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Degradation study of enniatins by liquid chromatography–triple quadrupole linear ion trap mass spectrometry

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ABSTRACT

Enniatins A, A_1, B and B_1 (ENs) are mycotoxins produced by *Fusarium* spp. and are normal contaminants of cereals and derivate products. In this study, the stability of ENs was evaluated during food processing by simulation of pasta cooking. Thermal treatments at different incubation times (5, 10 and 15 min) and different pH (4, 7 and 10) were applied in an aqueous system and pasta resembling system (PRS). The concentrations of the targeted mycotoxins were determined using liquid chromatography coupled to tandem mass spectrometry. High percentages of ENs reduction (81–100%) were evidenced in the PRS after the treatments at 5, 10 and 15 min of incubation. In contrast to the PRS, an important reduction of the ENs was obtained in the aqueous system after 15 min of incubation (82–100%). In general, no significant differences were observed between acid, neutral and basic solutions. Finally, several ENs degradation products were identified using the technique of liquid chromatography–triple quadrupole linear ion trap mass spectrometry.

1. Introduction

The *Fusarium* genus is the most prevalent toxin-producing fungi of the Northem temperate region (SCF, 2002). Several *Fusarium species as avenacum*, *moniliforme*, *proliferatum* and *subglutinans* are producers of some minor *Fusarium* mycotoxins called enniatins (ENs). These bioactive compounds are cyclic hexadepsipeptides formed by alternating of the D-α-hydroxy-isovaleric acid (HyLv) and different N-methylamino acid residues as valine (Val) and isoleucine (Ile). The ENs are classified as ionophoric compounds, forming stable molecules with a "sandwich" structure with alkali metals or alkaline earth metals, across human cell membranes (Jestoi, 2008).

In vitro studies have demonstrated that ENs evidenced cytotoxic activity in different cell lines, including rodent (V79), lepidopteran (SF-9), monkey (Vero) and human cells (Caco-2, Hep-G2, HT-29) (Behm, Degen, & Föllmann, 2009; Fornelli, Minervini, & Logrieco, 2004). Generally, the contamination levels by ENs evidenced in cereals collected in Mediterranean area is higher than the data evidenced in Central and Northern European regions, probably due to the different clime condition of these two different parts of the continent (Santini, Meca, Uhlig, & Ritieni, 2012). ENs have been detected in processed products containing essential cereals for adult and infant nutrition, such as breakfast cereals, rice, pasta, infant formula, bread mill and other derived products (Jestoi, 2008; Serrano, Font, Mañes, & Ferrer, 2013a).

Several studies have been published related to the mitigation strategies of mycotoxins in food, focalized principally on the reduction of the trichothecenes, fumonisins (FBs), aflatoxins (AFs), ochratoxin A (OTA), patulin (PAT) and zearalenone (ZEA) during food processing (Abramson, House, & Nyachoti, 2005; Bullerman & Bianchini, 2007; Cramer, Königs, & Humpf, 2008; Kushiro, 2008; Park, Scott, Lau, & Lewis, 2004; Ryu, Hanna, Eskridge, & Bullerman, 2003). At the moment, only two studies are available in the scientific literature on the thermal degradation of the minor *Fusarium* mycotoxins. In particular, Meca, Ritieni, and Mañes (2012) studied beauvericin (BEA) stability during several heat treatments, in a model system and also in homemade crispy bread, obtaining percentages of degradation variables from 20% to 90%. Vaclavikova et al. (2013) determined ENs levels during beer and bread production, concluding that ENs concentrations were reduced during breadmaking (from 71% to 79% in milling and from 50% to 60% in baking), whereas these mycotoxins were not detected in the final beer.

Nevertheless, other studies have focused on the identification of the mycotoxins degradation products formed after the treatments, as well as in the evaluation of the toxicity of these new identified compounds. Meca, Luciano, Zhou, Tsao, and Mañes (2012) studied the stability of BEA in a solution model and in wheat flour using allyl isothiocyanate (AITC) as a reactant. Two reaction products between the bioactive compounds employed in this study were identified by LC–MS-LIT, corresponding to BEA conjugates

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containing one or two AITC molecules. Bretz, Beyer, Cramer, Knecht, and Humpf (2006) studied the DON stability in a food system elaborated with different macronutrients as sugar, starch and proteins, at temperatures ranging from 150 to 200 °C using incubation times variables from 5 to 20 min. The DON reduction was temperature and time dependent. Also three DON degradation products were identified in commercial samples. The cytotoxicity of the degradation products was compared to DON by cell culture experiments. The results evidenced that DON degradation products were less cytotoxic than DON, and the heat treatments employed reduced the risk associated to DON intake. The reduction of the fumonisins has been evidenced by many studies during food processing and cooking (baking, frying, roasting, extrusion and heating). The studies demonstrated that FBs stability depends of several factors, such as temperature, time or sugar and water content. Moreover, different FBs degradation products have been identified during food treatments (Humpf & Voss, 2004).

Considering the lack of data related to the degradation of ENs during food processing, the aims of the study were: (a) to study the thermal stability of ENA, ENA_1, ENB and ENB_1 at different pH, in an aqueous system and in a food model simulating pasta composition (pasta resembling system) by liquid chromatography coupled to a triple quadrupole mass spectrometer detector (LC–MS/MS QqQ), (b) to identify and characterise ENs degradation products produced during heat treatments by liquid chromatography coupled to the mass spectrometry-linear ion trap (LC–MS-LIT).

