor on successive step changes of sterigmatocystin concentration in a continuous stirring electrolytic cell at 0.0 V. As Fig. 4 (B) shown, the response current increased linearly with the sterigmatocystin concentration in the range of 10 to 950 ng/ml (correlation coefficient of 0.9985) with a sensitivity of 2.64 $A \cdot g^{-1} \cdot ml \cdot cm^{-2}$ and a detection limit of 2 ng/ml (S/N=3). The 95% of the steady-state current can be obtained within 8 s by using the CS-AFO-SWCNTs/PB-CS/ Cys/Au electrode, indicating a fast response to sterigmatocystin change.

3.1.2 Rice samples analysis with enzymatic sensor

The bioelectrode has been used to determine the recoveries of 15 various concentrations of sterigmatocystin by standard addition in real corn samples. As Table 1 shown, satisfactory values between 82.0 and 115.0 % for sterigmatocystin were obtained for the recovery. This biosensor electrode is convenient in use with quick response and trustworthy results. Besides this merit, the uncomplicated procedure of the sample preparation may also appeal to users.

Sample number	Added (ng/mL)	Detected (ng/mL)	R.S.D (%)	Recovery (%)
1	10	11.5	4.9	115.0
2	15	13.2	9.2	88.0
3	20	17.1	4.3	85.5
4	25	21.4	6.4	85.6
5	30	27.8	3.8	92.7
6	35	28.7	4.5	82.0
7	40	34.6	4.6	86.5
8	45	47.9	8.3	106.4
9	50	55.1	4.2	110.2
10	60	62.2	5.8	103.7
11	70	64.2	8.5	91.7
12	80	73.9	10.8	92.4
13	90	98.9	4.2	109.9
14	100	97.2	10.6	97.2
15	150	161.7	8.6	107.8

Table 1. The detection of sterigmatocystin in rice sample using CS-AFO-SWCNTs/PB-CS/ Cys/Au electrode. The data reported in the table represents the average of four measurements.

3.2 Feasibility investigation on predictive detection of aflatoxin B_1

3.2.1 Versicolorin A and aflatoxin B_1 content time course for the pure culture of *A. flavus* and *A. parasiticus*

Pure cultures of *A. flavus* and *A. parasiticus* on different culture media revealed that versicolorin A can be detected in significant amounts after 7d while aflatoxin B_1 might not, depending on the culture conditions (Table 2). Similarly, versicolorin A and aflatoxin B_1 production in pure cultures of *A. parasiticus* on white rice demonstrated that versicolorin A but not aflatoxin B_1 was detected in early fungal contamination using TLC (Fig.5-1 and 5-2). However, analysis by HPLC revealed the existence of both metabolites on Day 3. Additionally, the amount of aflatoxin B_1 was significantly lower than that of versicolorin A in all samples (Fig. 6). Furthermore, HPLC analysis of versicolorin A and aflatoxin B_1 in commercial animal feeds demonstrated the same phenomena (Fig.7).

Fungus	Medium	Versicolorin A		Aflatoxin B_1	
		Day 7	Day 10	Day 7	Day 10
A. flavus	CAO	+	+	-	-
	YES	+	+	-	-
	PG	+	+	-	-
A. parasiticus	CAO	+	+	-	-
	YES	+	+	+	+
	PG	+	+	-	+

"+" denotes positive and "-" denotes negative; detection limit for aflatoxin B_1 is 5ng.

Table 2. Results of versicolorin A and aflatoxin B_1 production in pure cultures of *A. flavus* and *A. parasiticus* on different culture media incubated under 28 °C and ambient humidity without agitation in darkness and detected by TLC.

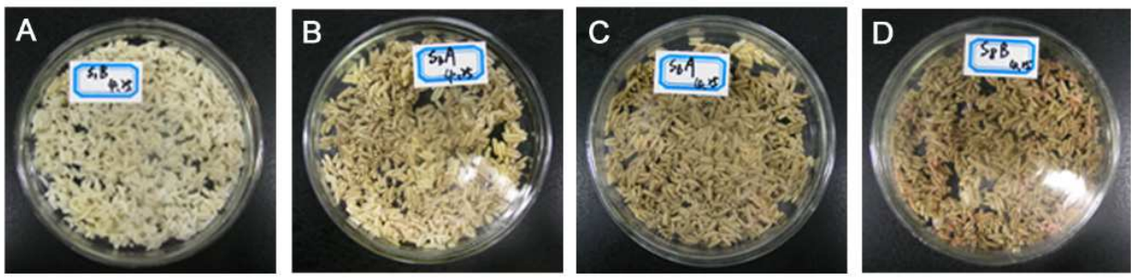


Fig. 5.1. Observation of versicolorin A and aflatoxin B_1 production in pure cultures of *A. parasiticus* on white rice under 35 °C and ambient humidity in darkness over 14 days by TLC. Photographs of rice samples taken on Day 2 (A), Day 5 (B), Day 7 (C) and Day 14 (D) after fungus inoculation.

