Note

Formation Ratios of Zearalanone, Zearalenols, and Zearalanols *versus* Zearalenone during Incubation of *Fusarium semitectum* on Sorghum and Ratios in Naturally Contaminated Sorghum

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We incubated *Fusarium semitectum* on sorghum and measured the production of zearalenone (ZEN) and ZEN-related compounds (zearalanone (ZAN), α -zearalenol (α -ZEL), β -zearalenol (β -ZEL), α -zearalanol (α -ZAL) and β -zearalanol (β -ZAL)) in the culture by LC-MS. Of the five ZEN-related compounds, ZAN and β -ZEL were mainly detected. The concentrations of ZEN and the five ZEN-related compounds increased until 9 days after incubation and then increased slightly or stayed constant between days 9 and 15. The ratios of α -ZEL, β -ZEL, α -ZAL and β -ZAL to ZEN decreased in a similar manner after 7 days, whereas the ratio of ZAN to ZEN remained constant after 5 days. Analysis of naturally contaminated sorghum by LC-MS/MS revealed that the production ratio of α -ZEL to ZEN was inconsistent with that of our *in vitro* incubation analysis. The results indicate that ZAN might not be suitable for use as an internal standard.

(Received May 21, 2015)

Key words: *Fusarium semitectum*; sorghum; zearalanol; zearalanone; zearalenol; zearalenone

Introduction

Zearalenone (ZEN), a mycotoxin produced by *Fusarium* fungi, contaminates maize, barley, oat, sorghum (milo), and other cereal grains worldwide¹⁻³⁾. Although ZEN has relatively low acute toxicity, it can exert estrogenic effects on the reproductive system, particularly in swine¹⁾. Several compounds related to ZEN have been identified: zearalanone (ZAN), α -zearalenol (α -ZEL), β -zearalenol (β -ZEL), α -zearalanol (α -ZAL, zeralanol) and β -zearalanol (β -ZAL, taleranol)³⁾ (Fig. 1). These ZEN-related compounds (ZRCs), except for β -ZEL, have stronger estrogenic activity than ZEN⁴⁻⁶⁾. In particular, the estrogenicity of α -ZEL is 92 times greater than that of ZEN, as estimated by an improved MCF7 human breast cancer cell proliferation assay⁶⁾.

The above-mentioned five ZRCs have been detected in urine, feces, and tissues^{3,7)}, as well as in processed foods^{8,9)}. In beer, β -ZEL, α -ZEL and ZAN are formed from ZEN by the *Saccharomyces cerevisiae* strains used in brewing¹⁰⁾. ZEL and ZAL were also detected in *Fusarium* cultures^{4,11,12)}. Therefore, these compounds are considered natural contaminants in grains; however, they commonly occur at lower concentrations than

ZEN^{11,13-17)}. JECFA set a provisional maximum tolerable daily intake of ZEN at 0.5 μ g/kg of body weight per day and recommends that the total intake of ZEN and its metabolites should not exceed this limit¹⁸⁾. However, little is known about the production of the 5 ZRCs during incubation of *Fusarium* or about their relative concentrations in grains.

	Abbreviation	C ₁ -C ₂	R	Relative estrogenicity
Zearalenone	ZEN	C=C	=O	1
α -Zearalenol	α -ZEL	C=C	····OH	92
β -Zearalenol	β -ZEL	C=C	◀OH	0.44
Zearalanone	ZAN	C-C	=O	2.5
α -Zearalanol	α -ZAL	C-C	····OH	18
β -Zearalanol	β -ZAL	C-C	◀OH	3.5

Fig. 1. Chemical structures and relative estrogenicity values of ZEN and the 5 ZRCs

The relative estrogenicity of each compound was reported by Shier *et al.*⁶⁾

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Sorghum is produced in areas where maize is not suitable for cultivation due to the hot or dry climate¹⁹⁾. Sorghum is one of the most important grains in the world, along with maize, rice, wheat and barley¹⁹⁾. Next to maize, sorghum is the main feed ingredient in Japan. We previously reported on ZEN contamination of sorghum²⁰⁾, and demonstrated that sorghum is a significant source of ZEN contamination, of which the primary causative fungus is *F. semitectum*. In this study, we examined the production of the 5 ZRCs during incubation of *F. semitectum* on sorghum and also measured their levels in naturally contaminated sorghums. This study is first report on *in vitro* production of ZRCs by *F. semitectum*. Although ZAN has been widely used as an internal standard, our results indicate that a different ZEN-related compound might be superior.

