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An Immunoassay for Ergotamine and Related Alkaloids†

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A competitive inhibition (CI) enzyme-linked immunosorbent assay (ELISA) for ergotamine was developed with use of polyclonal antibodies produced in rabbits by immunization with an ergotamine–albumin conjugate. The assay was found to be specific for the ergot alkaloids having a phenylalanine moiety in the peptide portion of the molecule. These include ergotamine, ergostine, and ergocristine, all of which are produced by the ergot fungi, *Claviceps spp.* The assay was able to detect ergotamine in spiked grain samples at a level of 10 ng/g. Target alkaloids were detected in ergot sclerotia of wheat and tall fescue and in fescue seeds infected with the endophytic fungus, *Acremonium coenophialum* Morgan-Jones and Gams.

Ergotamine is one of the ergot peptide alkaloids produced by fungi of the genus *Claviceps* and some related genera, which were responsible for the classic examples of ergot poisoning in Europe in the middle ages (Lamey et al., 1982; Stoll and Hoffman, 1965). As a human toxicosis, ergotism is no longer prevalent due to prevention of infected grain from entering food channels (Scott and Lawrence, 1980; Lamey et al., 1982). However, sporadic outbreaks of ergotism in livestock due to feeding ergot-infected grain still occur (Lamey et al., 1982; Riet-Correa et al., 1988). These alkaloids exert a number of pharmacological effects, one of the most dramatic being vasoconstriction, which may result in gangrene and loss of extremities (Floss et al., 1973). Other effects include reproductive disorders, such as lowered conception rates, stillbirth, and agalactia (Lamey et al., 1982; Floss et al., 1973). The striking similarity of the latter symptoms to the toxicosis caused by ingestion of tall fescue infected with the endophytic fungus *Acremonium coenophialum* Morgan-Jones and Gams led various investigators to the discovery of ergot peptide alkaloids in tall fescue as well (Porter et al., 1981, 1987; Yates et al., 1985; Yates and Powell, 1988). On a global scale, economic loss caused by ergot alkaloids in endophyte-infected forage grasses may even exceed that from *Claviceps* infestations in grain.

The same pharmacological effects making ergotamine and related alkaloids so toxic in some cases also make them pharmaceutically useful in others. Due to its vasoconstrictive effects, ergotamine is prescribed to control migraine headaches (Barnhart and Huff, 1985) and to

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stop postpartum bleeding. Other potentially useful properties of these compounds are related to their ability to induce labor contractions, inhibit lactation by prolactin suppression, act as an α-adrenergic blocking agent and serotonin antagonist, terminate early pregnancy, and inhibit mammary tumors (Floss et al., 1973).

Currently, methods used for detection of ergotamine or related alkaloids are thin-layer chromatography (TLC) (Svendsen and Verpoorte, 1983), high-pressure liquid chromatography (HPLC) (Yates and Powell, 1988), and tandem mass spectrometry (MS/MS) (Yates et al., 1985). These methods have the disadvantages of extensive sample preparation and the requirement of expensive equipment, as well as considerable skill in its use. An immunoassay for these alkaloids would obviate many of these disadvantages, resulting in an assay suitable for rapid screening of agricultural products for this class of alkaloids.

MATERIALS AND METHODS

Preparation of Protein Conjugates. A Mannich condensation reaction, following the protocol of Taunton-Rigby et al. (1973), was used to conjugate ergotamine (tartrate salt; Sigma Chemical Co., St. Louis, MO; E-6875) to bovine serum albumin (BSA; Sigma A-8022) for immunization and to ovalbumin (OVA; Sigma A-5503) for use as the coating antigen in the CI ELISA. Soluble protein in the conjugate was measured by the bicinchoninic acid method of Smith et al. (1985), and soluble alkaloid was measured by the colorimetric assay of Allport and Cocking (1932). Conjugates were also evaluated on 250-μm silica gel TLC plates developed in chloroform methanol (9:1, v/v) and visualized under UV light (365 nm) (Svendson and Verpoorte, 1983). The conjugates were dispensed in 500-μL aliquots and stored at −20 °C.

