Research Article

Safety and Efficacy Evaluation of Degradation of Aflatoxin B1 by Low-temperature Radio Frequency Plasma

1Lingling Zhao, 2Qijing Du, 1Shiqing Wang and 1Jingyi Sun
1Qingdao Key Laboratory of Modern Agricultural Quality and Safety Engineering, 2Laboratory of Food Risk Analysis and Control, College of Food Science and Engineering, Qingdao Agricultural University, Qingdao 266109, China

Abstract: The purpose of this study was to explore the application of Low-Temperature Radio Frequency Plasma (LTRFP) in the degradation of Aflatoxin B1 (AFB1). After AFB1 was exposed to LTRFP, the degradation rate was analyzed by HPLC and the acute toxicity of the degradation products was evaluated in Wistar rats and Human Hepatocellular carcinoma cells (HepG2). The results showed that AFB1 could be effectively degraded by LTRFP and the degradation rate decreased with initial AFB1 concentration rise. In initial concentration 0.2 mg/L, the degradation rate of AFB1 reached up to 95.34%; nevertheless, as the AFB1 concentration increased to 100 mg/L, the degradation rate declined markedly to 29.32%. The degradation product with degradation rate 29.32% was subjected to toxicity evaluation in rats and a LD50 of 29.41 mg/kg·bw with 95% confidence limit 25.15-34.40 mg/kg·bw was recorded, which was much higher than that of untreated AFB1 (0.5 to 10 mg/kg·bw). Similar results were also found in the HepG2 cells and the acute toxicity of the degradation product was reversely proportional to the degradation rate. It could be concluded that LTRFP was a potential alternative for the detoxification of AFB1 due to the reduced toxicity of the degradation product.

Keywords: Aflatoxin B1, degradation product, toxicity, low-temperature radio frequency plasma

INTRODUCTION

Aflatoxins (AFs) refer to a group of highly toxic, mutagenic and carcinogenic compounds. They are the secondary metabolites of Aspergillus flavus and Aspergillus parasiticus, which are found worldwide to infect both living and dead plants and animals (Asao et al., 1963; Turner et al., 2005; Méndez-Albores et al., 2005). To present, a large number of AFs have been identified, among which, aflatoxin B1 (AFB1) is the most teratogenic, mutagenic and hepatocarcinogenic (Ma et al., 2014, 2015) and has been classified as a group 1 carcinogen by the International Agency for Research on Cancer (1993), Chang et al. (2013) and Corcuera et al. (2015). To remove the toxins, multiple physical, chemical and biological approaches have been proposed (Samarajeewa et al., 1990; Magnoli et al., 2008; Alberts et al., 2009; Wu et al., 2009; Saalia and Phillips, 2010; Diao et al., 2013). However, these methods suffer many disadvantages such as inconvenient operation, high equipment cost and no fulfilling the food safety requirements, especially regarding the safety of the degradation products and safeguarding the nutritional properties of treated foods and feeds, which greatly limited their practical applications (Liu et al., 2011; Luo et al., 2014).

Plasma is a partially ionized, low-pressure gas that contains ions, electrons, UV photons and reactive neutral species with sufficient energy to break covalent bonds and initiate various chemical reactions. In a previous work, we found that AFB1 could be effectively degraded by Low-Temperature Radio Frequency Plasma (LTRFP). According to the structure-toxicity relationship of AFB1, we proposed that the degradation products should have reduced toxicity, which required further experimental confirmation (Wang et al., 2015). Hence, the purpose of this study is to investigate the toxicity of the LTRFP degradation products of AFB1 in Wistar rats and the HepG2 cell. We believe that this study could provide useful information for the practical application of LTRFP in the detoxification of AFB1.

