Distribution of nivalenol in milling fractions of severely *Fusarium*-infected Japanese soft winter wheat grains

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Summary

The fate of *Fusarium* mycotoxin nivalenol during milling of a Japanese wheat cultivar was investigated. Grain samples with two distinct nivalenol levels were test-milled to produce six flour fractions (Breaking flours: 1B, 2B, and 3B, Middling flours: 1M, 2M, and 3M) and two outer layer fractions (bran and shorts). Patent flour for human consumption was made from 1B, 1M, 2B, and 2M, while low-grade flour was made from 3B and 3M. These four samples; Patent flour, low-grade flour, bran and shorts were analyzed for the content of nivalenol by HPLC-UV. Two samples showed similar patterns of nivalenol distribution in milling fractions.

Key words : nivalenol; processing; flour; milling; HPLC-UV

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Introduction

Trichothecene mycotoxins frequently occur in cereal and cereal-based products and are secondary metabolites produced mainly by *Fusarium* head blight pathogens¹⁾. *Fusarium* toxins are harmful to human and animal health²⁾. The acute signs and symptoms of trichothecene poisoning are characterized by skin irritation, food refusal, vomiting, diarrhea, hemorrhage, neural disturbance, miscarriage, immunosuppression, body growth inhibition and death³⁻⁵⁾. The mycotoxin nivalenol (NIV) (Fig. 1) is a major *Fusarium* secondary metabolite that has been recorded worldwide⁶⁾. NIV is an analog of deoxynivalenol (DON) (Fig. 1), both often co-occur^{7,8)}. The distribution of *F. asiaticum* has been widely reported in the western region of Japan, resulting in more frequent occurrence of NIV than DON⁹⁾. Therefore, NIV is an important contaminant in Japan.

Milling is a processing step to remove outer layers of grain and obtain a high-grade flour or patent flour from the endosperm of grain¹⁰⁾. Previous studies reported that among the milling fractions, the outer layers of the grain (bran and shorts) contained higher concentration of mycotoxins¹¹⁻¹³⁾. Milling and cooking effects on

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Fig. 1. Chemical structures of nivalenol (NIV) and deoxynivalenol (DON) (R=OH:NIV, R=H:DON)

DON content have been studied worldwide¹⁴, and the effects of milling on grains of a Japanese wheat cultivar *Chikugoizumi* with medium (ca. 1 mg/kg), and high (ca. 5 mg/kg) levels of DON contamination were investigated in previous studies^{16,17}. In this study, we examined the effects of milling after sorting of wheat grains of the same cultivar contaminated with high (Sample A, ca. 5 mg/kg) and very-high (Sample B, ca. 10 mg/kg) levels of NIV, because information about the content of NIV in a Japanese wheat cultivar after milling is limited. A laboratory-scale test mill was used to prepare four types of semi-processed preparations: patent flour (for human consumption), low-grade flour, bran and shorts from a Japanese wheat cultivar with different levels of NIV contamination, and the concentration of NIV in each sample was analyzed using an HPLC-UV method.

Materials and Methods

Sampling Japanese soft red winter wheat cultivar *Chikugoizumi* aimed for noodles was used. It was planted and harvested in Kumamoto, Japan. Harvest time was June, 2006. The inoculation of *Fusarium* fungi to wheat plants were performed as described previously⁹. The average levels of NIV contained in the original (prior to clean) grains were 5.4 mg/kg and 9.5 mg/kg, respectively. The grains were cleaned using a small winnower (Type/model: PS, Ohyatanzo Seisakusyo & Co., Japan), which removed straw, husk and unhealthy grain using air flow. They were stored at 4 °C until milling.

Sample preparation Cleaned grains (2-5 kg) were tempered to 14.5 % water content. Milling was performed using a Bühler laboratory mill (MLU-202, Bühler AG, Uzwil, Switzerland) equipped with three break rolls and three middling rolls (Fig. 2). The mill produced eight fractions: 1B, 2B, 3B, 1M, 2M, 3M, bran and shorts. Milling speed and spacing between the rolls were based on the manufacturer's instructions. Patent flour for human consumption was made from a homogenous blend of fractions 1B, 1M, 2B and 2M. Low-grade flour was made by blending fractions 3B and 3M. All the milling fractions were kept in 4 °C prior to analysis.

Moisture content analysis Moisture contents were measured by the oven drying method¹⁵) (dried in an oven at 135 °C for 1 h).

Chemicals and standards NIV standard solution (100 µg/mL) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Acetonitrile for extraction was special grade, and for HPLC analysis, methanol, acetonitrile, and distilled water were of HPLC grade.

