Detection of Ergot Alkaloids from *Claviceps* Species in Agricultural Products by Competitive ELISA Using a Monoclonal Antibody†

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A competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) was developed which was able to detect ergot alkaloids in seed and flour at the picograms per gram level. A monoclonal antibody (MAb) directed against ergonovine was found to be sensitive to the secondary metabolites of *Claviceps* spp. having an intact ergoline moiety in wheat, bahiagrass, bluegrass, and tall fescue seeds. The assay could detect the alkaloids of *Claviceps purpurea* when sclerotia were diluted 10^{-5} by weight in whole wheat flour, or approximately one sclerotium in 20 kg of wheat. Ergonovine added to ergot-free wheat flour was detected at 10 ng/g. Total elapsed time for the assay is 1.5 h for 96 samples in the microplate format.

INTRODUCTION

Claviceps is a cosmopolitan genus represented by at least 10 species parasitic on over 200 species of grass and sedge hosts (Farr et al., 1989). As phytopathogens, they cause losses to crops that can be substantial (Craig and Hignight, 1991), but their impact is probably greatest as producers of mycotoxins. Vining (1973) listed 46 ergot alkaloids as having been identified in *Claviceps* species, including ergoline and lysergic acid derivatives. Since that time, the number of ergot alkaloids reported has grown as new members of the group have been added (Flieger et al., 1984, 1989). The ergot alkaloids include many with profound pharmacological activities which can be beneficial when administered in the proper context or derivatized or which can be toxic to humans and livestock when ingested in agricultural commodities (Floss et al., 1973). *Claviceps purpurea*, the most common ergot fungus, can cause gangrene, loss of extremities (Floss et al., 1973), and other animal health problems (Coppock et al., 1989; Riet-Correa et al., 1988). *Claviceps paspali*, the ergot fungus of *paspalum* species, causes symptoms known as "paspalum staggers" (Yamazaki, 1980) when livestock are allowed to graze infested fields.

Current chromatographic separation methods for these alkaloids are based on TLC or electrophoresis on silica gel plates (Agurell, 1965) and HPLC in reversed-phase columns (Scott and Lawrence, 1980; Ware et al., 1986; Rottinghaus et al., 1990). Detection is by fluorescence or, in the case of nonfluorescing ergot alkaloids on TLC, spraying with a chromagen such as *p*-(dimethylamino)-benzaldehyde. Mass spectrometry (Plattner et al., 1983; Yates et al., 1985; Porter et al., 1987) has also proven to be useful.

An earlier competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) based on a rabbit polyclonal antibody developed against ergotamine (Shelby and Kelley, 1990) proved to be sensitive when the target was ergopeptine alkaloids having a phenylalanine moiety as part of the cyclol peptide but was unreactive to most other ergot alkaloids. This resulted in an assay which was insensitive to some potentially toxic ergot alkaloids, such as ergonovine, clavines, and lysergic acid derivatives.

Our recently developed monoclonal antibody (MAb) directed against ergonovine (Shelby and Kelley, 1991) has proven to be useful in detection of the ergot alkaloids produced by *Acremonium coenophialum* in tall fescue seed and forage. This MAb, directed against ergonovine, also recognizes a broad spectrum of ergopeptines, clavines, and lysergic acid derivatives which can be produced by ergot fungi on various hosts. The present paper describes the use of this antibody, EN9F10, in the detection of ergot alkaloids produced by *Claviceps* spp. in various seeds and grain products.

MATERIALS AND METHODS

Synthetic Blending of Flours. Wheat ergot sclerotia were collected from infected fields or were isolated from lots of seed sent to us or our colleagues for routine analysis. Whole wheat flour was purchased from a local grocer and tested by the HPLC method of Rottinghaus et al. (1990, 1992) and ELISA to confirm the absence of ergot alkaloids for spiking studies. Ergot sclerotia were ground in a cyclone mill to pass a 1-mm screen and blended with flour to obtain mixtures which would approximate ergot sclerotial contamination at ratios of from 10^{-2} to 10^{-6} by weight.

Spiking Studies. Ergot-free whole wheat flour was spiked with ergonovine (maleate salt, Sigma E6500) by making dilutions in 10 mL of methanol and adding each to 10 g of flour. Methanol was evaporated in ambient atmosphere in darkness in a fume hood.

Other Grass Seed. Dallisgrass (*Paspalum dilitatum*) and bahiagrass (*Paspalum notatum*) seeds were identified as contaminated by the Alabama Department of Agriculture Seed Laboratory or in our laboratory from commercial seed lots. In these grasses, the predominant infection was the conidial or "honeydew" stage of the *Claviceps* life cycle. Contaminated seed were identified by macroscopic visual observation and confirmed by microscopic observation of conidia on the seed (Luttrell, 1977). Sclerotia were not observed in these *Paspalum* seeds but may have been present in small amounts. Fescue and bluegrass seeds were represented in this study by sclerotia isolated from infected seed. All seeds and sclerotia were milled as above prior to analysis.