MATERIALS AND METHODS

Materials: Standard AFB1 (2, 3, 6a, 9a-tetrahydro-4-methoxycyclopenta [c] fur [2, 3-4, 5] fur [2, 3-h]
chromene-1, 11-dione; C_{17}H_{12}O_{6}; purity>99% was purchased from Sigma (St. Louis, MO, USA). The human hepatocellular carcinoma cell line (HepG2) was obtained from Shandong Analysis and Testing Center (Shandong, China). Acetonitrile of UPLC grade was obtained from Merck (Merck KGaA, Germany). Standard AFB1 was dissolved in acetonitrile to make a stock solution of 100 mg/L. Dimethyl sulphoxide (DMSO) was purchased from Sigma (St Louis, MO, USA). Dulbecco’s Minimum Essential Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (10000 U/mL penicillin and 10000 U/mL streptomycin; Invitrogen), β-mercaptoethanol and methyl thiazolyl tetrazolium (MTT) were purchased from Gibco Life Technologies. Milli-Q deionized water with 18 MΩ/cm was used throughout the work and all other reagents were of analytical grade except otherwise specified.

Degradation of AFB1 by LTRFP:
Degradation procedure: The stock solution of AFB1 (100 mg/L) was transferred to a weighing bottle and dried by nitrogen purge. Then, the powder was exposed to the LTRFP as described in our previous work (Wang et al., 2015). Several minutes later, the powder was collected for degradation rate determination and toxicity evaluation.

Degradation rate determination: The degradation rate was determined by using an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA) equipped with a 4.6×250 mm Agilent Eclipse XDB-C18 column. The degradation product was dissolved in acetonitrile and the elution was carried out isometrically using a mixture of distilled water, methanol and acetonitrile in 50:40:10 (v/v/v) as the mobile phase. The elution lasted 10 min at room temperature in a flow rate of 0.8 mL/min and the absorbance at 365 nm was monitored.

The degradation rate of AFB1 was determined according to the variation of the peak area by using the following equation:

\[
\text{Degradation rate (%) =} \frac{\text{Peak area of AFB1 before treatment} - \text{Peak area of AFB1 after treatment}}{\text{Peak area of AFB1 before treatment}} \times 100\%
\]

Toxicity evaluation in Wistar rats: Forty male and 40 female 3-week Wistar rats, weighing about 60-70 g, were obtained from the Better Biotechnology Co., Ltd (Nanjing, China). Upon arrival, all the animals were examined for health condition and acclimatized for 5 d on the standard diet prior to experiment. The rear conditions were temperature 25±1°C, 12-h light/dark cycles, humidity 50±5% and free access to standard commercial diet and drinking water throughout the acclimation and experimental periods. The rats were randomly grouped into 8 groups with 5 males and 5 females each group. All the procedures for animal experimentation were carried out in strict compliance with China’s guidelines for animal care.

According to preliminary experiments, the actual toxicity of the degradation product was evaluated using the dosages 17.50, 22.68, 29.40, 38.10, 49.38, 64.00, 82.96 and 107.52 mg/kg·bw. The degradation product was dissolved in Dimethyl Sulphoxide Solution (DMSO) to yield an 11 mg/mL solution and administered intragastrically to the rat on the first day of the experiment. The rats were fasted for 6 hours prior to toxin administration, but were allowed free access to standard diet and tap water 2-3 h after drug administration. The experiment lasted 14 days in total and the rats were observed for behavioral changes, signs of toxicity, or death during the experiment. The acute toxicity of the degradation product was calculated using the Karber method (Zhang et al., 2012; Ahmed, 2015).

Toxicity evaluation in HepG2 cells:
Cell culture: The HepG2 cells were maintained in DMEM containing 10% fetal bovine serum, 1% penicillin-streptomycin solution and 5 mM β-mercaptoethanol. The cells were grown in 63-cm² cell culture dish coated with type 1 collagen at 37°C under 5% CO2 and 95% humidified environment. Prior to the experiment, the degradation product was dissolved in DMSO and applied to the HepG2 culture. The culture medium was refreshed every 3-4 d and subcultured a ratio of 1:4 once a week. After progressing to the logarithmic phase, the cells were exposed to difference concentrations of AFB1 or its degradation products dissolved in DMSO for toxicity evaluation. The concentration of DMSO in the medium did not exceed 1% (v/v) throughout the experiment.