2. Materials and methods

2.1. Materials

Acetonitrile (AcN) and methanol (MeOH) were provided by Merck (Darmstadt, Germany). Ammonium formate (99%) and formic acid (>98%) were supplied by Panreac Quimica S.A.U. (Barcelona, Spain). Deionized water (<18 $M\Omega\,cm^{-1}$ resistivity) was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore, Bedford, MA, USA). All solvents were filtered through a 0.45 μm HV filter provided by Scharlau (Barcelona, Spain) before use.

Gluten from wheat (⩾80% of protein), starch from potato (PhEur) and albumin from bovine serum (⩾98%, lyophilized powder) used for the preparation of the pasta resembling system were purchased from Sigma–Aldrich (Oakville, ON, Canada). The standards of ENA, ENA_1, ENB and ENB_1 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Standard solutions of ENs A, A_1, B and B_1 were prepared dissolving 1 mg of each compound in 1 ml of MeOH, obtaining stock solutions of the minor *Fusarium* mycotoxins of 1000 $mg\,l^{-1}$. The stock solutions were then diluted with pure MeOH in order to obtain the appropriate working solutions. All the solutions were stored in glass-stoppered bottles in darkness at −20 °C.

2.2. Sample treatment

2.2.1. Aqueous system

The model solutions were prepared in 100 ml Erlenmeyers at three different pH values (pH 4, 7 and 9). In order to adapt the experiments to the real cooking processes conditions, lemon juice and sodium bicarbonate marketed in Valencia were employed to reach the required pH. The acid (pH 4) and basic (pH 9) solutions were prepared adding 1.5 ml of lemon juice and 2 g of sodium bicarbonate to 50 ml of deionized water under continuous stirring. The experiments in the neutral solution (pH 7) were carried out using deionized water. The pH measurements were performed employing a GLP21 Crison pH-metre (Crison Instruments, S.A., Barcelona, Spain) with a Hamilton pH electrode (Fisher Scientific, Madrid, Spain). The model solutions were filtered through a 0.45 μm HV filter provided by Scharlau (Barcelona, Spain), and then 980 μl of each solution was contaminated with 20 μl of each EN (1000 $mg\,l^{-1}$) to obtain a final concentration of 20 $mg\,l^{-1}$. ENs reduction experiments were performed at 100 °C in a water bath SS40-2 (Gran Instruments, Cambridge, United Kingdom) at different boiling times (5, 10 and 15 min). Aliquots of each treatment were filtered through 13 mm/0.20 μm nylon filter (Membrane Solutions, Texas, USA) and injected into the LC–MS/MS and LC–MS-LIT systems.

2.2.2. Pasta resembling system (PRS)

The PRS was formulated by mixing 65 g of starch, 8 g of gluten and 2 g of albumin to obtain homogenous flour. The experiments were performed simulating the boiling process of pasta (100 g of pasta and 1 L of water) employing three aqueous solutions: acid, basic and neutral (see preparation in Section 2.2.1). For this purpose, 75 mg of PRS was contaminated with 20 μl of each EN (1000 $mg\,l^{-1}$) individually, and 1000 μl of aqueous solution was added to the vial. The final concentration of each EN in the vials was of 20 $mg\,l^{-1}$. Thermal experiments were performed at 100 °C in a water bath at different times (5, 10 and 15 min). Afterwards, mycotoxin extraction was carried out as pointed out in next paragraph.

2.3. Mycotoxin extraction

Treated samples from PRS, were extracted with 10 ml of AcN using a Ika T10 basic Ultra-Turrax (Staufen, Germany) for 3 min. The supernatant was evaporated to dryness by nitrogen at 35 °C using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After solvent evaporation, the extract was reconstituted with 1000 μl of AcN/MeOH 50/50 v/v, and filtered through 13 mm/0.20 μm nylon filter until the analysis in the LC–MS/MS and LC–MS-LIT systems.

2.4. Analysis

2.4.1. LC–MS/MS analysis

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) consisting of an autosampler, a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface and a Mass Lynx NT software version 4.1 were used for the MS/MS analyses. The separation was achieved by a Gemini-NX C_{18} (150 × 2 mm I.D., 3 μm particle size) analytical column supplied by Phenomenex (Barcelona, Spain), preceded by a security guard cartridge C_{18} (4 × 2 mm I.D.), using gradient elution that started at 90% of A (AcN) and 10% of B (20 mM ammonium formate in MeOH), increased linearly to 50% B in 10 min. Then, it was decreased linearly to 10% of B in 3 min. Afterwards, the initial conditions were maintained for 2 min. Flow rate was maintained at 0.2 $ml\,min^{-1}$. The analysis was performed in positive ion mode. The electrospray ionisation source values were as follows: capillary voltage, 3.50 kV; extractor, 5 V; RF lens 0.5 V; source temperature, 100 °C; desolvation temperature, 300 °C; desolvation gas (nitrogen 99.99% purity) flow, 800 $l\,h^{-1}$; cone gas 50 $l\,h^{-1}$ (nitrogen 99.99% purity).

The cone voltage selected was 40 V and the collision energy selected was 35 eV for all ENs. The analyser settings were as follows: resolution 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 0.5; entrance and exit energies, −3 and 1; multiplier, 650; collision gas (argon 99.995% purity) pressure, 3.83×10^{-3} mbar; interchanel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.1 ms. The mass spectrometer was operated in