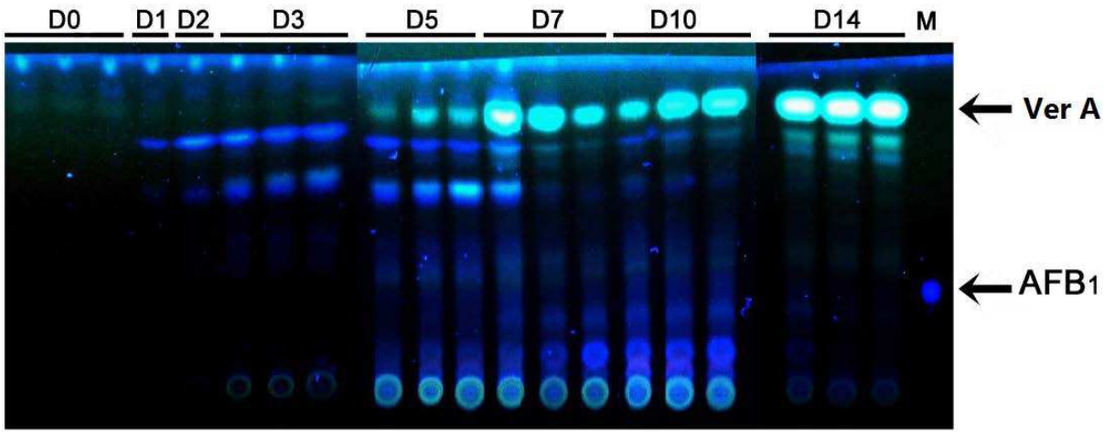


Fig. 5.2. Observation of versicolorin A and aflatoxin B_1 production in pure cultures of *A. parasiticus* on white rice under 35 °C and ambient humidity in darkness over 14 days by TLC. TLC detection of versicolorin A and aflatoxin B_1 in rice samples on respective days indicated above after fungus inoculation. Experiments were performed in triplicate.

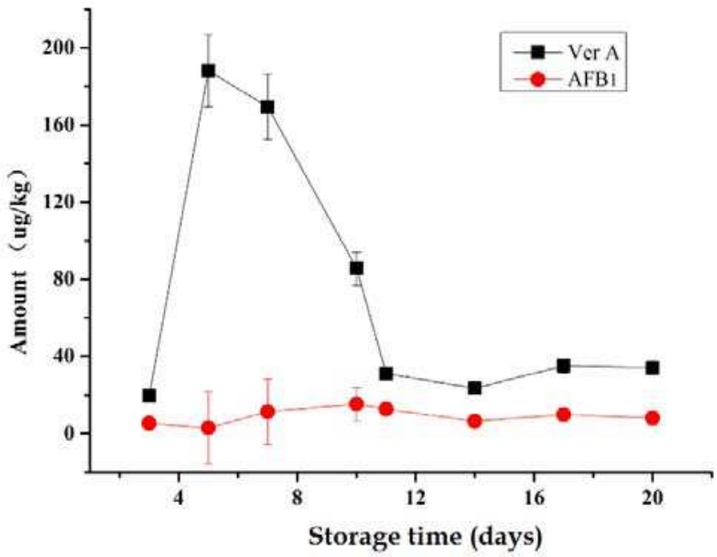


Fig. 6. Observation of versicolorin A and aflatoxin B_1 production in pure cultures of *A. parasiticus* on white rice at 28 ºC and 80% relative humidity in darkness over 20d by HPLC. All experiments were performed in triplicate.

