LWT - Food Science and Technology 56 (2014) 469–477

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Fate of enniatins and beauvericin during the malting and brewing process determined by stable isotope dilution assays

Ling Hu [a], Martina Gastl [b], Andrea Linkmeyer [c], Michael Hess [c], Michael Rychlik [a,d,*]

[a] Chair of Analytical Food Chemistry, Technische Universität München, Alte Akademie 10, D-85354 Freising, Germany
[b] Chair of Brewing and Beverage Technology (WZW), Technische Universität München, D-85354 Freising, Germany
[c] Chair of Phytopathology, Technische Universität München, D-85354 Freising, Germany
[d] BIOANALYTIK Weihenstephan, ZIEL Research Center for Nutrition and Food Sciences, Technische Universität München, Alte Akademie 10, D-85354 Freising, Germany

ARTICLE INFO

Article history:
Received 17 January 2013
Received in revised form
15 October 2013
Accepted 3 November 2013

Keywords:
Enniatins
Beauvericin
LC-MS/MS
Beer
qPCR

ABSTRACT

The fate of enniatins A, A1, B, B1 and beauvericin during the malting and brewing process was investigated. Three batches of barley grains were used as starting material, one was naturally contaminated, two were artificially inoculated with *Fusarium* fungi. Samples were taken from each key step of the malting and brewing procedure, the levels of the toxins were determined with stable isotope dilution assays using liquid chromatography–tandem mass spectrometry detection. Significant increases of the toxins were found during germination of two batches of barley grains, resulting in green malts contamination up to a factor of 3.5 compared to grains before germination. Quantitative PCR analyses of fungal DNA revealed in all batches growth of *Fusarium avenaceum* during germination. After kilning, only 41–72% of the total amounts of the toxins in green malts remained in kilned malts. In subsequent mashing stage, the toxins in kilned malts predominantly were removed with spent grains. In the final beer, only one batch still contained 74 and 14 μg/kg of enniatin B and B1, respectively. Therefore, the carryover of these enniatins from the initial barley to final beer was less than 0.2% with the main amounts remaining in the spent grains and the malt rootlets.

1. Introduction

Beer is a popular and widely consumed drink in the world. In some countries such as the Czech Republic, Germany and Austria, the annual per capita consumption exceeds 100 L (Beer statistics, 2010). Unfortunately, the major raw material for beer production, barley, is frequently infected with mycotoxin-producing fungi either in the field or during storage (Medina et al., 2006). Some residues of the mycotoxins accumulated in barley grains may survive the beer production chain and contaminate the final product.

A number of mycotoxins have been analyzed in beer, including aflatoxins, fumonisins, T-2 and HT-2 toxins, ocharatoxin A, zearalenone, zearalenol, deoxynivalenol, 3- and 15-acetyldeoxynivalenol, and deoxynivalenol-3-glucoside (Kostelanska et al., 2009; Romero-González, Vidal, Aguilera-Luiz, & Frenich, 2009; Zöllner, Berner, Jodlbauer, & Lindner, 2000). Among them, many were not detectable or existed only in traces, with the exception of deoxynivalenol and its derivatives, the levels of which were as high as 37 μg/L.

Some earlier studies have focused on the fate of aflatoxin, ochratoxin A, citrinin, zearalenone, fumonisins, as well as deoxynivalenol and 15-acetyldeoxynivalenol during the beer making process (Chu, Chang, Ashoor, & Prentice, 1975; Krogh, Hald, Gjertsen, & Myken, 1974; Schwarz, Casper, & Beattie, 1995; Scott, 1996), which mainly involves malting and brewing. In recent years, the predominance of deoxynivalenol and its derivatives in beer has drawn more attention of researchers to follow their fate during beer making in detail (Kostelanska et al., 2011; Lancova et al., 2008). The latter authors studied the influence of the key steps such as steeping, germination, kilning, mashing, and fermentation on the behavior of mycotoxins belonging to the deoxynivalenol group. They found DON-3-glucoside to be the most prevalent compound being transferred into beer with concentrations amounting to approx. 40 μg/L.