Measurement of cell viability: The cell viability was determined according to MTT assay. The cells were added at a density of 1.0×10⁴ cells per well on a 96-well plate and allowed to attach for 4-6 h. After complete attachment to the wells, AFB1 or its degradation products of different concentrations were transferred to the wells and incubated at 37°C for 24 h or 48 h. Then, 20 μL of 5 mg/mL MTT was added to each well and the contents were further incubated at 37°C in a 5% CO2 humidified incubator. Four hours later, the medium was removed and replaced with 150 μL DMSO to dissolve the purple crystals. The plate was shaken for 15 min at 150 rpm and the absorbance of each well was measured on a microplate reader at 570 nm.

The cell viability was calculated according to the following equation:

\[
\text{Cell viability (%) =} \frac{\text{MTT OD value of treated cells}}{\text{MTT OD value of untreated cells}} \times 100\%
\]
Table 1: Effect of LTRFP treatment on the content of AFB1

| Sample               | Time (min) | Peak area (mAU*min) | Peak height (mAU) | Peak width (mAU) | Symmetry factor | Degradation rate (%) |
|----------------------|------------|---------------------|-------------------|------------------|-----------------|----------------------|
| AFB1 before treatment| 8.72       | 6784.05             | 225.53            | 0.41             | 2.87            | 29.32%               |
| AFB1 after treatment | 8.80       | 4794.77             | 159.73            | 0.41             | 2.82            |                      |

*Peak area referred to the integral value of the peak height and retention time*

Fig. 1: Total ion chromatograms of AFB1 before (a) and after treatment by LTRFP (b)

**Optimization of degradation condition:** According to preliminary work, we found that the initial concentration of the AFB1 solution greatly affected the degradation efficiency. Hence, the effect of initial AFB1 concentration as well as the plasma generator power and the exposure duration the on the degradation rate was concerned in this study. The stock AFB1 solution was diluted to 0.2, 0.4, 0.5, 0.6, 0.8, 1.0 mg/L with acetonitrile and dried with nitrogen purge. Then, the powder was exposed to LTRFP under different input power (100, 200, 300, 400 W) and for different durations (2, 4, 6, 8, 10 min). The degradation rate was then determined.

**Statistical analysis:** All the measurements were performed in at least triplicate and all the values were expressed as the mean±SD. The comparisons among different groups were performed by analysis of variance using a Duncan test and SPSS 17.0 statistical software. The results were considered significant in p<0.05.

**RESULTS AND DISCUSSION**

**Degradation rate of AFB1:** The degradation rate after exposing 100 mg/L AFB1 to 300 W LTRFP for 8 min was given in Table 1. It could be seen that the degradation rate was only 29.32%, which was much lower than the values reported in our previous work (Wang et al., 2015) and could be possibly ascribed to the higher initial concentration of AFB1. The HPLC chromatograph of the degradation product was illustrated in Fig. 1. It could be seen that five products...
Table 2: LD₅₀ and 95% confidence limit of AFB₁ after degradation by LTRFP

| Group | Dose (mg/kg) | Logarithmic dose (x) | Mortality (D/T) | Mortality (%) | LD₅₀ values and 95% confidence limits (mg/kg) |
|-------|--------------|----------------------|----------------|--------------|---------------------------------------------|
| 1     | 17.50        | 1.24                 | 0/10           | 0            | LD₅₀ = 29.41 mg/kg; 95% confidence limits:  |
|       |              |                      |                |              | 25.15-34.40 mg/kg                           |
| 2     | 22.68        | 1.36                 | 4/10           | 40           |                                             |
| 3     | 29.40        | 1.47                 | 7/10           | 70           |                                             |
| 4     | 38.10        | 1.59                 | 8/10           | 80           |                                             |
| 5     | 49.38        | 1.70                 | 8/10           | 80           |                                             |
| 6     | 64.00        | 1.81                 | 9/10           | 90           |                                             |
| 7     | 82.96        | 1.92                 | 9/10           | 90           |                                             |
| 8     | 107.52       | 2.03                 | 10/10          | 100          |                                             |

D/T: dead/treated rats

were identified. This result was consistent with our previous work (Wang et al., 2015).