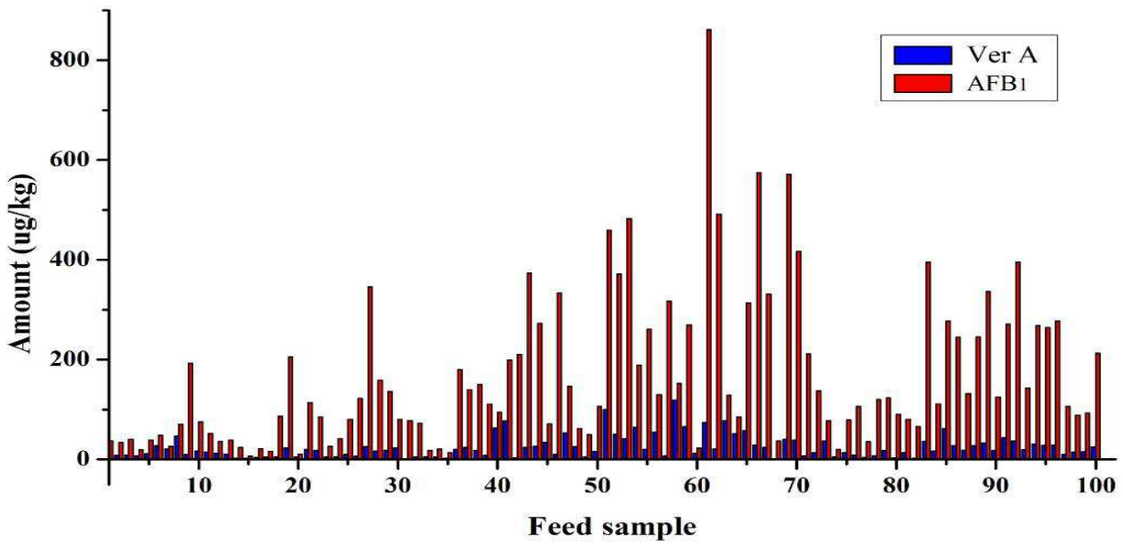


Fig. 7. Detection of versicolorin A and aflatoxin B_1 on commercial animal feeds by HPLC.

3.2.2 Statistical analysis of versicolorin A and aflatoxin B_1

From the 100 feed samples data, it's indicated that they are significantly logarithmic relative as Fig. 8 shown.

$$y = 0.658x + 1.240 \quad (y = \lg \text{Conc. } AFB_1,\ x = \lg \text{Conc.Ver A}) \qquad \text{(Equation 1)}$$

R=0.637，Rsq=0.405，P<0.001 (by SPSS13.0 soft ware)

Analyses of versicolorin A and aflatoxin B_1 in white rice contaminated with *A. parasiticus* and in commercial animal feeds purchased from the market revealed that the two metabolites were co-existent. We deduced that the observed phenomenon was caused by the immediacy in their biosynthesis and the heterogeneity of the fungal contamination. However, we could not rule out the possibility that aflatoxin B_1 production lags behind versicolorin A in other circumstances because of the complex pathway of aflatoxin biosynthesis. In addition, our investigations on different culture conditions of *A. flavus* and *A. parasiticus* demonstrated that toxin production differs under different nutritional compositions and culture temperatures. It is apparent that the time relationship between sequential product of aflatoxin B_1 metabolites depends on the choice of sample of interest and culture conditions.

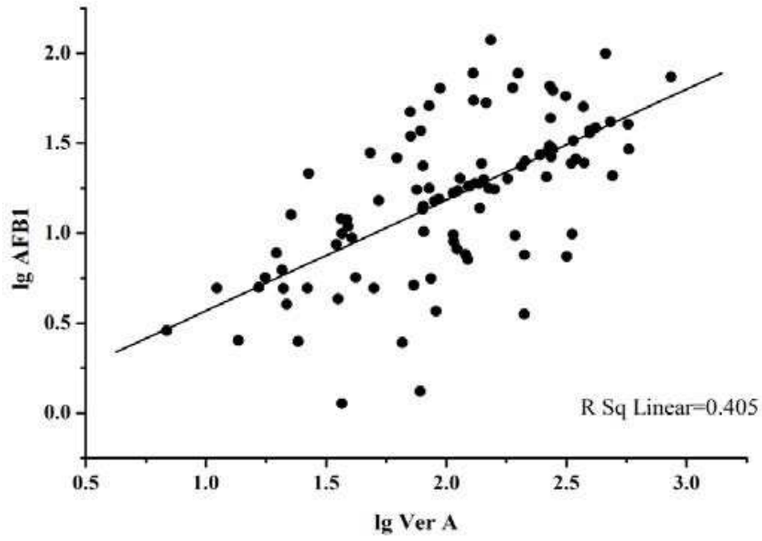


Fig. 8. Statistical analysis for content of versicolorin A and aflatoxin B_1 (sample pool:100)

In this study, pure cultures of *A. flavus* and *A. parasiticus* on different culture media revealed that versicolorin A was detected in significant amounts by TLC, but aflatoxin B_1 might not be detected under the same culture conditions. HPLC analysis of *A. parasiticus*- contaminated white rice on different days after fungal inoculation showed that versicolorin A was detected in amounts 2 to 28 times higher than that of aflatoxin B_1. Analysis of commercial 100 feed samples also showed that versicolorin A quantities were 1.2~59 times higher than that of aflatoxin B_1. Therefore, it could be concluded that versicolorin A existed concurrently and in significantly higher amounts as compared to aflatoxin B_1 in aflatoxin B_1-positive samples. The content of versicolorin A has shown significant relative to the content of aflatoxin B_1.