Competitive Inhibition ELISA. The CI-ELISA protocol was essentially as reported previously (Shelby and Kelley, 1991), except as noted below. All operations were carried out using disposable glass- or plasticware to avoid sample carryover and contamination. For extraction of alkaloids, milled samples (1.0 g) were weighed into sample cups, and 10 mL of phosphate-buffered saline (pH 7.4) plus 0.05% Tween 20 (PBST) was added. The mixture was stirred briefly and incubated at room temperature for 30 min. To test the effectiveness of prolonged extraction,

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Table I. Detection of Ergot (*C. purpurea*) Sclerotia Diluted in Flour Using Monoclonal Antibody EN9F10 in Competitive ELISA

	30-min extraction		overnight extraction	
dilution by wt	ELISA absorbance 490 nm[a]	SD[b]	ELISA absorbance 490 nm[a]	SD[b]
no ergot	1.3902	0.0487	1.3813	0.0878
10^{-6}	1.3775	0.0370	1.2400	0.0798
10^{-5}	1.3898	0.0363	1.1015	0.1100
10^{-4}	0.1365	0.0440	0.1355	0.0244
10^{-3}	0.1035	0.0045	0.1180	0.0240
10^{-2}	0.0663	0.0013	0.0013	0.0279
LSD ($P = 0.05$)	0.049		0.0628	

[a] Mean of four ELISA wells. [b] Standard deviation of four absorbance values.

Table II. Detection of Ergonovine Spike in Whole Wheat Flour Using Monoclonal Antibody EN9F10 in Competitive ELISA

	30-min extraction		overnight extraction	
spike, ng/g	ELISA absorbance 490 nm[a]	SD[b]	ELISA absorbance 490 nm[a]	SD[b]
0	1.4322	0.0279	1.268	0.0887
10	1.2553	0.0329	0.4540	0.0324
100	0.1530	0.0175	0.1078	0.0318
1000	0.0533	0.0045	0.1063	0.0364
LSD ($P = 0.05$)	0.0282		0.069	

[a] Mean of four ELISA wells. [b] Standard deviation of four absorbance values.

the incubation step was extended to overnight (12 h) at 6 °C. Except as noted, the shorter extraction time was used. After extraction, samples could be pipetted directly from the upper portion of the sample cup without filtration.

Ninety-six well polystyrene ELISA plates (Dynatech immulon 4) wer coated with lysergic acid–poly(L-lysine) conjugate (LA–PLL), diluted 1/4000 from stock solution in 0.05 M sodium carbonate buffer (pH 9.6) [approximately 825 ng/mL measured as poly(L-lysine)]. Unbound LA–PLL was removed by five washes of PBST with a hand-held wash bottle.

For the competitive inhibition step, 50 μL of sample extract was pipetted directly into the wells of coated plates. Antiserum in the form of hybridoma supernatant was diluted 1/200 in PBST plus 1% w/v nonfat dry milk (PBSTM), and 50 μL was added to the well. Following incubation at room temperature for 15 min, plates were washed as above with PBST. Bound antibody was measured using commercial goat anti-rabbit IgG (H+L) peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA, 170-6515), diluted 1/1000 in PBSTM. After incubation at RT for 15 min, the plate was washed as before with PBST. To each well was added 100 μL of the chromagen, *o*-phenylenediamene dihydrochloride (OPD, Sigma P-8287) dissolved at 1 mg/mL in 100 mM sodium citrate buffer (pH 5.0) plus 0.003% hydrogen peroxide. Color development at room temperature was stopped after 15 min with 50 μL/well of 3 M sulfuric acid. Optical density was measured at 490 nm using a Dynatech MR 580 ELISA plate reader.

RESULTS

Using the abbreviated (30-min) extraction protocol, the CI-ELISA was able to detect alkaloids of wheat ergot when sclerotia were diluted 10^{-4} by weight in flour (Table I). Extending the extraction to 12 h extended the limit of sensitivity to 10^{-5}. This represents the alkaloids produced by one sclerotium in approximately 20 kg of wheat flour. The ergonovine spiking data (Table II) showed a similar enhancement of sensitivity when extraction was extended to 12 h, but in either case the lowest spike level (10 ng/g) was easily distinguishable from the nonspiked control.