Materials and Methods

Standards and reagents

The standards of ZEN and the 5 ZRCs were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared at a concentration of 100 µg/mL (β -ZEL) or 50 µg/mL (the others) in acetonitrile and stored at -20°C . The solvents used for LC-MS and LC-MS/MS were of LC grade. Ammonium acetate (1 mol/L) solution was used for LC, and was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents used were of analytical grade.

Fungal strain studied

F. semitectum was isolated from sorghum as previously described²⁰⁾. Briefly, ground sorghum was homogenized in 0.1% peptone–water. The diluted solution was placed on a dichloran rose bengal chloramphenicol agar (Oxoid, Cambridge, UK) plate, and incubated at 25°C for 5 to 7 days. The *F. semitectum* isolates were purified by subculturing on potato-dextrose agar slants and carnation leaf agar, and were identified based on macroscopic and microscopic characteristics.

Determination of mycotoxin concentration in culture

Unground sorghum and rice were obtained commercially, and used as artificial inoculation substrates. For production of ZEN and the 5 ZRCs, *F. semitectum* was inoculated on 30 g of sterilized substrate and incubated at 25°C for 14 days. To monitor the time courses of production of ZEN and the 5 ZRCs, the fungi inoculated on the sorghum substrate were incubated at 25°C for 3, 5, 7, 9, 11, 13 and 15 days. The time course analysis was conducted in triplicate for days 3 to 11 and in quintuplicate for days 13 and 15.

ZEN and the 5 ZRCs were extracted and purified according to the ZEN production assay for *Fusarium* species as previously described²⁰⁾. In summary, ZEN and the 5 ZRCs were extracted by blending 25 g of the culture material with 100 mL of acetonitrile–water (21 : 4) for 5 min. The extract was loaded onto a multifunctional clean-up column (MultiSep 226 AflaZon+, Romer Labs, Union, MO, USA). The first 4 mL of eluate was discarded,

and 2 mL of the subsequent 3 mL was evaporated to dryness. The residue was redissolved in 1 mL of acetonitrile–water (1 : 1). The concentrations of ZEN and the 5 ZRCs in the purified solution were determined by LC-MS with an atmospheric pressure chemical ionization (APCI) interface according to the conditions of our previous report²⁰⁾ with the addition of monitoring of 5 ZRC ions (m/z 319 for ZAN and α - or β -ZEL, and 321 for α - or β -ZAL). Detection of ZEN or the 5 ZRCs by LC-MS was confirmed by LC-MS/MS with an electrospray ionization (ESI) interface. The LC-MS/MS conditions used were as previously described²⁰⁾. However, the following conditions were added in the present study: the collision energy for the 5 ZRCs was set to 20 eV, and the precursor and product ions were selected to be m/z 319 and 275, respectively, for ZAN and α - or β -ZEL, and 321 and 277, respectively, for α - or β -ZAL.

Determination of mycotoxin concentrations in naturally contaminated sorghum

Sorghum samples for detection of naturally occurring ZEN and the 5 ZRCs were collected in quantities of about 5 kg at a silo. All five samples were imported, three from Argentina, and the others from the United States. These samples were ground to particles less than 1 mm in diameter using an ultracentrifugal mill (ZM1, Retsch, Haan, Germany) in our laboratory and stored at 5°C prior to analysis.

ZEN and the 5 ZRCs in sorghum were detected using an official method for feed analysis in Japan^{*1} as follows: ZEN and the 5 ZRCs were extracted by shaking 50 g of the sample in a 300 mL flask with 100 mL of acetonitrile–water (21 : 4) for 60 min. The extract was centrifuged at $650\times g$ for 5 min and the supernatant was loaded onto a MultiSep 226 AflaZon+. The initial 4 mL of eluate was discarded, and the following 2 mL was collected. A portion of the collected solution was mixed with the same amount of water–acetic acid (100 : 1). The solution was ultracentrifuged at $5,000\times g$ for 5 min, and the supernatant was analyzed by LC-MS/MS. Some LC-MS/MS conditions were different from those used to determine the concentration in culture. LC separation was performed using an Acquity UPLC system (Waters, Milford, MA, USA), and mass detection was performed using a Xevo TQD triple quadrupole mass spectrometer (Waters) equipped with ESI. A 10 µL aliquot of sample was injected into and separated on a ZORBAX Eclipse XDB-C18 column (150×4.6 mm i.d., 5 µm; Agilent Technologies) at 40°C . The mobile phase consisted of 10 mmol/L aqueous ammonium acetate solution and acetonitrile at a flow rate of 0.2 mL/min. The gradient elution program was set as follows: 0–1 min hold on 10% acetonitrile, 1–20 min linear increase on 10–80% acetonitrile, and 20–35 min hold on 80% acetonitrile. The mass spectrometer was operated in selected reaction monitoring

*1 Notification 14729 (Apr. 1, 2008) Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, Forestry and Fisheries, Japan.