Assays for determination of aflatoxins are diverse. Aflatoxin B_1 is the major biomarker for aflatoxin contamination in food and feed. Aflatoxin B_1 determination methods include TLC, HPLC, ELISA, etc (Turner et al., 2009). However, each of these methods has their pros and cons (Jiang et al., 2005). For instance, TLC is fast and convenient but the detection limit is high. HPLC is more suitable for quantification but chemical derivatization and fluorescence detectors are required for high sensitivity (Kok, 1994). Additionally, cleanup with affinity columns is essential for a majority of food and feed samples (Jiang et al., 2005). On the other hand, versicolorin A can be detected by simple HPLC coupled with fixed wavelength UV detector (222nm or 288nm, or both of them if DAD detector is available). Moreover, it was found to exist

concurrently and in significantly larger quantities than aflatoxin B_1 in our studies. Thus, it offers the alternative to a sensitive and cost efficient indicator of aflatoxin contamination.

3.2.3 The content changed for storage of versicolorin A and aflatoxin B_1

The seventeen chosen samples with aflatoxin B_1 lower than 25ug/kg while versicolorin A more than 50ug/kg were stored under darkness with 22 ± 2 °C and relative humidity 70 ± 2% for 10 days. The content changed as shown by Fig. 9, 10. The trends of the decrease of versicolorin A with the increase of aflatoxin B_1 after storage are clearly presented.

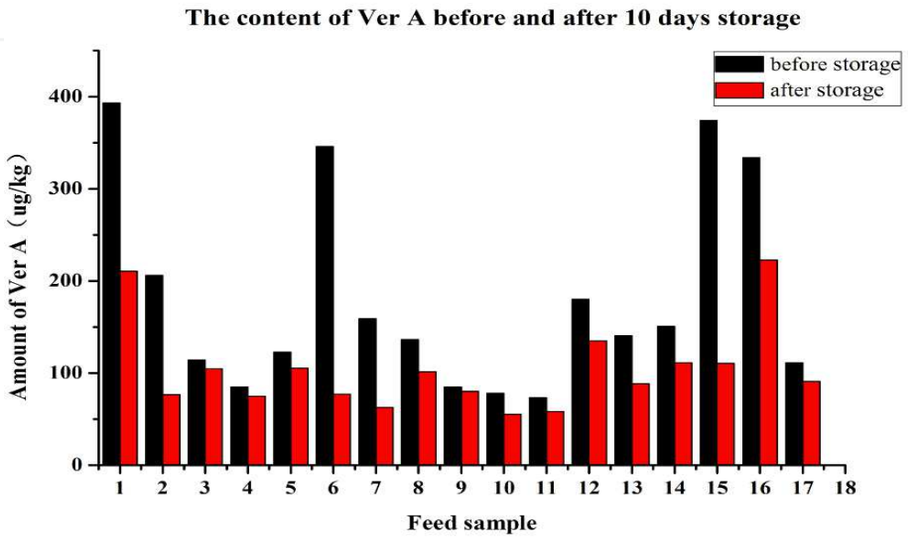


Fig. 9. The content of versicolorin A before and after 10d storage (darkness with 22 ± 2 °C and 70 ± 2% relative humidity)