Table III. Detection of Ergot Alkaloids of *Claviceps* Species in Various Grass Seed Lots Using Monoclonal Antibody EN9F10 in Competitive ELISA

species	infection status	ELISA absorbance 490 nm[a]	SD[b]
P. notatum (bahiagrass)	–	1.005	0.0057
	–	1.128	0.0245
	–	1.154	0.0178
	+[c]	0.516	0.0536
	+[c]	0.195	0.0300
	+[c]	0.307	0.0115
Poa pratensis (bluegrass)	–	1.279	0.0184
	–	1.385	0.0158
	–	1.423	0.0117
	+[d]	0.119	0.0044
	+[d]	0.516	0.0643
	+[d]	0.157	0.0190
Festuca arundinaceae (tall fescue)	–	1.275	0.0207
	+[d]	0.180	0.0340

[a] Mean of four ELISA wells. [b] Standard deviation of four absorbance values. [c] Honeydew or conidial infection. [d] Sclerotia present.

The CI-ELISA clearly detected the presence of ergot alkaloids of *C. paspali* conidial or honeydew infestation in bahiagrass seed lots (Table III). Confirmation of ELISA results with macro- and microscopic observations was difficult in the absence of obvious sclerotia, but conidia of *Claviceps* spp. were observed on most seeds of infected lots. In the case of bluegrass and fescue seeds, *C. purpurea* sclerotia could be observed in infested seed lots but typically were less than 0.1% by weight.

DISCUSSION

The previous immunoassay we developed for ergot alkaloids employed a rabbit polyclonal antibody developed against ergotamine conjugated to bovine serum albumin at the indole nitrogen of the ergoline group (Shelby and Kelley, 1989). This resulted in an antibody specific for the peptide portion of the molecule. As a result, the assay was specific for a few ergopeptine alkaloids having common amino acids. Typical ergot infestations, however, produce a spectrum of alkaloids, many not of the ergopeptine type. The total alkaloid contamination contributes to human and animal toxicosis, and any effective assay method designed to intercept contaminated food or feed should be able to detect a range of potential toxins within a group rather than a single molecular structure. The new MAb, EN9F10, has a broader spectrum of recognition, binding with any ergot alkaloids having an intact four-member ergoline ring structure (Shelby and Kelley, 1991). Therefore, the CI-ELISA based on MAb EN9F10 could best be described as one for total ergot alkaloids. EN9F10 is most sensitive to ergonovine, which was the immunogen used to develop the MAb. Lysergic acid derivatives appear to be a common product of ergot contaminations. Vining (1973) lists ergonovine as being produced by both *C. paspali* and *C. purpurea*, and Flieger et al. (1989) identified ergine derivatives in submerged cultures of *C. paspali*. Scott and Lawrence (1980) found ergonovine in all of the ergot-infested wheat and rye flour samples they tested by HPLC. Yates and Powell (1988) also found ergonovine in tall fescue seed infested with *A. coenophialum*. The sensitivity of the assay is probably enhanced by the relative water solubility of ergonovine. This relatively brief extraction releases only a portion of the total ergot alkaloids from the sample matrix, but the sensitivity of the CI-ELISA to ergonovine compensates for the poor recovery of the ergopeptines. In PBSTM, the 50% inhibition value for ergonovine was 460 pg/mL (Shelby and Kelley, 1991),

and we estimate the lower limit of detection to be approximately 10 pg/mL. Attempts to use other solvents, such as methanol, in the sample extraction step resulted in a less sensitive assay (data not shown).

The present assay offers several distinct advantages over the conventional methods of ergot alkaloid detection. Like any immunoassay, it has the advantages of being faster and less expensive than HPLC or mass spectrometry. Sensitivity is comparable to that of these other methods (Shelby and Kelley, 1991), and the immunoassay can be performed by technicians with relatively less training. The immunoassay, due to the cross-reactivity of the antibody, is able to detect ergot alkaloids which would be missed by other methods. HPLC with fluorescence detection, for example, would not be able to detect nonfluorescing clavine alkaloids with a double bond at the 8–9 position. The small analyte volume (50 μL) makes it possible to test smaller samples, such as single seeds, sclerotia, or fungal colonies from agar plates. Detection of ergot sclerotia in whole grains is quite easy with the naked eye, but once the sample has been milled, light microscopy must be used to detect sclerotial fragments (McClymont and Harwig, 1982). The CI-ELISA would be a convenient assay method in milled grains, and the sensitivity should be adequate to detect potentially hazardous ergoline-containing alkaloids where levels of ergot contamination are significant [0.3% ergot contamination accepted as the U.S. standard (Marasas and Nelson, 1987)]. Where accurate quantitative analysis of a specific ergot alkaloid is necessary, this assay could serve as an initial screen to eliminate those samples with no ergot alkaloids, to be followed by a quantitative method such as HPLC.

Although acute ergot toxicosis is a well-known syndrome in humans and animals, chronic ingestion of trace amounts of these toxins may have an effect yet unknown, which underscores the need for more extensive testing for ergot alkaloids in agricultural commodities.

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