カビ毒配糖体(マスクドマイコトキシン)に関する研究

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Research on mycotoxin glucosides (masked mycotoxins)

Hiroyuki Nakagawa[1]

[1]National Agriculture and Food Research Organization (NARO), National Food Research Institute (NFRI), 2-1-12 Kannon-dai, Tsukuba, Ibaraki 305-8642, Japan

Keywords
Fusarium; glucoside; LC-MS; masked mycotoxin; trichothecene

Correspondence
Hiroyuki Nakagawa, 2-1-12 Kannon-dai, Tsukuba, Ibaraki 305-8642, Japan
E-mail: hironkgw@affrc.go.jp

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Abstract

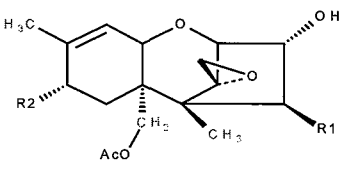
For the detection of new glucoside derivatives of mycotooxins (masked mycotoxins), screening based on the accurate mass was carried out through the high resolution mass spectrometry measurement with LC-MS (LC-Orbitrap MS). Consequently, mono-glucoside derivatives of type B trichothecenes (nivalenol and fusarenon-X) in wheat grains infected with *Fusarium* strains and those of the type A trichothecenes (T-2 toxin, HT-2 toxin, neosolaniol, diacetoxyscirpenol, and monoacetoxyscirpenol) in corn powder reference material were detected, respectively. Di-glucoside derivatives were also found for T-2 toxin and HT-2 toxin. These findings indicate that the presence of masked mycotoxins is not limited to some specific mycotoxins such as deoxynivalenol and zearalenone, but likely with the other *Fusarium* mycotoxins.

Fusarium fungi are plant pathogens infecting cereals such as wheat, barley, and corn, and some of them produce mycotoxins, such as trichothecenes (Fig. 1). and zearalenone (ZEN, Fig. 2). In Japan, the infection of grain by *Fusarium* fungi is occasionally serious, since those crops are usually planted during the rainy season. Among the *Fusarium* mycotoxins, deoxynivalenol (DON, Fig. 1), which belongs to the type B trichothecenes, is considered the most important[1] and many countries have set regulatory limits for DON in commodities and foods[2].

Recently, the existence of DON-3-glucoside (DON3G) (Fig. 3) in cereal grain and beer was reported [3),4]. Similar glucoside derivatives for other *Fusarium* mycotoxins including ZEN-14-glucoside (ZENG) (Fig. 3) were also found in plant[5),6]. Since these derivatives are usually not detected by the conventional analytical methods, they are called "masked mycotoxins"[7),8)]. The existence of ZEN-14-sulphate (ZENS) was reported in another study[9)], and also regarded as a masked mycotoxin. Hydrolysis of masked mycotoxins to their original aglycons has also been reported[7),8),9),10)], therefore it is suggested that they are suggested to present an additional, potential risks of mycotoxins.

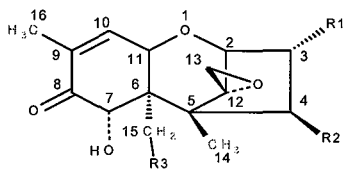
Thus far, reports on the natural contamination of masked mycotoxin were mainly limited to those of DONG and ZENG (Fig. 3). However, it seemed to be very likely that glucoside derivatives of other mycotoxins also should exist considering that *Fusarium* fungi produce several different type of mycotoxins (Fig. 1, 2).

Type A trichothecenes



	R_1	R_2
T-2 toxin (T2)	OAc	$COOCH_2CH(CH_3)_2$
HT-2 toxin (HT2)	OH	$COOCH_2CH(CH_3)_2$
Diacetoxyscirpenol (DAS)	OAc	H
Monoacetoxyscirpenol (MAS)	OH	H
Neosolaniol (NES)	OAc	OH

Type B trichothecenes



	R_1	R_2	R_3
Nivalenol (NIV)	OH	OH	OH
Deoxynivalenol (DON)	OH	H	OH
Fusarenon-X (FUX)	OH	OAc	OH
3-Acetyl deoxynivalenol	OAc	H	OH
15-Acetyl deoxynivalenol	OH	H	OAc

Fig. 1 Trichothecene mycotoxins

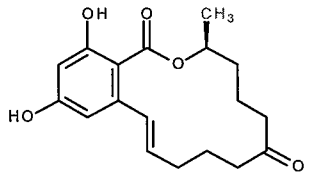


Fig. 2 Zearalenone (ZEN)

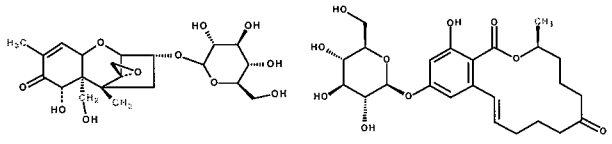


Fig. 3 Typical masked mycotoxins

From these backgrounds, the author's group started screening for new glucoside derivatives of mycotoxins (masked mycotoxins) focusing on the *Fusarium* mycotoxins.