Immunization of Rabbits. The ergotamine–BSA conjugate was homogenized with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI; 0638-60-7). Three New Zealand white does were immunized each with a subcutaneous injection of 2 mL of the injection homogenate, equally divided between two sites. This protocol delivered 1.05 mg of the conjugate/rabbit calculated on the basis of soluble protein. A booster of conjugate calculated to deliver 0.8 mg of soluble protein/rabbit was homogenized with Freund's incomplete adjuvant (Difco 0639-60-6) and injected at different sites after 15 days; this was repeated after an additional 63 days.

Rabbit Immune Response. Rabbit immune response to ergotamine was initially screened by CI ELISA (see below) with whole serum serially diluted in 0.1 M phosphate-buffered saline (pH 7.4; PBS) + 0.05% Tween-20 (PBST). Antibody specificity for ergotamine was determined by competitive inhibition of binding by a 1 μg/mL solution of ergotamine tartrate in PBST. The serum lot having the highest anti-ergotamine titer, based on lowered absorbance values in ergotamine-containing wells, was the serum taken 11 days after the final immunization. This serum lot was chosen for purification and further testing. Antiserum was purified by precipitation with 50% saturated ammonium sulfate, followed by reprecipitation with 35% ammonium sulfate (Hudson and Hay, 1976) and dialysis against three changes of PBS + sodium azide, 0.2 g/L, 12 h each at 5 °C. The antiserum was adjusted to 1 mg/mL with PBS + azide by measuring optical density at 280 nm.

Competitive Indirect (CI) ELISA. Ninety-six-well ELISA plates (Immulon 2; Dynatech Corp., Chantilly, VA) were coated with 100 μL of a 39 ng of soluble protein/mL solution of ergotamine–ovalbumin conjugate in 0.05 M sodium carbonate buffer (pH 9.6). Plates were incubated at 37 °C for 45 min and washed briefly five times with PBST with a hand-held wash bottle. For the competitive inhibition step, an alkaloid solution to be tested, which varied with the test and sample type (see below), was added in a volume of 50 μL. Antiserum was diluted to 5 μg of protein/mL in PBST, and 50 μL was added to the well. Following incubation at 37 °C for 15 min, plates were washed as above with PBST. Bound antibody was measured with commercial goat anti-rabbit IgG (H+L) peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA; 170-6515), diluted 1:2000 in PBST. After incubation at 37 °C for 15 min, the plate was washed as before with PBST. To each well was added 100 μL of the chromagen, *o*-phenylenediamine dihydrochloride (OPD; Sigma P-8287) dissolved at 1 mg/mL in 100 mM sodium citrate buffer (pH 5.0) + 0.003% hydrogen peroxide. Color development at room temperature was stopped after 5 min with 50 μL/well of 3 M sulfuric acid. Optical density was measured at 490 nm with a reference wavelength of 570 nm on a Dynatech MR 580 reader.

Cross-Reactivity. Authentic standards of alkaloids for spiking, cross-reactivity, and controls in quantitative CI ELISA were stored desiccated at 5 °C protected from light. Stock solutions were prepared in methanol at 1 mg/mL and stored at −20 °C, protected from light. Since the ergot alkaloids are known to be unstable, particularly to form C_8 epimers (Scott and Lawrence, 1980), cross-reactivity experiments were begun within 24 h of making methanol dilutions of the standards. Epimerization of ergotamine was monitored by TLC on silica gel plates in chloroform–ethanol (95:5, v/v) and visualized with UV light (365 nm) and Dragendorf's reagent (Svendsen and Verpoorte, 1983). To calculate cross-reactivity, stock alkaloid solutions were diluted to 1 μg/mL in PBST and serial 1:2 dilutions were made directly on the horizontal axis of the ELISA plate.

Spiked Samples. Samples of cracked wheat, whole rye, and millet, designated for human consumption, were purchased locally. Ergotamine tartrate was diluted from stock solutions in methanol and added to the commodities to obtain 10, 10^2, and 10^3 ng/g. After being air-dried in the dark at room temperature overnight, the unground samples were extracted by shaking briefly with methanol–PBST (1:1, v/v) and then allowed to settle at −2 °C for 30 min. A ratio of 5 g of sample to 10 mL of extract buffer was used. Control samples for each commodity, to which no ergotamine was added, were treated as above. Supernatants were decanted into 1.5-mL polypropylene centrifuge tubes and centrifuged at 14000*g* for 10 min in a benchtop microcentrifuge. A 50-μL portion of the supernatant was then placed directly into an ELISA well, and the CI ELISA protocol described above was followed. Samples were replicated by placing three aliquots of each sample in adjacent wells on the plate.