Acute toxicity in rats: The 14-day acute toxicity of the degradation product of AFB₁ was presented in Table 2. It could be seen that the mortality increased progressively as the dose increased from 17.50 to 107.52 mg/kg. On day 14, the survival rate of the rats in the eight group were 100, 60, 30, 20, 20, 10, 10 and 0% respectively. During the experiment, we found that the rats showed irreversible signs of toxicity, including asthenia, piloerection, ataxia, anorexia, syncope, urine yellow, weight loss and finally death. Such symptoms have been reported in similar researches (Stanley et al., 1993).

Many works have showed that AFB₁ could reduce the activity of some pancreatic enzymes, inhibit the synthesis of enzymes and other hormones and hinder the absorption and metabolism of nutrients in the body, resulting in impaired digestion, loss of appetite, weight gain and growth and development of stagnation (Peters and Teel, 2003; Williams et al., 2004; Supriya et al., 2014). A similar variation, including decreased feed and water intake as well as declined body weight was also recorded in this study (data not shown). LD₅₀ is not only the main indicator of chemical toxicity, but also the most important quantitative index in acute toxicity evaluation. From Table 2, we could see that the LD₅₀ of the AFB₁ degradation product with degradation rate 29.32% was calculated to be 29.41 mg/kg·bw for rats. It has been reported that LD₅₀ of untreated AFB₁ ranged from 0.5 to 10 mg/kg·bw (Costanzo et al., 2015), which was much lower than that of treated AFB₁. Hence, LTRFP degradation could effectively reduce the toxicity of AFB₁ in Wistar rats.

Preliminary toxicity evaluation in HepG2 cell: To evaluate the toxicity of the degradation product in HepG2 cells, a preliminary research was carried out. The AFB₁ degradation product with degradation rate 29.32% was applied to the HepG2 cells and the cell viability was measured on 24 h and 48 h using the MTT assay. In Fig. 2, the survival rate of the HepG2 cells decreased with degradation product concentration increase, but was always higher than that of untreated AFB₁. For example, in toxin concentration 0.78 μmol/L, the survival rate of cells in the degradation product group was 98.41%, whereas that of the native AFB₁ group was only 94.46%. When the toxin concentration increased to 100 μmol/L, the viability of the degradation product group decreased significantly to 65.76%, but was still higher than that of the native AFB₁ group (p<0.05).

The effect of LTRFP treatment on the 24-h and 48-h acute toxicity of AFB₁ in HepG2 cells was shown in Fig. 3. It could be seen that the survival rates of the HepG2 cell in the native AFB₁ group on 24 h and 48 h were significantly lower than that on 0 h (p<0.05), indicating that both the untreated AFB₁ and the degradation product possessed certain toxicity. Compared with the native AFB₁ group, the degradation product group displayed significantly higher cell viability.
Fig. 3: Effect of exposing 100 μmol/L AFB₁ to 300 W LTRFP for 8 min on the 24-h and 48-h acute toxicity of AFB₁ in HepG2 cells. Letters in lowercase indicated the comparisons between the values of the three groups in the same exposure duration and those in uppercase represented the comparisons between the values of the same group in different exposure durations.

Fig. 4: Effect of initial AFB₁ concentration on its degradation by LTRFP viability (p<0.05), indicating that LTRFP degradation effectively reduced the toxicity of AFB₁. This result was consistent with the toxicity evaluation results in rats.

Effect of initial AFB₁ concentration on AFB₁ degradation by LTRFP: As revealed in Table 1, exposing 100 μmol/L AFB₁ to 300 W LTRFP for 8 min resulted in a degradation rate of only 29.32%. To explore the relationship between the degradation rate and toxicity of the degradation product, the effects of various parameters, including initial AFB₁ concentration, plasma generator power and exposure duration, on the degradation rate were carried out in this study.

AFB₁ solutions with initial concentration 0.2, 0.4, 0.6, 0.8 and 1.0 mg/L were exposed to 300 W plasma for 8 min and the degradation rates were shown in Fig. 4. It could be seen that the degradation rate declined along with the increase of initial AFB₁ concentration. In initial AFB₁ concentration 0.2 mg/L, the degradation rate of AFB₁ reached up to 95.34%, which was significantly higher than that of other groups (p<0.05). This result disagreed with the photo degradation pattern of AFB₁, in which the AFB₁ degradation was not affected by its concentration (Liu et al., 2011). The difference could be related to the collision and reaction between particles. When the plasma generation power was constant, the number of active ions generated by the plasma was essentially the same and was sufficient for the degradation of only limited amount of AFB₁. Hence, when the moiety of AFB₁ increased, the degradation rate declined accordingly; whereas in photogradation, sufficient reactive ions were available and consequently the substrate moiety was no longer a limiting factor.

