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Reaction of zearalenone and α-zearalenol with allyl isothiocyanate, characterization of reaction products, their bioaccessibility and bioavailability *in vitro*

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ABSTRACT

This study investigates the reduction of zearalenone (ZEA) and α-zearalenol (α-ZOL) on a solution model using allyl isothiocyanate (AITC) and also determines the bioaccessibility and bioavailability of the reaction products isolated and identified by MS-LIT. Mycotoxin reductions were dose-dependent, and ZEA levels decreased more than α-ZOL, ranging from 0.2 to 96.9% and 0 to 89.5% respectively, with no difference ($p \leqslant 0.05$) between pH 4 and 7. Overall, simulated gastric bioaccessibility was higher than duodenal bioaccessibility for both mycotoxins and mycotoxin-AITC conjugates, with duodenal fractions representing $\geqslant$63.5% of the original concentration. Simulated bioavailability of reaction products (α-ZOL/ZEA-AITC) were lower than 42.13%, but significantly higher than the original mycotoxins. The cytotoxicity of α-ZOL and ZEA in Caco-2/TC7 cells was also evaluated, with toxic effects observed at higher levels than 75 μM. Further studies should be performed to evaluate the toxicity and estrogenic effect of α-ZOL/ZEA-AITC.

1. Introduction

Zearalenone (ZEA) and its derivative α-zearalenol (α-ZOL) are non-steroidal estrogenic mycotoxins produced by fungi belonging to the genus *Fusarium* and *Gibberella* (Yang, Wang, Liu, Fan, & Cui, 2007). These fungal species contaminate pre and post-harvest cereal crops such as corn, barley, wheat, rice and oats mainly from temperate and warm regions (Zinedine, Soriano, Molto, & Mañes, 2007). Several studies have demonstrated hepatotoxic, haematotoxic, immunotoxic, genotoxic and teratogenic effects of these mycotoxins to a number of mammalian species (Zinedine et al., 2007). ZEA elicit estrogenic response upon binding to the estrogen receptor (Drzymala et al., 2015). Moreover, it can be metabolized in the human body to α-ZOL, which possesses three to four times higher estrogenic activity (Wang et al., 2014).

There are some strategies proposed for the detoxification and biodegradation of ZEA in foods through chemical/enzymatic methods using ozone (McKenzie et al., 1997), H_2O_2 (Abd Alla, 1997) and lactonohydrolase (Takahashi-Ando, Kimura, Kakeya, Osada, & Yamaguchi, 2002); biological methods using lactic acid bacteria (Mokoena, Chelule, & Gqaleni, 2005), *Aspergillus niger* strain FS10 (Sun et al., 2014) and *Lactobacillus plantarum* Lp22, Lp39 and Lp4 (Zhao et al., 2015); and physical methods such as the use of adsorbent materials (Avantaggiato, Havenaar, & Visconti, 2003; Ramos, Hernández, Plá-Delfina, & Merino, 1996) or extrusion (Cetin & Bullerman, 2005). However, there is little information concerning the metabolites produced through these processes and their potential toxicity.

Glucosinolates (GLs) are a group of phytochemicals found in vegetables of the *Brassicaceae* (*Syn. Cruciferae*) family, which includes broccoli, cauliflower, mustard and horseradish (Meca, Luciano, Zhou, Tsao, & Mañes, 2012). Damage to the plant tissue leads to the hydrolysis of GLs by endogenous myrosinase, producing numerous biologically active compounds, including isothiocyanates (ITCs), thiocyanates and nitriles (Borges, Simões, Saavedra, & Simões, 2014). ITCs have several biological activities including plant defense (against insects and microbial infections) (Luciano & Holley, 2009; Mansour et al., 2012; Santos, Faroni,

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Sousa, & Guedes, 2011), benefits to human health (chemopreventive and anti-angiogenic properties) (Cavell, Sharifah, Donlevy, & Packham, 2011; Fimognari, Turrini, Feruzzi, Lenzi, & Hrelia, 2012; Zhang, 2004) and might be used as natural food preservatives (Borges et al., 2014; Saavedra et al., 2010). Allyl isothiocyanate (AITC) is one of the most common ITC, which has been reported as potent antimicrobial (Luciano & Holley, 2009). Previous studies have also demonstrated its capacity to react with mycotoxins such as beauvericin (BEA) (Meca, Luciano, et al., 2012) and fumonisins (FBs) (Azaiez, Meca, Manyes, Luciano, & Fernández-Frazón, 2013) in buffered solutions and in food matrices. AITC was able to react with both mycotoxins forming adducts, which may reduce their toxicity.

Toxins ingested through food products can be degraded or modified by metabolic processes of the human body, and only a fraction of the initial content may be accessible for absorption (Angelis, Monaci, Mackie, Salt, & Visconti, 2014). In this sense, bioavailability is defined as the portion of ingested contaminant that reaches the bloodstream (Kabak & Ozbey, 2012). These studies in combination with cell models can provide important information concerning the impact of these compounds on human health (Meca, Mañes, Font, & Ruiz, 2012). Bioavailability and toxicity evaluated through cellular systems has been widely used by rapid and cost-effective assays of easy standardization, which reduce the use of experimental animals and enables the investigation of specific mechanisms using different cultured cells (Fernández-Garcia, Carvajal-Lérida, & Pérez-Gálvez, 2009).