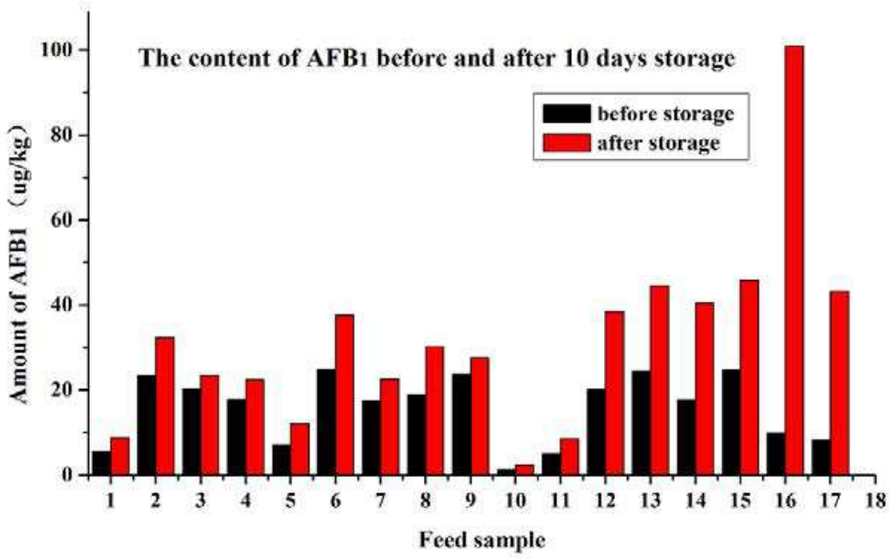


Fig. 10. The content of aflatoxin B_1 before and after 10d storage (darkness with 22 ± 2 °C and 70 ± 2% relative humidity)

3.2.4 Statistical analysis of the versicolorin A before storage and aflatoxin B_1 after storage

To reveal whether the versicolorin A content is meaningful of subsequent contamination of aflatoxin B_1, statistical analysis of the original versicolorin A against with aflatoxin B_1 after-10d-storage (22 ± 2 °C and 70 ± 2% relative humidity) has been performed. Results indicated that they are significantly relative in a negative reciprocal relationship shown as Fig. 12 and equation 2 display.

$$\text{Conc. } AFB_1^{subs.} = -2890.631\ (1 / \text{Conc.Ver } A^{ori.}) + 50.919 \qquad \text{(Equation 2)}$$

R = 0. 791，Rsq = 0. 626 (by SPSS13.0 soft ware)

(10D storage with 22±2°C and relative humidity 70±2%)

Fig. 11 shows a threshold for the original versicolorin A about 67 μg/kg. From the equation 2, it can be calculated that if the original versicolorin A level were about 67 μg/kg or 132 μg/kg, after 10d storage (darkness with 22 ± 2 °C and 70±2% relative humidity) the aflatoxin B_1 content were approximately 10 μg/kg or 30 μg/kg, respectively.

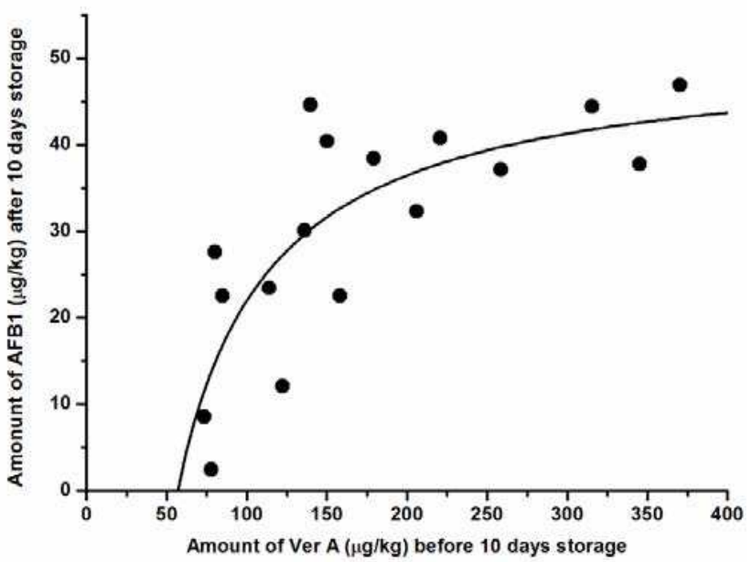


Fig. 11. Statistical analysis of original versicolorin A and subsequent aflatoxin B_1 after 10d storage (darkness with 22 ± 2 °C and 70 ± 2% relative humidity)

Another group of the same chosen samples have been investigated under the 4d storage at 28 °C with relative humidity 80% for. Results were showed in Fig. 12, 13 and equation 3. Under the fungi growth optimum condition (28 °C with relative humidity 80%), the subsequent aflatoxin B_1 showed a linear relationship with the original versicolorin A content.

These storage investigation results suggested the contamination progress rate may be various depending on the storage conditions, and to investigate the content of original versicolorin A and subsequent aflatoxin B_1 after-storage may reveal the various contamination pattern for a certain storage condition.