Standard curves were generated for each sample type by serially diluting ergotamine tartrate from the stock solutions in the control sample extract on adjacent wells of the same plate, as described for the cross-reactivity experiments. Since no other measure of ergotamine levels was attempted for these presumed ergotamine-free samples, data reported are readings above background. However, ELISA absorbance values of samples designated for human consumption did not differ appreciably from buffer blanks.

Regression equations for [ergotamine] vs absorbance at 490 nm were generated, and nanograms per gram ergotamine in each sample were calculated from absorbance means. Regression coefficients (r^2) were >0.90 for all samples.

Measurement of Ergotamine in Ergot Sclerotia and Infected Samples. Ergot (*Claviceps purpurea*) sclerotia were removed from lots of infected seed of wheat and fescue. Sclerotia were milled to an average particle size of 1 mm and subjected to the extraction and CI ELISA procedure used for spiked samples. To be within the range of the standard curve, ergot samples were diluted 1:100 to 1:1000 in methanol–PBST after extraction. Standards were diluted in extracts of ergot-free wheat or fescue seed milled and extracted in the same manner, and regressions were calculated as for spiked samples. To test the ability of the assay to detect ergot contamination in flour, milled wheat ergot sclerotia were diluted in whole wheat flour to 0.1% by weight and then extracted and assayed as for other samples. Fescue seed previously analyzed for percent endophyte by microscopy was treated in the same manner as ergot sclerotia and screened by CI ELISA. Ten seed lots each of high and low endophyte levels were chosen at random without regard to cultivar, seed age, origin, or other variables. Standards were diluted in one endophyte-free seed lot extract as other samples.

RESULTS AND DISCUSSION

A useful indicator of the success of conjugation of ergotamine to BSA and OVA was obtained by evaluation of

Table I. Cross-Reactivity of Anti-Ergotamine Antibody with Ergot Alkaloids

alkaloid	ng/mL 50%[a]	% cross-reactivity[b]
ergotamine	0.985	100.0
ergotaminine	3.45	28.5
ergostine	3.67	26.8
ergostinine	12.04	8.2
ergocristine	4.42	22.3
ergosine	50.63	1.9
ergosinine	38.33	2.6
ergovaline	1533.0	<1.0
α-ergokryptine	345.0	<1.0
ergonovine	3720.0	<1.0
ergocornine	3775.0	<1.0
ergonine	1937.0	<1.0
ergoptine	238.0	<1.0
elymoclavine	4361.0	<1.0
pyroclavine	17151.0	<1.0
festuclavine	2995.0	<1.0
regulovasine	7338.0	<1.0
setoclavine	3121.0	<1.0

[a] Concentration of alkaloid causing a 50% reduction in CI ELISA values when compared to buffer-only controls. [b] % CR = [(ng/mL 50% inhibition ergotamine) X 100]/(ng/mL 50% inhibition analogue).

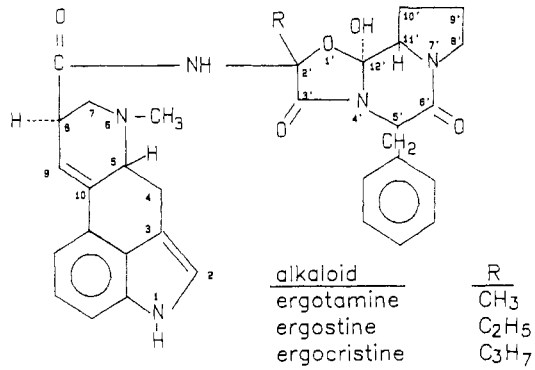

Figure 1. Structures of cross-reactive alkaloids.

the extensively dialyzed preparations on TLC plates. The conjugates exhibited intense fluorescence at the origin, while protein controls remained at the origin but did not fluoresce. Ergotamine controls had an R_f value of 0.67 in this solvent system. Colorimetric measurements of the alkaloid and protein content of the conjugates indicated the average number of ergotamine residues per molecule of carrier protein to be 7.7 for BSA and 1.3 for OVA.