On the other hand, the existence of the emerging *Fusarium* mycotoxins enniatins and beauvericin in beer has rarely been reported except one very recently published study on changes of enniatins during beer making by Vaclavikova et al. (2013). Enniatins and

* Corresponding author. BIOANALYTIK Weihenstephan, ZIEL Research Center for Nutrition and Food Sciences, Technische Universität München, Alte Akademie 10, D-85354 Freising, Germany. Tel.: +49 8161 71 3153; fax: +49 8161 71 4216.
E-mail address: michael.rychlik@tum.de (M. Rychlik).

0023-6438/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.lwt.2013.11.004

beauvericin are cyclic hexadepsipeptides consisting of three D-2-hydroxycarboxylic acid and *N*-methylamino acid moieties, they are often found in cereals such as wheat, barley, maize, and oats, as well as cereal-based products, and contamination levels of several hundred mg/kg have been reported (Mahnine et al., 2011; Ritieni et al., 1997; Uhlig, Torp, & Heier, 2006).

Enniatins and beauvericin possess a wide range of biological activities. They are toxic to brine shrimp, with enniatin B showing an acute (6 h) LC_{50} of 21 μg/mL (Hamill, Higgens, Boaz, & Gorman, 1969; Tan, Flematti, Ghisalberti, Sivasithamparam, & Barbetti, 2011). Their insecticidal activity towards adults of the blowfly *Calliphora erythrocephala* and larvae of the mosquito *Aedes aegypti* have also been reported (Grove & Pople, 1980). Besides, they are known to have inhibitory effects on acyl-CoA: cholesterol acyltransferase (ACAT), which is involved in cholesterol storage, with beauvericin exhibiting an IC_{50} of 3.0 μM (Tomoda et al., 1992). Furthermore, they are reported to be toxic to cell lines of human origin such as hepatocellular carcinoma-line Hep G2 and fibroblast-like foetal lung cell line MRC-5, IC_{50} values for enniatins A, A1, B1, and beauvericin were all in the lower micromolar-range (Ivanova, Skjerve, Eriksen, & Uhlig, 2006).

Recently, we biosynthesized the $^{15}N_3$-labeled enniatins and beauvericin and developed stable isotope dilution assays for their determination in cereals and related food samples (Hu & Rychlik, 2012). In the current study, we applied the stable isotope dilution assays of enniatins and beauvericin to monitor their fate during the whole beer production process on a laboratory scale. The aims of this study were to elucidate the behavior and transfer of enniatins and beauvericin from barley grains through malts to beer by using accurate and precise stable isotope dilution assays, and to assess the risk of enniatins and beauvericin contamination in beer.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (MeCN), chloroform, isoamylalcohol, ethanol and sodium chloride were purchased from Merck (Darmstadt, Germany), MeCN was of analytical-reagent grade. CTAB, Tris base, polyvinylpolypyrolidone-40, EDTA, malt extract, peptone were obtained from Sigma (Steinheim, Germany). Water for HPLC was purified by a Milli-Q-system (Millipore GmbH, Schwalbach, Germany). BEA was obtained from AnaSpec (San Jose, USA), ENN B was obtained from Bioaustralis (New South Wales, Australia), and ENNs A, A1, B1 were purchased from Enzo Life Sciences (Lörrach, Germany). The internal standards $[^{15}N]_3$-enniatin A, $[^{15}N]_3$-enniatin A1, $[^{15}N]_3$-enniatin B, $[^{15}N]_3$-enniatin B1, and $[^{15}N]_3$-beauvericin were synthesized as reported recently (Hu & Rychlik, 2012).