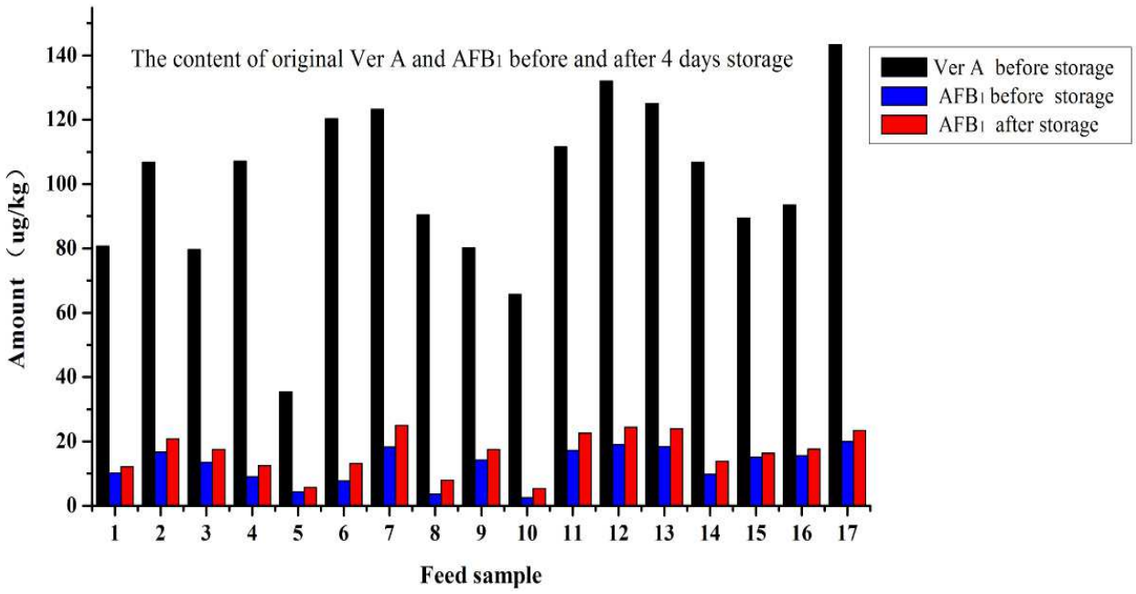


Fig. 12. The content of original versicolorin A and aflatoxin B_1 before and after 4 days storage (darkness with 28 °C and relative humidity 80%)

$$\text{Con. } AFB_1{}^{\text{subs.}} = 0.216 \text{ Con.Ver } A^{\text{ori.}} - 4.731 \quad \text{(Equation 3)}$$

R=0.885, Rsq=0.784, P<0.001 (statistics significant)
(For 4D storage with 28°C and relative humidity 80%)

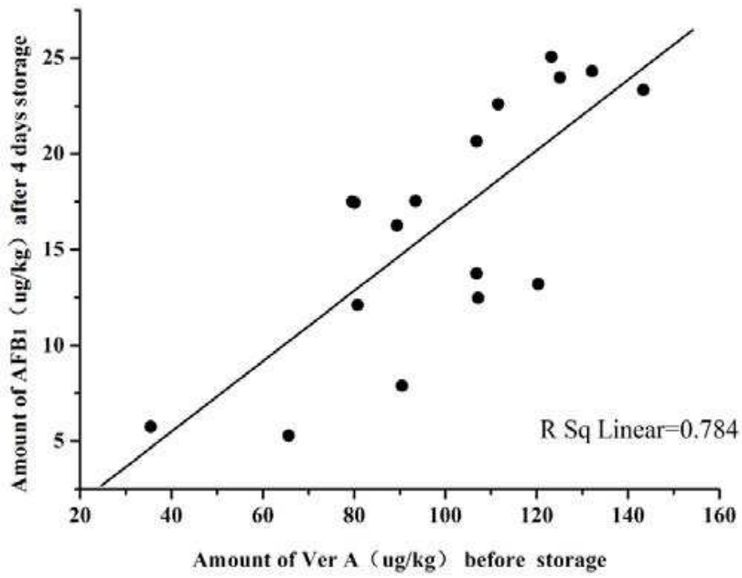


Fig. 13. Statistical analysis for original content of versicolorin A and aflatoxin B_1 before and after 4d storage (darkness with 28 °C and relative humidity 80%)

3.2.5 Mutagenicity tests

Results of the Ames tests with with *Salmonella typhimurium* TA97, TA98, TA100 and TA102 tester strains demonstrated that VerA exhibited mutagenicity on the TA98 tester strains at the concentration of 0.6μg/plate and above. (Figure 14).

