Effect of Cooking Process on the Deoxynivalenol Content and Its Subsequent Cytotoxicity in Wheat Products

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The retention of deoxynivalenol in noodles and bread made from naturally-contaminated flour was examined by a chemical analysis (HPLC) and bioassays. The retention level of deoxynivalenol obtained from both assays was reduced by boiling process, although only the bioassays showed it to have been reduced by baking. This study is the first to estimate the exposure to deoxynivalenol from the consumption of the final products of wheat flour in Japan.

Key words: bread; cytotoxicity; deoxynivalenol; high-performance liquid chromatography; noodle

Deoxynivalenol (DON), a kind of trichothecene mycotoxin, is a frequent contaminant of cereal crops worldwide.1) DON and other trichothecene mycotoxins affect animal and human health. The effects include feed refusal, a decrease in body weight, immunomodulation such as the enhancement of IgA production and reduction of host resistance against infection.2,3) Thus, an effective treatment for eliminating DON from food products is essential to minimize exposing humans to the toxin. In terms of the treatment methods, heating is known to have little effect in reducing the DON content due to its thermal stability, as has been observed in baked bread and cookies.4–6) In contrast, boiling could be expected to be an effective means of reducing the DON level because the toxin is water-soluble.

However, it is doubtful that this reduction in DON level would result in equally reduced harmful effects to health because food processing methods such as heating and boiling can themselves produce new toxic compounds. It has recently been reported that new DON and fumonisins compounds were found in food products.7,8) These compounds were mycotoxin-food matrix complexes generated by chemical and biological reactions during food processing. Howard et al.9) have found new compounds generated from fumonisins in the final corn product and examined their toxicity toward experimental animals. Yumbe-Guevara et al.8) have found a heat-induced derivative of DON in roasted barley. This derivative showed strong cross-reactivity to an antibody against acetylated DONs, but could not be detected by GC–MS.

As these reports indicate, cooking processes have the possibility of generating new compounds which are structurally different to the native compounds, but whose toxicity is unknown. However, such common analytical methods as HPLC, GC–MS, TLC and ELISA could not detect these compounds. To evaluate the potential health damage from food products containing mycotoxins, it is necessary to conduct assays by chemical and biological methods.

In this study, we measured the residual DON level in food products by chemical and biological methods. We chose noodles and bread as food products made from wheat because these are the major wheat products consumed in Japan.

The flour used for cooking the noodles and bread in this study was milled from naturally contaminated domestic wheat harvested in 2001, in which the DON concentration was 0.71 mg/kg for bread and 0.86 mg/kg.
for noodles. DON-free flour for blanks was a commercial product (8.5%, protein content, Nissin Food Products Co., Osaka, Japan) that was a mixture of Australian-standard wheat and Japanese domestic wheat. This flour contained less than 0.1 mg/kg of DON, the detection limit for an HPLC analysis.

Bread was prepared by mixing the following ingredients consisting of DON-contaminated flour (Haruyo-koi, 13.0%, protein concentration, 14% moisture basis) or blank flour, water (196 ml), sugar (14.0 g), salt (5.6 g), yeast (2.8 g), non-fat dried milk solids (5.6 g), and shortening (14.0 g). The baking procedure involved mixing the dough to an optimum stage in a mixer, resting for 20 min, kneading and leavening, resting for a further 108 min, repeating this step twice, and then baking at 160 °C for 35 min. The Japanese-style noodle dough commonly used in food such as “udon” was prepared by mixing the DON-contaminated flour (Hokushin, 8.0–9.0% protein concentration) or blank flour, sodium chloride (4% by weight of flour) and water (45% by weight of flour). The dough was then trimmed with a noodle-making machine for home use (UD-10 model, Izumiseiki Seisakusho Co., Nagano, Japan) and stored at −30 °C prior to use. The boiling treatment involved each noodle equivalent to 50 g of the flour being boiled for 10 min in 1 liter of tap water. The noodles were dried at 40 °C for 3 h to remove any remaining water and the boiling water solid, and were then freeze-dried. The lyophilized samples were stored at −30 °C until needed for the DON analysis.

The bread, noodle dough, boiled noodles and boiling water solids were ground for 5 min with a Waring blender in 200 ml of an extraction solution (acetonitrile: water, 85:15). The naturally contaminated and blank flour samples were extracted with the same solution by shaking at 250–300 rpm for 30 min at room temperature. Each extract was cleaned by passing through a multifunctional column (Autoprep® MF-T 1500, Showa Denko., Tokyo, Japan), as described by Truckess et al.10 before being subjected to the HPLC analysis or bioassays.