Analytical method Each ground or milled sample (5.0 g) was weighed accurately in a 50 mL polypropylene centrifuge tube, and 20 mL of acetonitrile:water (85 : 15) was added. Extraction was done using a

Fig. 2. Milling diagram of Sample A and Sample B in a Bühler laboratory mill. B.R.: breaking roll, M.R.: middling roll, 1B, 2B, 3B: breaking roll flour fractions, 1M, 2M, 3M: middling (smooth) roll flour fractions

laboratory homogenizer (Polytron PT3100, Kinematica, Lucerne, Switzerland) and purification was done by the same method as described previously¹⁶. HPLC-UV analysis was performed using a reverse-phase C18 column, Shodex, C18M 4E (Showa Denko, Tokyo, Japan), 250 mm × 4.6 mm (i.d.) with 5 μm particle size; The composition of the mobile phase was acetonitrile:methanol:water (5:5:90 v/v/v) and the flow rate was 0.90 mL/min. The sample was diluted or concentrated if the NIV concentration was outside of the range of the calibration curve. Standard NIV solution (10 μg/mL) was prepared in acetonitrile, evaporated under gentle nitrogen flow at 40 °C and re-dissolved to the desired concentration in acetonitrile:methanol:water (1:1:18 v/v/v) solution. Analysis was done in triplicate (n = 3, replicate from the weighing).

Statistical analysis Average values of triplicate determinations are expressed. An analysis of variance using grain or milled sample as a variable was performed using SPSS software (SPSS, Chicago, IL). Tukey's multiple range tests were performed to test for significant differences for each variable.

Results and Discussion

The average NIV levels of uncleaned samples were 5.4 mg/kg (Sample A) and 9.5 mg/kg (Sample B), while those of cleaned samples were 2.2 mg/kg (Sample A, Table 1) and 4.3 mg/kg (Sample B, Table 2). Therefore, sorting was effective to reduce NIV levels by half. NIV contents in the cleaned grain and milled samples from two different levels of contamination of Chikugoizumi wheat are shown in Tables 1 and 2. NIV recovery (%) for Sample A and Sample B were 115 % and 119 % respectively, both being reasonable values as mass balance. The milling properties were similar except for higher weight (%) observed in shorts of Sample B.

Sample A and Sample B showed similar patterns of NIV distribution in milling fractions (Tables 1 and 2, capital letters). Among the milled fractions, bran contained the maximum NIV concentration, followed by shorts, and flours, regardless of the contamination level. These are in agreement with previous findings

Table 1. Milling properties and distribution of NIV in Sample A

Grain or milled samples	Wt		Concentration (mg/kg) ^{a, b}	NIV mass balance ^c	Relative Concentration (%)
	g	%			
Cleaned grain	5,000		2.2 A ± 0.1	11,200	100
Patent flour	2,880	60.0	0.8 B ± 0.0	2,390	37
Low-grade flour	248	5.2	0.8 B ± 0.0	203	37
Bran	1,359	28.3	6.8 C ± 0.2	9,173	301
Shorts	314	6.5	3.5 D ± 0.2	1,112	158
Total Milled	4,802	100		12,878	

^a Values are mean ± standard deviation (n = 3).

^b Among sample types, values with different capital letters are significantly different by Tukey's multiple range test (p < 0.05).

^c Mass balance: [Weight (g) x Concentration (mg/kg)].

Table 2. Milling properties and distribution of NIV in Sample B

Grain or milled samples	Wt		Concentration (mg/kg) ^{a, b}	NIV mass balance ^c	Relative Concentration (%)
	g	%			
Cleaned grain	1,738		4.3 A ± 0.2	7,508	100
Patent flour	895	56.5	2.6 B ± 0.2	2,354	61
Low-grade flour	66	4.2	2.6 B ± 0.1	171	60
Bran	434	27.4	11.4 C ± 0.7	4,948	264
Shorts	188	11.9	7.9 D ± 0.2	1,489	183
Total Milled	1,583	100		8,961	

^a Values are mean ± standard deviation (n = 3).

^b Among sample types, values with different capital letters are significantly different by Tukey's multiple range test (p < 0.05).

^c Mass balance: [Weight (g) x Concentration (mg/kg)].

reported by Lee et al.¹¹⁾. The relative concentrations of NIV in patent flour of Sample A and Sample B were 37% and 61%, respectively, indicating lower retention of NIV in patent flour of Sample A compared to Sample B. The penetration of NIV toxin into the endosperm seemed to be higher according to the increase of the contamination level in cleaned grains.

Our previous studies showed that the reduction of DON after milling was not achieved for medium (ca. 1 mg/kg) and high (ca. 5 mg/kg) level DON contaminated cleaned grain of the same cultivar, because similar levels of DON among flour fractions and cleaned grain were observed^{16, 17)}. In the present study we found that the reduction in NIV concentration was achieved by milling with significant differences between the concentrations in cleaned grain and flours (Tables 1 and 2, capital letters). We considered that the difference between the previous study and the present study might be derived from the differences between the milling conditions in the two studies (screens for the middling rolls; 10XX, 10XX and 11XX (previous study), 8XX, 8XX and 9 XX (this study, Fig. 2)), and the differences between the NIV concentrations in whole grains. The grain samples were also different in harvest times. The samples used in the previous were harvested in June, 2008, while those in the present study were harvested in June, 2006. The differences of the toxin concentration in patent flour between previous and present study could be due to the timing of fungal infection (pre-, mid-, or post-anthesis).

The milling condition in the present study seemed to be effective for the removal of NIV from severely

Fusarium-infected grains. Comparison of the effect of milling conditions on the removal of NIV will be required. The effects of primary processing on the content of NIV, highly contaminated in grains of a Japanese wheat cultivar, were examined for the first time in this study. Further investigation with more grain samples and analysis of fungal biomass will clarify the retention properties of NIV and fungus inside of grains.

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