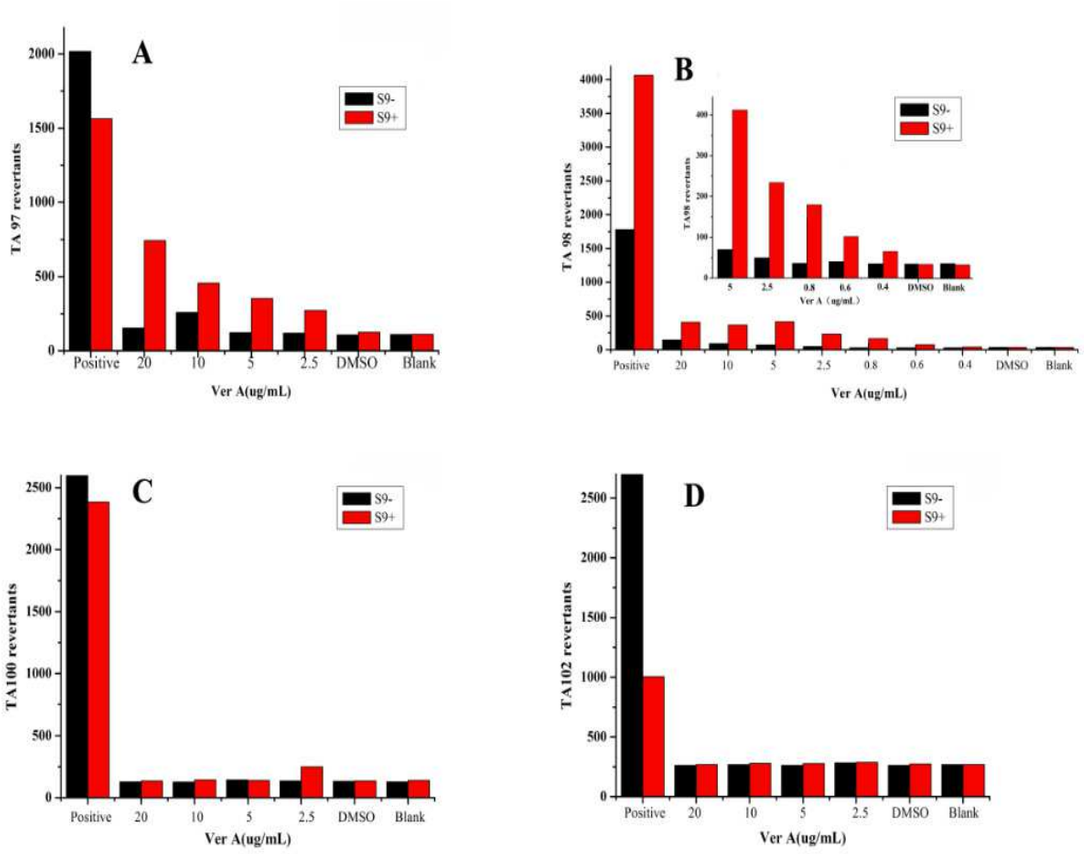


Fig. 14. Ames tests results of VerA with *Salmonella typhimurium* (A) TA97, (B) TA98 and (inset) at VerA concentrations between 5 and 0.4 μg/plate, (C) TA100, and (D) TA102 tester strains. All experiments were repeated twice in triplicate.

On the other hand, the human peripheral lymphocytes test indicated genotoxicity for VerA at the concentration of 1.6μg/mL, which is 25 times of Mitomycin C ($P<0.01$) (shown as Fig. 15). Hence, VerA may be confirmed to be a mutagen towards humanbeings.

4. Conclusions

4.1 Enzymatic sensory detection of sterigmatocystin

Due to the low detection potential (0.0 V) and the role of selective recognition by the enzyme, the biosensor exhibited sensitive and creditable response in corn samples analysis with resistant to glucose, methanol, oleic acid, phenol, L-tryptophan and ascorbic acid. The sensor has given values of recovery in the range of 82.0% - 115.0% and RSD of 4.2% - 10.8% with a simple two-step sample-preparation of 80% methanol extraction followed by centrifugation.