HPLC analysis was performed in an Inertsil® ODS-3 column (250 mm × 4.6 mm i.d., 5 μm; GL Sciences., Tokyo, Japan) held at 40 °C with a mobile phase of acetonitrile:methanol:water (5:5:90). The sample injection volume was 20 μl. DON was detected at 220 nm with a UV detector. The bioassays were carried out by using Swiss mouse 3T3 fibroblasts (ICRB9019; Health Science Research Resources Bank, Osaka, Japan). Cytotoxicity was determined with the BrdU and WST-8 bioassays. The BrdU assay assesses DNA synthesis, while the WST-8 assay assesses metabolic activity. The cells were cultured in DMEM containing 10% FCS, 4 mmol l-1-glutamine and antibiotics, and maintained in a humidified incubator at 37 °C under a 5% CO₂ atmosphere. The cells were seeded in each well of a 96-well plate (Corning, NY, USA) at a density of 5 × 10⁴ cells/well. The culture medium contained a DON standard solution and was replaced with a fresh medium (100 μl/well) after 24 h of culture, before being incubated for another 24 h. To obtain a standard curve, a pure DON solution (Sigma Chemical Co., St. Louis, MO, USA) or the DON-free flour extract spiked with DON was serially 2-fold diluted, and 100-μl aliquots of each dilution were added to the cell culture in triplicate. The samples for the assay were diluted, and 100-μl aliquots of each sample added to the cell culture in triplicate, resulting in a final sample concentration of 250 mg/ml. As a negative control, the DON-free noodle extract (DON-free extract) was diluted to 250 mg/ml. A BrdU ELISA cell proliferation kit (Roche Molecular Biochemicals, Basel, Switzerland) was used for the BrdU assay according to the manufacturer’s instructions. A cell counting kit (CCK-8; Dojindo Laboratories, Kumamoto, Japan) were used for the WST-8 assay containing WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophyl)5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt), a water-soluble tetrazolium salt which is reduced by the cellular dehydrogenase activity of viable cells to produce a yellow formazan dye. The cell density value in the BrdU assay was 666 ± 50 x 10⁴ cells/well. After 24 h of incubation, the sample and DON standard media changed to a fresh medium with the CCK-8 solution, before incubating for 1 h at 37 °C. The absorbance of each well was measured at 450 nm, with a reference wavelength of 620 nm, by using an automatic microplate reader (Spectra Max 340; Molecular Device, Sunnyvale, CA, USA). The results from the chemical and cytotoxicity assays were subjected to on statistical analysis (one-way analysis of variance (ANOVA)).

Widestrand et al.11 have developed a cell culture technique for screening a low concentration of Fusarium mycotoxins, including DON, by using Swiss 3T3 mouse fibroblasts. They found that a DNA synthesis assay (BrdU assay) was more sensitive for detecting the toxic effect of these toxins than the metabolic activity assay based on the ability of mitochondrial dehydrogenase (MTT assay) and the damage assay (LDH assay). They have recently applied the bioassay to detect Fusarium trichothecenes in real cereal samples and showed that the IC₅₀ value in the BrdU assay was 666 μg/kg of DON in a wheat extract.12 In the present study, a metabolic activity assay (WST-8 assay) was found to have the same sensitivity as the BrdU assay for detecting DON (data not shown), so we evaluated the cytotoxicity at each stage of cooking noodles and baking bread by using the BrdU assay and WST-8 assay to assess whether or not the cooking process generated new toxic compounds from DON. The standard curves obtained from pure DON and the DON-free extract spiked with DON showed a dose–dependent response in both bioassays (data not shown). There was no significant difference between these standard curves, indicating that the flour matrix did not affect the cytotoxicity of DON (data not shown). The IC₅₀ values from these assays with the DON-free flour extract spiked with DON were 623 μg/
kg of DON for the BrdU assay and 555 µg/kg of DON for the WST-8 assay. These results show that both bioassays of the flour extract had similar sensitivity to the BrdU assay of the wheat extract reported by Widerstrand et al.\textsuperscript{12} In addition, since the bioassay could detect the cytotoxicity of various trichothecene mycotoxins, it would be applicable to the detection of possible new toxic compounds derived from DON during the cooking process. In contrast, such chemical analyses as GC–MS and HPLC would have been unable to detect such new compounds because their structures were unknown. Therefore, the bioassays provided a unique and effective means of detecting possible new toxic compounds produced during food processing.