Table 1. LC-MS/MS conditions, LOD and LOQ

Mycotoxin	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)		Cone voltage (V)	Collision energy (eV)		LOD ($\mu\text{g/kg}$)	LOQ ($\mu\text{g/kg}$)
		Target	Qualifier		Target	Qualifier		
ZEN	317	175	131	52	24	32	0.3	1
ZAN	319	205	275	62	26	24	2	4
α - and β -ZEL	319	160	275	62	28	24	3	8
α - and β -ZAL	321	277	303	56	24	24	2	4

Table 2. Production of ZEN and the 5 ZRCs during incubation of *F. semitectum* on sorghum or rice

Substrate	Fungus No.	ZEN (mg/kg)	Ratio relative to ZEN (%)				
			ZAN	α -ZEL	β -ZEL	α -ZAL	β -ZAL
Sorghum ^{a)}	M-4-2	19400	0.80	0.05	0.52	<0.01	0.01
	M-8-1	10600	1.1	0.02	0.11	<0.01	<0.01
	M-8-3	1530	1.6	0.06	0.60	<0.01	0.03
	M-8-4	6850	1.2	0.58	24	0.07	0.59
	M-8-5	2170	1.5	0.04	0.22	<0.01	0.01
	M-158-1	1640	2.2	0.17	2.8	0.01	0.12
	M-158-4	9290	1.2	0.02	0.15	<0.01	0.01
	M-480-3	4560	1.0	0.15	3.2	0.01	0.09
Rice ^{b)}	M-8-1	83.5	2.4	1.9	3.6	0.06	0.22
	M-158-4	124	1.5	0.48	4.1	0.01	0.14

^{a)} One substrate per strain was incubated at 25°C for 14 days.

^{b)} Five substrates per strain were incubated at 25°C for 14 days and the mean value is shown.

(SRM) mode at negative polarity. The capillary voltage was set to 0.6 kV, and the source block and desolvation gas temperatures were set to 150 and 400°C, respectively. The desolvation and cone gas were nitrogen at 800 and 50 L/h, respectively, and the collision gas used was argon. The monitoring ions, cone voltages, and collision energies for ZEN and the 5 ZRCs are shown in Table 1. Stock solutions were mixed to prepare standard solutions for calibration. The mixed solution was diluted in acetonitrile–water (21 : 4) to different concentrations and then mixed with the same amount of water–acetic acid (100 : 1) for LC-MS/MS. The peak signal areas of ZEN and the 5 ZRCs were plotted against those of standard solutions of different concentrations, and the concentration of ZEN or the 5 ZRCs in the sample solution was calculated from the calibration curve. The limit of detection (LOD) and the limit of quantification (LOQ) in the sample are shown in Table 1.

Results and Discussion

The amount of ZEN and the formation ratio of each of the 5 ZRCs to ZEN produced by incubation of *F. semitectum* on sorghum or rice are shown in Table 2. *F. semitectum* is the primary causative fungus of ZEN contamination on sorghum²⁰⁾. Therefore, sorghum was used as the primary substrate, and rice was employed as a reference substrate for comparison. The results revealed that ZAN and β -ZEL were dominant among the ZRCs on both substrates. ZAN is sometimes used as an internal standard for the analysis of ZEN and other mycotoxins, including trichothecene and aflatoxins^{8), 21)–23)}. Our study revealed that ZAN was formed dominantly among the 5 ZRCs,

suggesting that it may be unsuitable as an internal standard. The amount of β -ZEL relative to ZEN produced on the sorghum substrate was the highest among the 5 ZRCs at 24%. The ratio of α -ZAL to ZEN was consistently the lowest of the 5 ZRCs in all strains and substrates, with its highest value being 0.07%. Some strains of *F. semitectum* studied did not produce ZEN or the 5 ZRCs, although previous studies have reported production of the 5 ZRCs upon incubation of *Fusarium* fungi on medium. Bottalico *et al.* examined the production of α - and β -ZEL by *Fusarium* species associated with corn stalk rot¹¹⁾. In that study, incubation of *Fusarium* fungi on potato glucose agar resulted in the production of α - and β -ZEL. Their formation ratios by *F. culmorum* were 0.03–0.65% and 0.02–0.43% relative to ZEN, respectively. *F. equiseti* gave ratios of 3.3–4.8% and 13–29%, respectively. These findings are similar to those observed in our study, although different *Fusarium* fungi were used. Additionally, Richardson *et al.* also investigated the production of α - or β -ZEL and α - or β -ZAL by incubation of *Fusarium* fungi isolated from finished feeds and forage plants¹²⁾. When *F. graminearum* and other fungi were grown on rice culture, α -ZEL, β -ZEL, α -ZAL, and β -ZAL were produced at ratios of 1.3–20%, 0.34–110%, 0–13%, and 0–14% relative to ZEN, respectively. Although β -ZEL was not dominant among the 5 ZRCs in that study, the ratios of the 5 ZRCs to ZEN were similar to those observed in our study. Furthermore, we observed higher levels of α - and β -ZEL than α - and β -ZAL, which is also consistent with their results. In relation to the estrogenic activity of ZEN, the activities of 5 ZRCs cannot be ignore, because α -ZEL and ZAN were pro-