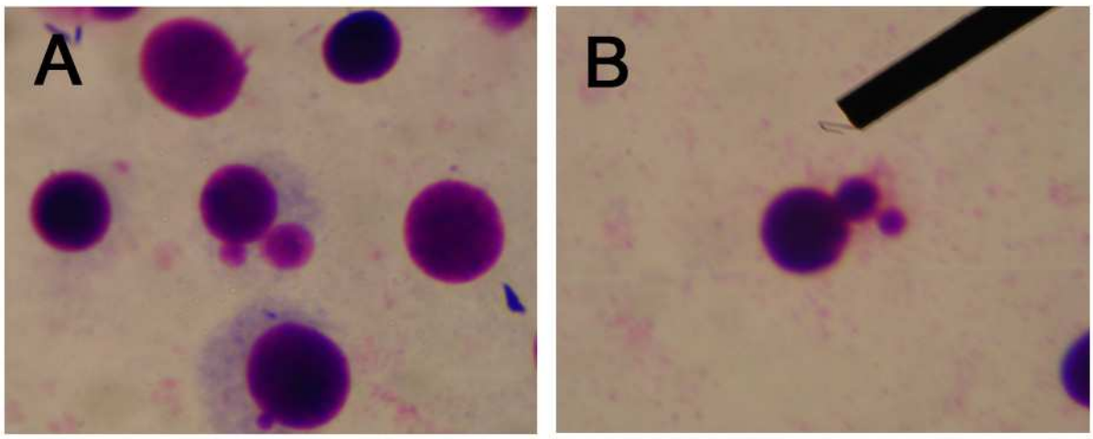


Fig. 15. Photographs of the human peripheral lymphocytes test. (A) Human peripheral lymphocytes from 8 donors were incubated with different concentrations of pure VerA for 24h and stained with 3% Giemsa. (B) A micronucleus-containing lymphocyte is indicated by the pointer. Nucleus was shown in red and cytosol in blue.

4.2 Feasibility investigation on predictive detection of aflatoxin B_1

Based upon the results of this investigation, we conclude that versicolorin A may exist prior to or concurrent with aflatoxin B_1. Although in other cases, in various cereals at diverse conditions, it would be rational to suggest that they are closely relative. In case of versicolorin A detected (even if aflatoxin B_1 not found or at very low level) in some samples, to stop the storage is highly recommended and timely treatment is required.

The mutagenicity test results manifested that Versicoloring A exhibited mutagenicity with the minimum VerA concentration causing mutagenicity in the study was 0.6μg/plate at an induction factor of 3.4 as compared to the negative control. This value is lower than the minimum dose of 0.8μg/plate reported previously (Wong et al., 1977). Nevertheless, Versicoloring A exhibited lower mutagenic effect as compared to 25ng/plate for AFB_1 (Green et al., 1982). On the other hand, Versicoloring A induced significant micro-nuclei at the concentration of 1.6μg/mL in the human peripheral lymphocytes test, which is 25 times that of positive control Mitomycin C ($P<0.01$). Notwithstanding, it manifested mutagenicity in absence of S9 mix in concentration of 5.0 μg/plate in the TA98 test, which implied Versicoloring A, when it is at a high concentration, may toxic without oxidative active by animal liver.

Besides, with the known of mutagenetic toxicity of versicolorin A (Dunn et al., 1982; Mori et al., 1985), requisite detection of versicolorin A is recommended in food and feed safety regulatory guidelines. Versicolorin A should be considered in food and feed safety guidelines and could also be monitored as a prediction indicator of aflatoxin B_1 contamination.

5. Acknowledgement

The authors thank Key Project of National Development (863) Program (2007AA100605), and Guangdong Provincial Technologies R&D Program (2005B20401004) for financial support of this research.

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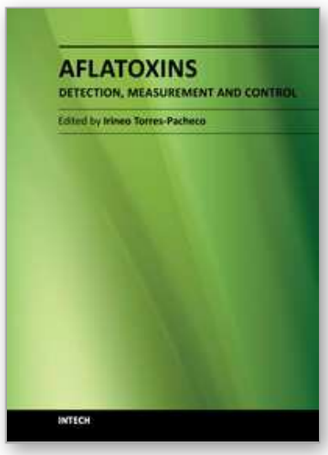
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Aflatoxins - Detection, Measurement and Control

Edited by Dr Irineo Torres-Pacheco

ISBN 978-953-307-711-6
Hard cover, 364 pages
Publisher InTech
Published online 21, October, 2011
Published in print edition October, 2011

This book is divided into three sections. The section called Aflatoxin Contamination discusses the importance that this subject has for a country like the case of China and mentions examples that illustrate the ubiquity of aflatoxins in various commodities The section Measurement and Analysis, describes the concept of measurement and analysis of aflatoxins from a historical prespective, the legal, and the state of the art in methodologies and techniques. Finally the section entitled Approaches for Prevention and Control of Aflatoxins on Crops and on Different Foods, describes actions to prevent and mitigate the genotoxic effect of one of the most conspicuous aflatoxins, AFB1. In turn, it points out interventions to reduce identified aflatoxin-induced illness at agricultural, dietary and strategies that can control aflatoxin. Besides the preventive management, several approaches have been employed, including physical, chemical biological treatments and solvent extraction to detoxify AF in contaminated feeds and feedstuffs.

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