Meanwhile, LC-MS is often the method of choice for the quantitative analysis of food contaminants, such as mycotoxins and pesticides. For instance, LC-MS/MS with a triple quadrupole mass spectrometer can provide very specific and selective detection, and is useful for the targeted screening of discrete sets of analytes when used in the selected reaction monitoring (SRM) mode. On the other hand, for the detection of unidentified compounds whose chemical standards are not available, a mass screening using the full scan mode is recommended because SRM is applicable only for the analytes whose chemical standards are available. Through a series of studies conducted by the author and co-workers, detection of new masked mycotoxins was conducted with the high resolution mass spectrometry measurement with an LC-Orbitrap MS instrument (Fig. 4). This machinery is a type of ion trap mass spectrometer that routinely achieves a mass resolving power of up to 100,000 FWHM (full width at half maximum) (m/z 200), and maintains excellent mass accuracy (<5 ppm) even without the use of continuous internal mass correction[11].

For the screening of new masked mycotoxins, cereal samples contaminated with *Fusarium* mycotoxins at higher concentration levels were recommended, since lower concentration of those toxins may result in the failure of detection. From 2008 to 2012, a governmental research project ensuring food safety from farm to table was on going by the administration of the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan, and wheat grain contaminated with DON and NIV was prepared by artificial inoculation with *F. graminearum* in an experimental field in Koshi, Kumamoto, Japan (Fig. 5). Inoculation with *F. graminearum* was performed using 'colonized maize kernel inoculum' as previously described[12]. The infected wheat was harvested at maturity (40 days after anthesis), and the grains were collected[12]. The grains were ground by a mill, Waring Blender Model 7012S with MC3 container (Waring Laboratory, Torrington, CT, USA), and used for the screening.

As another material for the screening of masked mycotoxins, corn powder reference material contaminated with *Fusarium* mycotoxins (batch number MTC-9999D) was purchased from Trilogy Co. Ltd (Washington, MO, USA). The material was certified to be contaminated with T-2 toxin (T2), HT-2 toxin (HT2), and DON, and those manufacturer-labeled concentrations were 300 ± 57 μg/kg (T2), 510 ± 83 μg/kg (HT2) and 2,200 ± 600 μg/kg (DON), respectively.

The obtained cereal powder (wheat or maize, 10 g) contaminated with *Fusarium* mycotoxins, was mixed with acetonitrile/water (80:20, v/v, 40mL) and acetic acid (0.4 mL), and homogenized with a POLYTRON PT3100 homogenizer (Kinematica AG., Lucerne, Switzerland), and centrifuged at 2,000 x *g* for 10 min. A portion of the supernatant (7 mL) was loaded onto a Bond Elut Mycotoxin column (Agilent Technologies, Santa Clara, CA, USA). After the initial eluent (3 mL) was discarded, successive eluent (2 mL) was collected. From the collected eluent, 1.5 mL was taken into a glass tube, and the solvent was evaporated under the nitrogen gas stream at 40°C. The residue was dissolved in 0.3 mL of acetonitrile/water/acetic acid (5:94:1, v/v/v) and subjected to the LC-MS analysis. Detection and identification of new masked mycotoxins were performed with a high resolution LC-MS (LC-Orbitrap MS) "Exactive" (ThermoFisher Scientific, Bremen, Germany) (Fig. 4). Chromatographic separation was performed on a HyPurity C_{18} column (250 x 3 mm i.d., 5 μm particle size) at 40°C. The carrier solvent was composed of water/acetic acid (99.9:0.1, v/v) containing 0.5 mM ammonium acetate (eluent A) and acetonitrile/acetic acid (99.9:0.1, v/v) (eluent B). Elution was conducted at the flow rate of 0.3 ml/min with a linear gradient of acetonitrile. MS parameters for the ionization with a heated electrospray interface (HESI-II) were optimized as reported previously[13),14),15),16]. The system was operated in the full spectral acquisition mode in the mass range of 70-1,000 m/z at the ultra high resolving power of 100,000 FWHM (200m/z), and accurate mass/high resolution (AM/HR) full scan (Scan event 1) and all ion MS/MS spectrum acquisition with collision energy (Scan event 2) were simultaneously performed in a single run. Fragmentation was achieved by an optional equipment HCD (Higher Collision Dissociation) cell, with appropriate collision energy (Scan event 2). Each scan event was performed in either negative or positive polarity, depending on the target compound. The external mass axis calibration without the use of the specific lock mass was employed. For the mass accuracy estimation, the mass at the apex of the chromatographic peak obtained as the extracted ion