2.2. Raw materials

For malting and brewing experiments, grain of the spring barley variety Quench (Syngenta Seeds, Bad Salzufflen, Germany) was used. Barley was grown under field conditions in Weihenstephan, Freising (Germany). A basic fungicide treatment was applied at the end of stem elongation to control foliar leaf diseases. In a completely randomized experimental design including three variants with four replicates, 12 square meter plots were artificially inoculated at flowering with macroconidia suspensions of highly aggressive single spore isolates of *Fusarium avenaceum* (Fa002) and *Fusarium culmorum* (Fc002) in a density of 75×10^6 conidia qm^{-1}. Control plots remained un-inoculated and were exposed to natural infestation. At dead ripening, grains of the core of each plot were harvested individually to avoid cross-contamination. Four repeated plots were mixed to the batches QC (control), QFc (inoculated with *F. culmorum*) and QFa (inoculated with *F. avenaceum*). As for malting and brewing more than 1 kg of barley was required, only one malting and brewing trial was possible for each batch. However, as the results of the different batches (contents of fungal DNA, decrease of mycotoxins during malting and brewing) are sound, the trials were considered to be representative. Bottom fermenting yeast *Saccharomyces cerevisiae* W 34/70 was supplied by the brewery Hofbräuhaus Freising. Hallertau Hallertauer Select hop (5.1% alpha acids) was purchased from Simon H. Steiner GmbH (Mainburg, Germany).

2.3. Malting process

Malting was performed according to the standard MEBAK procedure: steeping and germination time: 6 days, germination temperature: 14.5 °C, steeping degree: 45% (Anger, 2006). The germinated barley grains, i.e. the green malts, were then kilned at 50 °C for 16 h, followed by kilning at 60 °C for 1 h, at 70 °C for 1 h, and finally at 80 °C for 5 h. At the end of kilning, the brittle rootlets were removed from the kilned malts.

2.4. Brewing process

For each batch of malt, the wort production was carried out in a 10 L (scale: 10 L cast-out wort) scale pilot brewing plant. Kilned malt (1.2 kg) was milled with a two-roll mill using a 0.8 mm gap. The temperature profile of the infusion mashing was 62 °C for 30 min, then 72 °C for another 30 min, and finally 76 °C for 5 min. The malt/liquor ratio was 1.2 kg:5 L. No adjuncts were used according to the German "Reinheitsgebot". The wort was boiled for 90 min at atmospheric pressure. Hop addition was done at the beginning of wort boiling with Hallertau Hallertauer Select hop (5.1% alpha acids) in order to reach 20 bitter units (BU) in beer. The sweet wort was boiled until the wort reached 11.5 °P (degree Plato, specific gravity of the extract, equivalent to grams of sucrose in 100 g solution at 20 °C). After the whirlpool rest of 20 min, the trub (i.e. the precipitate) was separated from the hopped wort and the wort was cooled. To the latter, 70 g of yeast (equivalent to 15×10^6 yeast cells/mL) was added, and the subsequent 6 d fermentation took place at 12 °C. At the end of fermentation, the brewing tanks containing the green beer were kept at 16 °C for 3 d, followed by 10 d at 0 °C for maturation.

Fermentation and storage time is displayed in Fig. 1. After maturation, the beer was filtered through a filter sheet SEITZ-KS 80 (Pall Filtersystems GmbH, Bad Kreuznach, Germany). Thereafter, bottling was done with a single-organ long-tube filler with CO_2-flushing and pre-evacuation.

2.5. Sampling

Samples were taken during each key step of the malting and brewing processes (Fig. 1), including barley grains, first and second steeping water, green malt, kilned malt, rootlets, sweet wort, spent grains, cool wort, and trub. During the fermentation period, samples were taken every day. In addition, samples were taken after the three-day maturation at 16 °C, as well as after the ten-day maturation at 0 °C of the green beer. Filtered beer, yeast sediment, and hop were also analyzed.

2.6. Extraction for mycotoxin analysis

The green malt samples were sterilized with 70% ethanol and then dried at room temperature for 2 d before being ground and extracted. The rest of solid samples were ground and homogenized before extraction. The liquid samples were used directly. The three trub samples, which were separated from the boiled wort by precipitation, were dried at 80 °C in an oven for 12 h before extraction,