Reduction of deoxynivalenol and nivalenol content during roasting of naturally contaminated barley

Blanca E. YUMBE-GUEVARA and Takumi YOSHIZAWA

Department of Biochemistry and Food Science, Faculty of Agriculture, Kagawa University
(Miki, Kagawa 761-0795, Japan)

Summary

Decomposition of deoxynivalenol (DON) and nivalenol (NIV) during roasting of naturally contaminated barley was determined by GC-MS, and by ELISA using a monoclonal antibody which recognizes the partially acetylated derivatives of DON and NIV (3,15-diacetyl-DON and 3,4,15-triacetyl-NIV). As determined by GC-MS, DON and NIV were decomposed with the extent of reduction dependent upon temperature and processing time. By ELISA, a clear decrease in the toxin level was observed above 170 °C, whereas at 150 °C the toxin level increased after 5 and 30 min heating in spite of a slight decomposition observed by GC-MS. This phenomenon was also confirmed when the standard toxins were heated at 150 °C, indicating that heat-induced derivatives of the toxins may have a stronger cross-reactivity against the antibody.

Key words : deoxynivalenol, nivalenol, roasting, GC-MS, ELISA

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Introduction

With the common occurrence of deoxynivalenol (DON) and nivalenol (NIV) in cereals, it is important not only to understand the factors that lead to their occurrence, but also to determine management strategies to minimize their impact and occurrence in food products ¹⁾. Milling studies of wheat naturally contaminated with *Fusarium* mycotoxins indicated that the mycotoxins were differentially distributed in the kernel, with relatively higher levels in the dockage, outer bran fractions and shorts, and lower levels in the inner flour fractions ^{2,3)}. DON was not decomposed on making Egyptian bread, western style bread or cookies baked from hard wheat flour ^{2,4,5)}. In contrast, the effect of baking on DON in non-yeast products was reported as variable, ranging from no effect to 69 % reduction ^{6,7)}. Moreover, during bread baking, DON and NIV were reduced by 49 and 57 %, respectively ⁸⁾. Our experience also indicates that considerable reduction of DON and NIV could be achieved by roasting contaminated barley and wheat kernels ⁹⁾.

All these investigations suggest that, depending on the conditions in which the treatment is performed, the elimination of trichothecenes from contaminated cereals is possible; however, information on the decomposition products remained in the commodities is insufficient. Therefore, as an attempt to elucidate the characteristics of the heat-induced compounds, decomposition patterns of

DON and NIV in naturally co-contaminated barley were determined by two different methods; GC-MS and ELISA. The results presented here are some basis for the characterization of the compounds produced during heat treatment of DON and NIV.

Materials and Methods

Chemicals and Mycotoxins Standards Unless otherwise noted, all reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Organic solvents were of reagent grade and distilled in a glass apparatus before use. The methyl eicosanoate used as internal standard in GC-MS analysis was obtained from Tokyo Kasei Kogyo Co. For the derivatization of DON and NIV to trimethylsilylated (TMS)-ether, a formulation of *N*-TMS-imidazole, *N,O*-bis-TMS-acetamide and TMS-chloride (3:3:2, v/v/v) was used. Each trichothecene standard solution was prepared by dissolving the toxin in ethanol at 1 mg/ml level and then kept at 4 °C for further dilution.

For ELISA, ovalbumin (OVA) and 2, 2'-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt (ABTS powder and buffer) were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Boehringer Mannheim (Mannheim, Germany), respectively. PBS-Tween solution utilized for plate washing consists of 10 mM sodium phosphate buffer (pH 7.4) containing 140 mM NaCl (PBS) and 0.05 % (v/v) Tween 20.

Heating Treatments

1) Kernel Samples Barley kernels (200 g) were introduced into the gas-roaster, preheated for about 12 min up to 150, 170, 180 or 220 °C, and roasted for 5, 15, 30, 45 or 60 min. The gas level was hand-controlled to maintain a constant temperature during the process. Once the heating time was over, the sample was immediately removed and cooled to room temperature. For mass loss quantification, samples were weighed before and after heating.

2) Standard Mycotoxins An aliquot of each standard solution (equivalent to 100 µg) was evaporated to dryness in a small glass vial (8 mm i.d. x 50 mm, 1 ml) under air stream and heated in a convection oven at 150, 160 or 200 °C for 5, 10, 15, 20, 30, 45 or 60 min. Each treatment was performed in triplicate.

