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Detection of diacetoxyscirpenol and the related toxins from alfalfa infested with *Fusarium* spp. by gas-liquid chromatography

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Summary

DAS is considered as one of the most toxic trichothecene mycotoxins produced from *Fusarium* species. We tried to examine the best way in the analysis of the toxin in alfalfa infested by *Fusarium* spp..

From June to August, 1992, six samples were collected from the infected epigeal portion (leaf and stem) of alfalfa infested with *Fusarium* spp. and were screened for diacetoxyscirpenol (DAS), 3α-acetyldiacetoxyscripenol (3α-AcetylDAS), 15-acetoxyscirpenol (15-ASP) and scirpentriol (STO).

These toxins were extracted with ethyl acetate from 250 g of fresh alfalfa and were cleaned by a SEP-PAK Florisil cartridge.

After derivatization to their trimethylsilylated analogs, they were analyzed using a gas chromatograph equipped with a hydrogen-flame ionization detector. A DB-17 capillary column (30 m × 0.25 mm) was used, and the column oven temperature was programed to increase from 170 to 200°C at the rate of 3°C/min, then from 200 to 260°C at 5°C/min, to hold at 260°C for 10 min. DAS was detected in one of the five samples at the level of 5.9 μg/g.

High performance liquid chromatography (HPLC) was done using gradient programs. But it was difficult that each of toxin peaks could be separated from the peaks of alfalfa origin.

Though we tried to make monoclonal and polyclonal antibodies, we failed to obtain specific anti-DAS antibodies.

GLC is a better method for detecting DAS and the related toxins in alfalfa at this point.

Introduction

Trichothecenes are the second metabolites[1),12)] produced by a number of fungi, such as *Fusarium* spp., *Myrothecium* spp., *Trichothecium* spp. and certain others.

The group is characterized by a 12, 13-epoxytrichothec-9-ene ring system. Diacetoxyscirpenol (DAS) belongs to the group that does not contain a carbonyl

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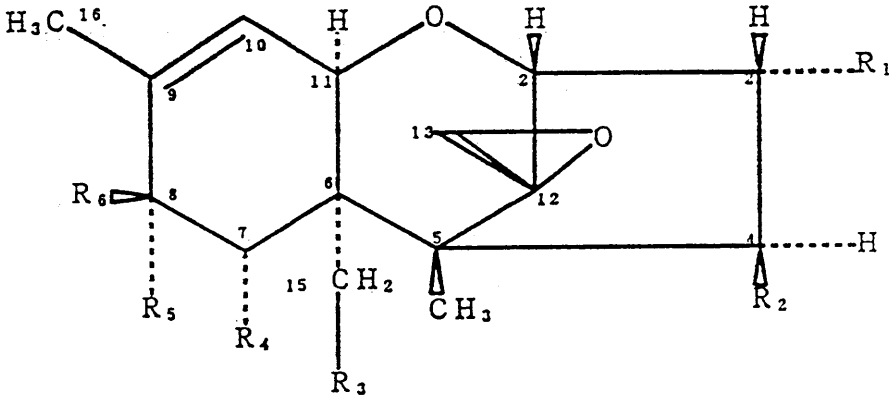

Fig. 1. Structure of trichothecene.

Table 1. Side chain residue of diacetoxyscirpenol and the related toxins

Name	Side chain residue					
	R1	R2	R3	R4	R5	R6
DAS	-OH	-OAc	-OAc	-H	-H	-H
3α-acetylDAS	-OAc*	-OAc	-OAc	-H	-H	-H
15-ASP	-OH	-OH	-OAc	-H	-H	-H
STO	-OH	-OH	-OH	-H	-H	-H

* Ac: acethyl group (-$COCH_3$).

fraction at C_8 conjugated with a double bond in 9-10[1),11),12)]. The structure of trichothecenes is shown in Figure 1, and the side chain residues of DAS, 3α-acetyldiacetoxyscirpenol (3α-AcetylDAS), 15-acetoxyscirpenol (15-ASP) and scirpentriol (STO) are shown in Table 1.

DAS is one of the more toxic trichothecenes, having an LD_{50} of 0.75 mg/kg after intraperitoneal administration to rats[3)]. Experimental exposure[10)] to DAS has been shown to cause effects such as skin necrosis and oedema. The toxin at high levels shows an adverse effect on the haematopoietic system of mice. In virto studies have also shown DAS to be cytotoxic[10)]. The natural occurrence of this trichothecene has been associated with a haemorrhagic bowel syndrome in swine[8)].