Fig. 4 LC-Orbitrap MS instrument

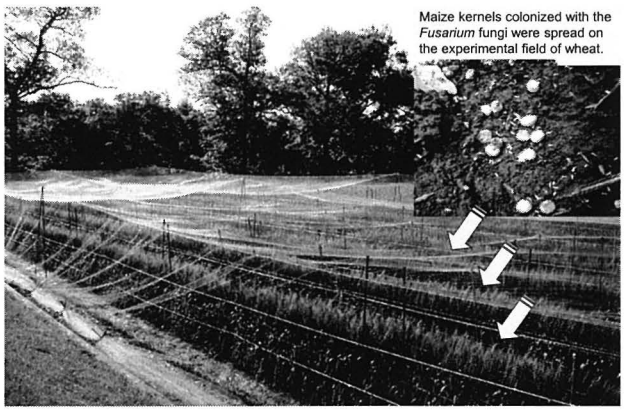


Fig. 5 Experimental field used for the inoculation with *F. graminearum* on wheat

chromatogram was used. According to European Commission guideline (European Commission 2009)[17], mass accuracy <5ppm is regarded as the criteria for compound identification. Therefore, mass deviation <5ppm from the calculated value was used as the criteria for the identification.

Through the screening with the high-resolution LC-MS (LC-Orbitrap MS) instrument, a new *Fusarium* mycotoxin glucoside, fusarenon X-glucoside (FUXG), was found in wheat grain that was artificially infected with *Fusarium* fungi (Fig. 6)[13]. Another mycotoxin glucoside, nivalenol-glucoside (NIVG) (Fig. 6) was also found in the same wheat grain sample[13]. Although the absolute structures of FUXG and NIVG were not clarified by LC-MS, 3-OH glucosylation seems to be the most probable based on the fragment profile while referring to the fact that DON3G reported as the predominant glucoside derivative of DON was simultaneously detected in the identical sample[13]. On the other hand, through the screening for masked mycotoxins derived from type A trichothecenes, mono-glucoside derivatives of (T2, HT2, neosolaniol (NES), diacetoxyscirpenol (DAS), and monoacetoxyscirpenol (MAS)) (Fig. 6) were detected in the corn powder reference material[14),15),16]. In addition, di-glucoside derivatives of T2 and HT2 (Fig. 7) were also found in the identical material[15]. In the case of T2G, the structure was fixed as T2-3-glucoside because there was only one OH residue in T2 molecule. Concerned with HT2G, HT2-3-glucoside may also be the most probable structural configuration considering that DON3G was also detected in the identical maize powder[14]. It was reported that a UDP-glucosyltransferease was responsible for the detoxification of DON, and that glucosylation occurs at C-3 position[18]. Similar enzyme(s) may exist in maize that were involved in the formation of T2G and HT2G. The proposed structures of masked mycotoxins found in the authors' study is shown in Fig. 6 and Fig. 7.

Another question concerns the amounts of these masked mycotoxins. Due to the unavailability of appropriate chemical standards, it was only possible to estimate the amounts of these compounds by extrapolation. If the molar ratios of the glucosylated/parent compounds were the same as that for DON3G/DON, then the content of NIVG and FUXG in the wheat grain was calculated as 178 and 17 μg/kg, respectively[13]. In the same manner, it is estimated that approximately 24 μg/kg of T2G and 41 μg/kg of HT2G were produced in the maize powder[14]. These findings indicate that the presence of masked mycotoxins is not limited to some specific mycotoxins such as DON and ZEN, but likely with a variety of *Fusarium* mycotoxins.

Zachariasova *et al.* (2012) reported the existence of di-glucosides, tri-glucosides, and more highly glucosylated forms of DON[19]. Based on the structural similarities to DON, such oligo-glucosides could also be found for other trichothecenes. This suggests the possibility that a kind of "masked mycotoxin cluster" (composed of mycotoxin mono-, di-, tri-, and poly-glucosides) can be produced in plants. These considerations bring us to recognize that the existence of masked mycotoxins as a potential risk is not negligible, since they could be transformed to the corresponding aglycons under certain conditions, for example during various food processing operations[20], and/or in the digestive tract of swine and rat after ingestion[7),10]. The availability of analytical standard (such as DON3G) is essential for the other masked mycotoxins to promote those researches related to them.