Table 1 shows the concentration of DON and the cytotoxicity at each stage of the noodle cooking process assessed by the HPLC analysis and the bioassays. The DON concentration corresponding to the cytotoxicity of a sample was calculated from a standard curve that had been established by using 3T3 cells exposed to the flour extract spiked with DON. The raw flour was contaminated with 0.86 ± 0.03 mg/kg of DON, and a similar concentration was found in the dough after conducting three assays. After boiling, the DON concentration in the noodles was 0.26 ± 0.04 mg/kg, and the DON retention in this boiled product was calculated to be at 30.52 ± 4.08% based on the HPLC results. In respect of the cytotoxicity, the WST-8 assay showed the DON concentration to be 0.30 ± 0.01 mg/kg and the retention ratio to be 34.53 ± 1.29%. The BrdU assay showed a 0.25 ± 0.04 mg/kg DON concentration and a 28.88 ± 5.02% DON retention ratio. There was no significant difference in DON concentration between these three assays. The DON concentration in boiling water, assayed by HPLC was 0.37 ± 0.04 mg/kg, which corresponds to 42.89 ± 4.58% of the raw DON concentration. The DON retention corresponding to the cytotoxicity assessed by the BrdU assay was similar to that by HPLC. However, the DON retention corresponding to the cytotoxicity assessed by the WST-8 assay was higher than that obtained from either the HPLC analysis or the BrdU assay. These results demonstrate that boiling reduced both the DON concentration and its cytotoxicity, and that the residue of DON leached into the boiling water. Although it is possible that a new compound having metabolic cytotoxicity was generated in the boiling water solids this seems not to be a serious problem for health because the boiling water is commonly discarded.

Nowicki et al.\textsuperscript{13} have also investigated the effects of processing and cooking on the DON level in noodles prepared from naturally contaminated Canadian Western Red Spring wheat flour. Although the protein concentration was unknown, they have shown that the reduction in level of DON by the chemical analytical method was 40% in Japanese-style noodles after boiling. The difference in reduction between their study and ours probably resulted from the kind of flour, protein concentration or other factors regarding the matrix structure.

Table 2 shows the concentration of DON and the cytotoxicity of raw flour and bread assessed by the bioassays and the HPLC analysis. The DON level in raw flour ranged from 0.69–0.78 mg/kg in the three assays. After making bread, the DON level assayed by HPLC was 0.77 ± 0.06 mg/kg and the retention ratio was 108.42 ± 8.45%. However, the DON levels corresponding to the cytotoxicity obtained from the WST-8 assay and the BrdU assay were 0.58 ± 0.03 mg/kg (84.05 ± 4.34%) and 0.72 ± 0.08 mg/kg (92.30 ± 1.03%), respectively. These results suggest that the DON level in bread was not reduced as a chemical compound but that...
rather the biological toxicity was significantly reduced. This fact indicates the possibility that a new complex produced in bread during cooking, such as a DON-binding protein or DON-binding carbohydrate had less cytotoxicity than DON itself. Many studies on the reduction of DON in the baking process have demonstrated that baking could not reduce the level of DON, and the DON level was simply measured by chemical analytical methods.\textsuperscript{14–16}) The present study is the first report to show the possibility that the baking process reduces the cytotoxicity of DON.

To estimate the risk of exposure to DON in Japan, we calculated the total retention level of DON based on the consumption of wheat products in Japan. A national nutritional survey has shown that 50% of wheat was consumed as bread and other 50% as noodles in Japan.\textsuperscript{17}) When the DON retention level in each final product was assayed by a chemical or biological analysis, its exposure level from the final wheat products consumed in Japan was calculated as 69.5% by the chemical analysis, 59.3% by the WST-8 assay and 60.6% by the BrdU assay of the DON level in raw flour (Fig. 1).

In conclusion, this study is the first to report the DON retention level at each cooking stage in noodles and bread determined by a chemical analysis and bioassays. The experiments with naturally contaminated wheat flour revealed that boiling significantly reduced not only the DON concentration, but also its cytotoxicity. In contrast, baking maintained the DON concentration at the same level as that of raw flour although reducing its cytotoxicity. The risk of exposure to DON was estimated 69.5% or less of the DON level in raw flour if people eat noodles and bread half and half as the final wheat products, which reflects the current consumption of final wheat products in Japan.

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