Alfalfa is a perennial leguminous plant cultivated all over the world, as it contains a high level of protein and is edibile to domestic animals. However, in Hokkaido 3 to 4 years after sowing, alfalfa blight occurs due to root rot and wilting by *Fusarium* spp.[10)]. It is assumed that DAS and the related toxins might be in the alfalfa infested by *Fusarium* spp..

These chemical compounds have been measured by TLC, HPLC, GLC and mass spectrometry. Recently, biospecific analysis methods have been developed and they are of high sensitivity and specificity.

This paper deals with a chemical method (HPLC and GLC) and a biospecific method (ELISA) to analyze DAS and its related toxins in fresh alfalfa from a field infested with *Fusarium* spp.. The aim of this experiment was carried out for the best method of the analysis of DAS and the metabolites in alfalfa plants.

Materials and Methods

Extraction of toxins

The infected epigeal portion (leaf and stem) of alfalfa infested by *Fusarium* spp. was collected from farmland of Rakuno Gakuen University six times in 1992. The collection period same was from the later part of June to the later part of August in the same year. Two-hundred fifty grams of alfalfa was added to 4 liters of distilled water and smashed with a blender for 5 min. The mixture was allowed to stand overnight in a cold (4°C) room and was filtrated with gauze. The filtrate was centrifuged at 11,000 rpm for 30 min to remove chlorophyll. The precipitate was discarded. The water solution was adjusted to pH 4.5 with acetic acid and incubated with 1% β-glucuronidase/arylsulfatase (Boehringer Mannheim GmbH Co., 20 mℓ/ℓ) at 37°C for 5 hours to release the toxins from their glucuronic conjugate in the alfalfa[6]. Then, the treated solution was filtrated with filter paper under reduced pressure. The filtrate was evaporated to 200 mℓ at 55°C and shaken with 100 mℓ n-hexane for 1 min for defatting. The water layer was extracted three times with 100 mℓ ethyl acetate for 15 min each time. The combined ethyl acetate solution was evaporated to 4 mℓ at 55°C. A 20 mℓ of n-hexane was added to the solution and mixed. After a SEP-PAK Florisil cartridge (Waters Co.) was rinsed with 20 mℓ of n-hexane was passed through the cartridge to eliminate impurities using 20 mℓ n-hexane. The solution eluted with 30 mℓ of mixed solution of chloroform and ethanol (9 : 1, v/v) was evaporated to dryness at 55°C. The residue regarded as containing crude toxins was dissolved with 5 mℓ of methanol.

Standards

All the referenced mycotoxin standards (DAS, 3α-AcetylDAS, 15-ASP and STO) were purchased in analytically pure form from Sigma Chemical Co.. The stock standard solution was made as a mixture containing 0.5 mg of each mycotoxin per mℓ in methanol, and stored in light exclusion containers at the neccesary −20°C.

High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) was carried out on a L-4200 equipped with a UV-detector and a L-6200 intelligent pump. The samples were separated with GL-Science Inertsil PH, Hitachi ODS and Hitachi WHC-18 columns (each 4φ×250 mm). The HPLC analysis was done using a gradient program. It was started at 40% methanol and was linearly increased to 60%. The other HPLC conditions were as follows: wavelength of detector, 210 nm; flow rate of solvent, 0.8 mℓ/min; sample volume, 5 μℓ.

Gas-liquid chromatography

The sample extracts as well as the mycotoxin standard mixtures were trimethylsilylated with the TMSI-C reagent (GL-Science Co.). A 100 μℓ of the standard mixture and 2 mℓ of the sample extracts were transferred to 5 mℓ reaction vials fitted with teflon septum stoppers. After being dried up by nitrogen gas. 100 μℓ of the TMS reagents was added to each vial. The preparations were shaken well and left to stand 30 min at room temperature.

Gas chromatography was performed on a Hitachi G-3000 gas chromatograph equipped with a hydrogen-flame ionization detector and a J & W Scientific DB-17 fused silica capillary column (30 m × 0.25 mm). The column oven temperature was programmed from 170 to 200°C at 3°C/min, then from 200 to 260°C at 5°C/min, and held at 260°C for 10 min. The other conditions were: injector temperature, 200°C; detector temperature, 260°C; flow rate of nitrogen gas, 30 mℓ/min; sample amount, 1 μℓ using a splitless injection method. The calibration table was based on one point with an integrator (Hitachi D-2500).

Preparation of monoclonal and polyclonal antibodies (against DAS and DAS-BSA conjugate)

DAS and DAS-BSA conjugate (Sigma) was used as the immunogen. Four BALB/C female mice were given intraperitoneal injections of 25~50 μg of immunogen in Freund's complete adjuvant (3 mℓ), followed by two subsequent immunizations with the immunogen in Freund's incomplete adjuvant.