The authors' findings are unique and clarified that the glucosylated derivatives (masked mycotoxins) are formed not only for the type B trichothecenes, but also for the type A trichothecenes. Among the type A trichothecenes, T2 and HT2 are the most focused due to their higher prevalence in crops, and the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM) established a group of tolerable daily intake (TDI) of 100 $ngkg^{-1}$ body weight for the sum of T2 and HT2[21]. Although the existence of masked mycotoxins is not counted yet with the risk

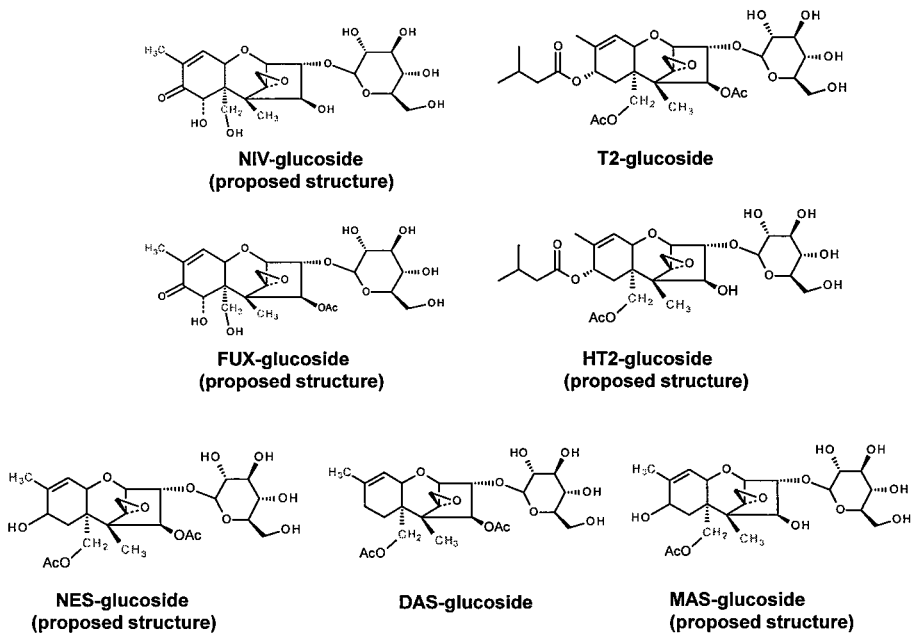


Fig. 6 Masked mycotoxins (mono-glucoside derivatives) found in this work

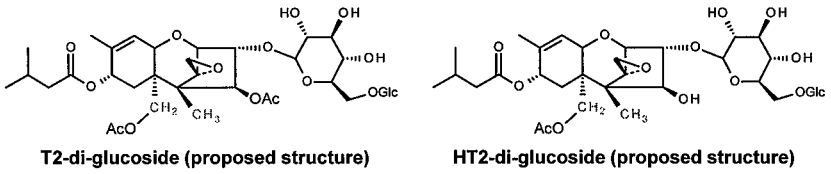


Fig. 7 Masked mycotoxins (di-glucoside derivatives) found in this work

evaluation, more analytical and toxicological studies on them are needed to determine the prevalence in foods and the relevance of the masked forms for human health.

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カビ毒配糖体（マスクドマイコトキシン）に関する研究

中川 博之[1]

[1]（国）農業・食品産業技術総合研究機構（〒305-8642　茨城県つくば市観音台2-1-12）

新規カビ毒配糖体（マスクドマイコトキシン）の検出を目的として，高分解能LC-MS（LC-Orbitrap MS）による精密質量を指標としたスクリーニングを行った．その結果，フザリウム菌感染小麦玄麦試料においてタイプBトリコテセン（ニバレノール，フザレノン-X）由来のモノグルコシド，およびトウモロコシ粉末認証標準物質試料においてタイプAトリコテセン（T-2トキシン，HT-2トキシン，ネオソラニオール，ジアセトキシスシルペノール，モノアセトキシスシルペノール）由来のモノグルコシド体がそれぞれ検出された．T-2トキシン，HT-2トキシンに関してはジグルコシド体も検出された．これらの知見から，マスクドマイコトキシンがデオキシニバレノールやゼアラレノンのような特定のマイコトキシンのみではなく，他のフザリウムトキシンについても存在することが示された．

キーワード：トリコテセン；配糖体；フザリウム；マスクドマイコトキシン；LC-MS