Chemical Analysis

1) GC-MS analysis Ground samples were extracted with CH₃CN-H₂O (3:1) and cleaned up using a Florisil mini-column. The concentration of DON and NIV in all samples was determined as TMS-ether by GC-MS (Shimadzu GCMS-QP 5000, Shimadzu Co., Kyoto) with selected ion monitoring mode under the following conditions: fused silica capillary column 30 m x 0.25 mm i.d. (J&W, CA, USA), carrier gas He at 2 ml/min, column temperature held for 5 min at 120 °C and then increased to 280 °C at 8 °C/min, injector temperature 280 °C, interface temperature 250 °C, ionizing voltage 70 eV, ionizing current 300 µA, scanning rate 1 scan/s, and sampling rate for selected ion monitoring 5 points/s. Selected ions were m/z 422 and 407 for DON, m/z 379 and 289 for NIV, and m/z 326 and 283 for methyl eicosanoate (internal standard)¹⁰. The recoveries and variation coefficients (in parentheses) of barley powder containing 1 µg/g of each toxin were 97 % (2.6) and 98 % (4.4) for DON and NIV, respectively. At 0.5 µg/g the recoveries were 96 % (2.6) and 95 % (3.8), and at 0.2 µg/g they were 91 % (2.5) and 93 % (6.8) for DON and NIV, respectively.

2) HPLC analysis Unheated and heated standards were analyzed by HPLC with UV detection under the following conditions: Shimadzu SCL-6A system connected to a Shimadzu SPD-M10A_{VP} diode array detector interfaced to a Shimadzu CBM-10A communications bus module and Shimadzu CLASS-LC10 model FMV-6300 DX 2c computer, column Shim-pack CLC-ODS, 150 mm x 6 mm i.d. (Shimadzu Co., Kyoto), oven temperature 40 °C, wavelength 220 and 270 nm, mobile phase water-methanol-acetonitrile (70 : 7 : 3, v/v/v) at a flow rate of 1.2 ml/min¹¹⁾.

ELISA Analysis ELISA analysis was conducted according to the method reported by Ikeda *et al.* (The 52nd Meeting of the Japanese Association of Mycotoxicology, Tokyo, January 4, 2002). Ground samples (5 g) were extracted with methanol - water (3:1, v/v, 20 ml) and stirred regularly for 1.5 hr. An aliquot (2 ml) of the filtrate was diluted with methanol (2 ml), cooled in ice for 4 hr and centrifuged (1400 x g, 15 min). A portion (160 µl) of the supernatant was evaporated to dryness and reacted with dried pyridine (50 µl) and acetic anhydride (25 µl) at 45 °C for 40 min. The reaction solution was evaporated to dryness under air stream without heating, dissolved in 100 µl ethanol and then diluted with 900 µl PBS-Tween. Under these conditions DON and NIV are partially acetylated to yield 3,15-diacetyl-DON (3,15-DADON) and 3,4,15-triacetyl-NIV (3,4,15-TANIV), respectively.

A 96-well ELISA plate (F96 Maxisorp, Nunc-Immuno Plate, Nalge Nunc International Co., Tokyo) was coated with 4,15-diacetyl-NIV-3-O-hemisuccinate-OVA (4,15-DANIV-3-O-HS-OVA) in 0.1 M NaHCO₃-Na₂CO₃ buffer (0.4 µg/ml, pH 9.6, 100 µl/well), and incubated at room temperature for 2 hr. The plate was washed 3 times with PBS-Tween, blocked with skim milk (1 mg/ml in PBS, 300 µl/well), and kept with this solution until use (stable for 2 months at 4 °C). The samples were diluted to a convenient amount (*ca.* 2 ng toxin/well) prior to application (50 µl/well), followed by addition of peroxidase-labeled antibody (KTM-240-POD) in PBS containing 1 % OVA (300 ng antibody/ml, 50 µl/well) for the simultaneous detection of 3,15-DADON and 3,4,15-TANIV. Then, the plate was incubated at room temperature for 1 hr and washed 6 times (3 times twice). An aliquot of ABTS substrate solution (1 mg/ml in ABTS buffer, 50 µl/well) was added, incubating at room temperature for 30 min prior to measurement with an immuno-microplate reader NJ-2300 (Inter Med Japan, Tokyo) at 414 nm. Each sample was analyzed in triplicate.

Results and Discussion

During roasting of naturally contaminated barley, DON and NIV were decomposed with the extent of reduction dependent upon temperature and processing time, for which the greatest effect occurred at the highest temperature. Although the final remaining amount of the toxin was different with each temperature, in all cases the reduction pattern determined by GC-MS was the same; rapid at the early stage followed by no change or slight decrease. After partial acetylation, the same naturally contaminated barley samples were analyzed by ELISA. Roasting at above 170 °C resulted in a clear decrease of total trichothecenes level (3,15-DADON + 3,4,15-TANIV) in the samples analyzed by ELISA as shown in Fig. 1A. The reduction pattern was the same as GC-MS, however, at 150 °C the toxin level increased after 5 and 30 min heating (57 and 62 %, respectively), in contrast to the GC-MS analysis, for which a slight decomposition was observed as shown in Fig. 1B.

Two different possibilities were proposed to explain the increase in toxin level; firstly,

Fig. 1. Effect of roasting at (A) 170 °C and (B) 150 °C on total DON and NIV concentration in naturally contaminated barley determined by GC-MS (-----) and ELISA (——). Each point represents the mean of three replications.