Serum was collected 2 and 4 weeks after immunization and was checked with its titer by the indirect ELISA method[2),13),14)]. Cell fusion was carried out as described by Pauly[2)] and Hack *et al*[13)]. The preparation of polyclonal antibody was carried out as described Mills *et al*[14)]. Two New Zealand white male rabbits were given multiple intra-muscular injections (in the back) 100 μg immunogen in Freund's complete and incomplete adjuvants (each 3 mℓ). Titer check was done 5 and 36 weeks after immunization.

Result and Discussion

Four toxins in the standard mixture could be separated clearly by HPLC using Inertsil-PH column and the gradient program of solvent flow. The approximate retention times of STO, 15-ASP, DAS and 3α-acetylDAS were 4.3, 8.6 15.6 and 23.6 min, respectively.

Six alfalfa samples were analysed on Inertsil-PH column by HPLC. However, it was difficult that each of the toxin peaks could be separated from the peakes of alfalfa origin.

We tried to make monoclonal and polyclonal antibodies for DAS, but failed to get hybridoma that produced anti-DAS monoclonal and anti-DAS IgG-polyclonal antibodies. The value of the antibodies was raised to BSA, because 10 mol DAS conjugated with 1 mol BSA was used, as an immunogen. It was assumed that the value of the antibodies to BSA was raised. In future studies, it might be possible to get a specific anti-DAS antibody by changing the carrier protein (HSA and BSA hemiglutarate) conjugated with DAS. We could not get good results in both HPLC and ELISA, so we used GLC.

Four toxins in the standard mixture could be clearly separated by GLC. Their approximate retentions were 15.1, 18.2, 20.8 and 24.8 min. For the recovery test, 1 mg of each of the four toxins was added to 250 g fresh alfalfa and was extracted as mentioned above. The recoveries of DAS, 3α-acetylDAS, 15-ASP and STO were 82.6, 52.1, 39.7 and 922.0%, respectively. The recovery of STO was so high, was crossed with each other. Therefore, more purification was needed to quantify STO.

Table 2. Detection of diacetoxyscirpenol and the related toxins from alfalfa infested with *Fusarium* spp. by GLC

Sample No.	Amount of mycotoxins		
	15-ASP	DAS	3α-AcethylDAS
1	ND*	ND	ND
2	ND	ND	ND
3	ND	ND	ND
4	ND	5.9**	ND
5	ND	ND	ND
6	ND	ND	ND

* ND=not detected.
** Values reprecent μg/g.

DAS has been found in barley[1),5)], maize[1),5),7)], wheat[1),5),10)], and mixed feed[1),5),9)] of several countries. Levels of contamination in several European countries[5)] reached a maximum of 1.6 ppm, except for one sample of 31.5 ppm. However, little information has been available on the presence of DAS in alflfa. Our experiments showed that DAS was detected at 5.9 μg/g in sample No. 4 of the 6 samples, and that other analogues showed less than the detection limit (Table 2).

The best method of DAS analysis was GLC among the three method, and the metabolites of DAS in alfalfa plants were not detected in this experiments. To increase the sensitivity, the application of the electron capture detector[11)] or mass spectrometry[4),11)] in GLC method was needed.

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要　　約

1992年6月から8月に *Fusarium* spp. に感染したアルファルファ (葉と茎) を採取し diacetoxyscirpenol (DAS), 3α-acetyldiacetoxyscirpenol (3α-acetylDAS), 15-acetoxyscirpenol (15-ASP), scirpentriol (STO) の検出法についてガスクロマトグラフィー (GLC), 液体クロマトグラフィー (HPLC) および酵素免疫測定法 (ELISA) の比較検討を行った。採取したアルファルファ 250 g から酢酸エチルでこれら毒素を抽出し，SEP-PAK Florisil cartridge 夾雑物の除去を行った。得られた試料をシリル化した後，水素炎検出器を装着したガスクロマトグラフで定量分析を行った。カラムは，J & W Scientific DB-17 キャピラリーカラム (30 m×0.25 mm) を使用した。分析は，170～200°C まで 3°C/min, 200～260°C まで 5°C/min で昇温し，260°C で5分間保持する条件で行った。定量分析の結果 DAS は，採取した6サンプルの内1サンプルから 5.9 μg/g 検出された。

HPLC による分析は，グラージェントプログラムを用いて行った。しかし，マイコトキシンピークとアルファルファ由来ピークの分離が困難であった。

さらに ELISA での分析のために，DAS に対するポリクローナル，モノクローナル抗体の作製を試みたが，DAS に対する特異抗体は得られなかった。

現段階では，アルファルファ中の DAS およびその関連物質の検出には，ガスクロマトグラフィーによる分析が有効であると思われる。