duced in artificial culture and they both possess higher estrogenic activities than ZEN (Fig. 1).

To examine the time courses of production of ZEN and the 5 ZRCs in culture of the M-8-4 *F. semitectum* strain (Table 2), the fungus was grown on sorghum for 3 to 15 days. The concentration changes of ZEN and the 5 ZRCs are shown in Fig. 2. All compounds increased until 9 days after the start of the incubation period, and then further increased slightly or stayed constant from 9 to 15 days. Narita *et al.* examined the production of ZEN, α -ZEL, and β -ZEL during incubation of *F. graminearum* on potato dextrose agar medium²⁴⁾, and reported that the production profiles of α -ZEL and β -ZEL were similar to that of ZEN with three of the four strains tested, but a different production profile was observed with the other strain. Our results also indicated that the production of the 5 ZRCs increased in a manner similar to ZEN.

To examine the production of the 5 ZRCs in more detail, a time course analysis of the production ratios of the 5 ZRCs relative to ZEN was performed (Fig. 3). The ratios of β -ZEL, α -ZAL, and β -ZAL decreased from 3 to 15 days. However, the ratio of α -ZEL reached its maxi-

mum at 7 days, and thereafter decreased similarly. ZAN increased until 5 days, after which its production remained constant. These results suggest that the contribution of the five ZEN-related compounds to the estrogenic activity of ZEN will decrease over time. Although the production of β -ZEL amounted to 24% relative to ZEN (Table 2), the time course analysis did not reveal a similar phenomenon (Fig. 3).

To observe the natural occurrence of the 5 ZRCs in sorghum, we examined the levels of ZEN and the 5 ZRCs in five imported sorghums collected at a silo (Table 3). ZAN and α - and β -ZEL were detected. This result, like the *in vitro* incubation analysis, also brings into question the suitability of using ZAN as an internal standard. The ratios of ZAN and β -ZEL to ZEN, observed in naturally contaminated sorghum, were similar to those observed in culture of *F. semitectum*. However, the ratio of α -ZEL to ZEN in sorghum was larger than that observed in culture. Of the 5 ZRCs, only α -ZEL contamination levels in sorghum have been previously re-

Fig. 2. Time course analysis of the production of ZEN and the 5 ZRCs

The M-8-4 strain of *F. semitectum* was incubated on the sorghum substrate. The data represent the mean of triplicate (3 to 11 days) or quintuplicate (13 and 15 days) values $\pm$ the standard error of the mean.

Fig. 3. Time course of the production ratios of the 5 ZRCs relative to ZEN

The M-8-4 strain of *F. semitectum* was incubated on a sorghum substrate. The scale for β -ZEL production ratio is on the right Y-axis and that of the other ZRCs is shown on the left Y-axis. The data represent the mean of triplicate (3 to 11 days) or quintuplicate (13 and 15 days) values $\pm$ the standard error of the mean.

Table 3. ZEN and the 5 ZRCs in naturally contaminated sorghum

Sorghum No.	ZEN ($\mu\text{g/kg}$)	Ratio relative to ZEN ^{a)} (%)				
		ZAN	α -ZEL	β -ZEL	α -ZAL	β -ZAL
1-128	313	2.7	ND	3.1	ND	ND
2-35	303	1.4	1.7	ND	ND	ND
3-116	178	ND	2.4	2.4	ND	ND
3-170	194	5.5	3.5	4.5	ND	ND
3-253	211	ND	2.5	3.0	ND	ND

^{a)} Concentrations of LOD or more were used in the calculation.

ported²⁵). That study demonstrated that, similar to our findings, α -ZEL was detected in two of five sorghums at ratios relative to ZEN of 5.3 and 2.8%. Studies investigating the contamination of the 5 ZRCs in other crops^{11,13–17} show that the levels of the 5 ZRCs are lower than that of ZEN, and ZAL was lower than the other ZRCs, as also observed in our study. It is difficult to reconstruct the *in situ* contamination ratio precisely from the *in vitro* incubation analysis, but *in vitro* analysis might be helpful as an indicator of possible contamination.

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