Fig. 2. Effect of heating at (A) 200 °C and (B) 150 °C on DON standard determined by GC-MS (-----) and ELISA (——). Each point represents the mean of three replications.

compounds derived from DON and NIV during roasting may have stronger cross-reactivity than the parent toxins against the antibody (KTM-240-POD) and secondly, heat-induced products of food components may have some affinity against the antibody. To clarify these possibilities, heat treatment on DON and NIV standards, as to eliminate the matrix effects, was carried out.

In GC-MS analysis, concentration of DON standard was found to be decreased by 21, 28 and 93 %

after heating for 30 min at 150, 160 and 200 °C, respectively. ELISA analysis also showed a clear decrease of DON standard heated at 160 and 200 °C, whereas at 150 °C an apparent increase of 8 and 5 % occurred after 10 and 20 min heating, respectively, as shown in Fig. 2. The same phenomenon was also observed for NIV standard (data not shown).

Thus, the above mentioned results clearly indicate that heat-treated DON and NIV produce certain heat-induced compounds with stronger cross-reactivity than the parent toxins against the used antibody. If the heating process continues, the parent and the heat-induced compounds were found to be completely decomposed, suggesting that roasting is an effective method to eliminate DON and NIV in co-contaminated cereals such as barley.

The characteristics of the heat-induced compounds with stronger cross-reactivity than the parent toxins against the antibody were of special concern. Taking into account that the antibody KTM-240-POD can recognize specifically the partially acetylated derivatives (3,15-DADON and 3,4,15-TANIV), it is suggested that a structural change due to the rearrangement of A-ring in the trichothecenes probably occurred. Greenhalgh *et al.*¹²⁾ reported the presence of a DON-related compound in bread made from contaminated flour. This compound (isoDON) has an 8-hydroxy-8-en-7-one moiety with λ_{max} at 277 nm. The λ_{max} of DON and NIV (220 nm) corresponds to the absorption of an α,β -unsaturated ketone with an α -alkyl substituent; however, if the α or β substituent is a hydroxyl group the λ_{max} * absorption shifts above 270 nm¹³⁾. As expected, in the HPLC analysis of DON standard (retention time [t_R] 12.7 min) heated at 150 °C for 20 min (Fig. 3), a decomposition product was detected at t_R 14.8 min when monitored at 277 nm. Furthermore, in NIV standard (t_R 6.7 min) heated at the same condition, a product at t_R 8.0 min was observed (Fig. 4). Fig. 5 shows the UV spectra with the maximum absorbance (220 nm) of DON and NIV shifted to 277 nm in the respective decomposition products, suggesting the possible rearrangement of the 7-hydroxy-9-en-8-one moiety in the parent

Fig. 3. HPLC chromatogram of (A) DON unheated standard and (B) DON standard heated at 150 °C for 20 min monitored at 220 and 277 nm. The arrow indicates the decomposition product.

toxins to an 8-hydroxy-8-en-7-one moiety in the products.

Although the results presented here do not fully explain the toxin augment observed in roasted barley samples because the toxins in naturally contaminated cereals are in a more complex environment, the structural rearrangement that take place with DON in processed cereal samples ¹²⁾ may be also observed for NIV.

Undoubtedly roasting offers a promissory alternative for the reduction of mycotoxins in contaminated cereals. However, when this treatment is conducted at low temperatures, part of this reduction is possibly a structural rearrangement. Bearing in mind that DON and NIV toxicity is

Fig. 4. HPLC chromatogram of (A) NIV unheated standard and (B) NIV standard heated at 150 °C for 20 min monitored at 220 and 277 nm. The arrow indicates the decomposition product.

Fig. 5. UV spectra for (A) DON and NIV, and (B) their respective decomposition products. The spectra were obtained with a diode array detector by scanning the peaks in Fig. 3 and 4.

———— DON and its product
 - - - - - NIV and its product

mainly determined by the presence of the 12, 13-epoxy ring, it can be assumed that under these conditions the toxicity of the mycotoxins or their heat-induced compounds in commodities will not decrease. The final confirmation of the structure of heat-induced compounds, the determination of their cross-reactivity and toxicity will be the subject of a further publication.

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焙煎による自然汚染大麦中のデオキシニバレノールとニバレノールの減少

ユンベ・ブランカ, 芳澤宅實 : 香川大学農学部 (761-0795 香川県木田郡三木町池戸)

焙煎による自然汚染大麦中のデオキシニバレノール (DON) 及びニバレノール (NIV) の分解について, GC-MS またはモノクローナル抗体を用いた ELISA で検討した。DON と NIV が加熱温度と加熱時間に依存して分解されることが GC-MS で確認された。しかし, 150 で 5 分あるいは 30 分の加熱条件下では, GC-MS 分析では僅かな減少にもかかわらず, ELISA では増加することが認められた。この現象は両トキシンの標準品を 150 で加熱した際にも確認され, トキシンの加熱生成物がモノクローナル抗体に対して高い交差反応性を示すことが示唆された。

キーワード : デオキシニバレノール, ニバレノール, 焙煎, GC-MS, ELISA