Effect of plasma generator power on AFB₁ degradation by LTRFP: AFB₁ in initial concentration 0.5 mg/L was exposed to 100, 200, 300, or 400 W LTRFP for 8 min and the resultant degradation rates were illustrated in Fig. 5. It could be seen that the degradation rate of AFB₁ increased significantly along with plasma generator power rise (p<0.05). In generator power 100 W, the degradation rate was 64.35%; when the power was raised to 400 W, the degradation rate increased by 41.34% and reached up to 90.95%. The density of reactive ions in plasma was proportional to the generator power. When the quantity of AFB₁ was constant, more reactive ions could lead to more complete substrate degradation.
**Effect of radiation time on AFB₁ degradation by LTRFP:** AFB₁ in initial concentration 0.5 mg/L was exposed to 300 W for different times and the degradation rates were demonstrated in Fig. 6. It could be seen that, similar to the effects of plasma generation power, the degradation rate increased significantly with exposure time elongation (p<0.05). When the exposure time was 2 min, the degradation rate of AFB₁ was as low as 32.56%; when the exposure time was elongated to 10 min, the degradation rate amounted up to 89.13%, indicating that the exposure time was a critical time that affected AFB₁ degradation.

**Toxicity evaluation in HepG2 cells:** The degradation product with degradation rate 95.34% was subjected to acute toxicity evaluation in HepG2 cells. The effects of degradation product concentration on the viability of HepG2 cells were shown in Fig. 7. It could be seen that the cell viability decreased with degradation product concentration increase and that of the degradation product group was always lower than the AFB₁ group. When the degradation production concentration increased from 0.78 μmol/L to 100 μmol/L, the cell viability declined slightly from 98.24% to 93.37% without significant difference (p>0.05), whereas that of
untreated AFB$_1$ decreased markedly from 97.78% to only 63.72% (p<0.05).

The 24-h and 48-h acute toxicity of the AFB$_1$ degradation product in initial concentration 100 μmol/L was shown in Fig. 8. We could see that exposure to the degradation product for 24 h and 48 h resulted in survival rate of 93.37 and 91.11%, respectively, which was slightly lower than the control, but was significantly higher than that of the AFB$_1$ group, whose cell viabilities were 63.72 and 33.12%, respectively (p<0.05). These results were consistent with the toxicity evaluation results in Wistar rats, that is, degradation by LTRFP reduced the toxicity of AFB$_1$.

**CONCLUSION**

The degradation efficiency of AFB$_1$ by LTRFP and the acute toxicity of the degradation products in Wistar rats and HepG2 cells were explored in this study. It was found that AFB$_1$ could be effectively degraded by LTRFP and the exposure of 100 mg/L AFB$_1$ to 300 W plasma for 8 min could result in a degradation rate of 29.32%. The degradation product was subjected to acute toxicity evaluation in Wistar rats and the estimated median lethal dosage (LD$_{50}$) was found to be 29.41 mg/kg·bw with 95% confidence limit 25.15-34.40 mg/kg·bw, which was much higher than that of untreated AFB$_1$. The initial AFB$_1$ concentration, plasma generator power and exposure duration greatly affected the degradation efficiency of LTRFP and the degradation rates in the selected experimental conditions ranged from 29.32 to 95.34%. The toxicity of the degradation products with the lowest and highest degradation rates were evaluated in HepG2 cells. LTRFP degradation significantly reduced the toxicity of AFB$_1$ in HepG2 cells and the 24-h and 48-h acute toxicity was inversely proportional to the degradation rate. It was concluded that LTRFP was potential alternative to the detoxification of AFB$_1$ contaminated products due to its high degradation efficacy and reduced toxicity of the degradation product.

**ACKNOWLEDGMENT**

This study was supported by the National Natural Science Foundation of China under Grant Nos. 31101391 and 31271963.

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