The objective of the present study was to assess the potential of AITC to react with α-ZOL and ZEA in buffered solutions and to determine the bioaccessibility and bioavailability *in vitro* of the reaction products.

2. Material and methods

2.1. Materials and apparatus

ZEA (MW = 318.36 g/mol; ⩾98% purity) and α-ZOL (MW = 320.38 g/mol; 97% purity) standards, AITC (MW = 99.15 g/mol; 95% purity), formic acid (HCOOH), potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulfate ($NaSO_4$), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amylase, hydrochloric acid (HCl), pepsin, pancreatin and bile salts were obtained from Sigma–Aldrich (St. Louis, MO, USA). The stock solutions were prepared in methanol and kept at −20 °C. Acetonitrile, methanol and ethyl acetate of LC–MS grade were purchased from Fisher Scientific (New Hampshire, USA). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Cell culture materials including Dulbecco's modified Eagle's medium (DMEM), penicillin, amphotericin B, HEPES, no essential aminoacids (NEAA), streptomycin, phosphate buffer saline (PBS), Hank's balanced salt solution (HBSS) and dimethyl sulfoxide (DMSO) were also provided by Sigma-Aldrich. Fetal calf serum (FCS) was purchased from Cambrex Co. (Belgium).

2.2. Reduction of α-ZOL and ZEA with AITC in vitro

ZEA and α-ZOL standards were diluted to 78 μM in phosphate buffer at pH 4 or 7. AITC at 2, 20, 100 or 200 mM was added to the reaction vials (final volume of 1 ml), which were tightly closed, shaken with the use of a vortex for 1 min and kept at room temperature. Aliquots were draw after 0, 4, 8, 24 and 48 h of reaction for further analyses. Assays were carried out in triplicate and compared with a standard curve ranging from 0.3 to 300 μM. The results were expressed in percentage (%) of reduction of mycotoxins based on a control sample prepared with the mycotoxin standard.

2.2.1. HPLC analysis

ZEA and α-ZOL were determined using Merk HPLC with a diode array detector (LC-DAD) L-7455 (Merk, Darmstadt, Germany) at 236 nm and Hitachi Software Model D-7000 version 4.0 was used for data analysis. A Gemini C_{18} column (Phenomenex, Torrance, USA) 4.6 × 150 mm, 3 μm particle size was used as the stationary phase. The isocratic mobile phase was consisted of water/acetonitrile (55:45, v/v) with a flow rate of 0.7 ml/min. The samples were filtered through 0.22 μm nylon membrane and 20 μl was injected into HPLC system. There was a new peak identified on LC-DAD chromatograms corresponding to the reaction product of AITC and either α-ZOL or ZEA. The structures of these compounds were confirmed by a linear ion trap spectrometer (MS-LIT). Assuming that 1 mol of AITC and 1 mol of α-ZOL or ZEA produces 1 mol of adducts, the molecular weight (MW) was considered 418.7 and 418.4 g/mol of ZEA-AITC and α-ZOL-AITC respectively. This ratio was used to calculate its theoretical concentration.

2.2.2. MS-LIT characterization of a-ZOL and ZEA-AITC adducts

A 3200 QTRAP™ linear ion trap mass spectrometer (AB SCIEX Concord, Ontario, Canada) coupled to a Turbo Ion Spray source was used. This instrument is based on a triple-quadrupole path (QqQ) in which the third quadrupole also operates as a linear ion trap (QqLIT) with improved performance. In the QqLIT configuration, Q TRAP™ operates in enhanced resolution (ER) and enhanced product ion (EPI) scan modes. Applied Biosystem/MDS SCIEX Analyst software version 1.3.2 was used for data acquisition and processing.

The electrospray ionization mass spectrometry (ESI-MS) analyses were performed in positive ion mode. The equipment was set as follows: ESI needle voltage at 5500 V, curtain gas at 35 (arbitrary units), GS1 and GS2 were set to 35 and 40 °C, respectively, and probe temperature at 350 °C. Nitrogen served both as turbo-gas and collision gas. The method was optimized based on mycotoxin reduction, and α-ZOL/ZEA-AITC reaction products were obtained from the combination of 200 mM of AITC and 78 μM of each mycotoxin (α-ZOL or ZEA). Products were extracted from the buffer solution with 3 ml of ethyl acetate (99.9%). The extraction was repeated with another 3 ml of ethyl acetate and the solvent was evaporated using nitrogen flow. The isolate was diluted in 1 ml of methanol and infused into the ion source at a flow rate of 20 μl/min introduced via a model 11 Harvard infusion pump. Full-scan spectra were analyzed for the identification of products formed through the reaction between ZEA or α-ZOL and AITC. Spectra were preliminarily recorded by connecting the Harvard infusion pump to the interface. The characterization of isolated compounds were performed using the modality of ER scan, the mass range from 200 to 500 Da to obtain the general spectra of the molecule. The utilization of the mass spectrometry associated at the detection with the linear ion trap allowed the total characterization of the isolated compounds.

2.3. In vitro digestion model

The static *in vitro* digestion model used was performed according to Gil-Izquierdo, Zafrilla, and Tomás-Barberá (2002) with some modifications. All digestive solutions were warmed to 37 ± 3 °C before the experiment. Methanol solutions (1 ml) of α-ZOL, ZEA or adducts (α-ZOL/ZEA-AITC) were added to polyethylene tubes