Competitive inhibition in CI ELISA (Table I) was greatest for the target alkaloid, ergotamine, and was significant only for ergostine and ergocristine, the two other ergot peptide alkaloids having the MePh moiety at the 5′-position (Figure 1). The nontarget alkaloids, ergostine and ergocristine, differ from the target alkaloid ergotamine by only one or two methyl groups, respectively, at the 2′-position (Figure 1). In addition, the antibody appears to be unable to distinguish between two C_8 epimers of reactive alkaloids tested (ergotaminine and ergostinine; Table I). Further evidence of epimer cross-reactivity was indicated by aged standards. As predicted by other investigators (Scott and Lawrence, 1980; Ware et al., 1986), our methanol solutions of standards slowly epimerized even when stored at −20 °C. This was obvious on thin-layer chromatograms developed in chloroform–ethanol (95:5, v/v), a solvent system that separates these C_8 epimers (Svendsen and Verpoorte, 1983). CI ELISA using aged standards yielded essentially the same results even after several months of storage when epimerization had reached equilibrium.

Although thin-layer chromatograms of aged methanol solutions of ergotamine revealed the presence of only these two C_8 epimers, other decomposition products of ergotamine are reported to occur spontaneously (Kreilgard, 1977): i.e., aci inversion at C-2 under acid conditions; lumni compounds in light; and oxidation in the presence of oxygen. No attempt was made to isolate these decomposition products, so their cross-reactivities with the antibody are not known.

Table II. Competitive Indirect ELISA of Spiked Grain Samples

grain	added ergotamine, ng/g	abs (490 nm)[a]	measd ergotamine,[b] ng/g	% recovery
cracked wheat	0	1.303	0	
	10	0.925	2.6	26
	10^2	0.377	140	140
	10^3	0.100	1046	105
whole rye	0	1.492	0	
	10	1.200	6.0	60
	10^2	0.567	221	221
	10^3	0.140	2208	221
whole millet	0	1.449	0	
	10	1.187	6.2	62
	10^2	0.515	360	360
	10^3	0.191	2555	255

[a] Mean of three wells. Overall mean CV = 7.8%. [b] Calculated by regression (see text).

The Mannich condensation reaction to produce conjugates for immunization links the alkaloid to the carrier protein through the indole nitrogen of the ergoline moiety of the alkaloid. This directs the antibody specificity to the portion of alkaloid distal to the carrier protein, in this case the peptide portion of ergotamine. Similar specificity of polyclonal antisera produced by a Mannich reaction was reported for lysergic acid (Arens and Zenk, 1980) and for LSD (Taunton-Rigby et al., 1973; Ratcliffe et al., 1977; Castro et al., 1973), while other conjugation methods involving the sites distal to the indole nitrogen produced antisera more cross-reactive with a variety of related alkaloids (Van Vunakis et al., 1971; Loeffer and Pierce, 1973).

Combined levels of ergotamine and ergocristine have been reported to account for 66, 77, and 85% of the total alkaloids of ergot sclerotia of fescue, barley, and rye, respectively (Porter et al., 1987). These percentages were similar in ergot-infected flours of rye (Scott and Lawrence, 1980). Our CI ELISA proved to be an effective indicator of ergot infection in these grain samples. Ergotamine CI ELISA measurements of spiked samples (Table II) detected ergotamine at 10 ng/g, which is within the range detected by Scott and Lawrence (1980) using HPLC on commercial flours. As in other quantitative mycotoxin CI ELISA assays (Warner et al., 1986; Dixon et al., 1987), our ELISA tended to underestimate the target alkaloid at lower spiking concentrations and overestimate at higher concentrations. Some error no doubt arises from incomplete extraction of the spiked alkaloid in our samples due to the relatively brief extraction in a mild solvent. Other errors in recovery could result from sample decomposition, since elaborate safeguards were not employed to prevent decomposition in the spiking process.

Estimates of ergot peptide alkaloids in ergot sclerotia by CI ELISA (Table III) are generally within the range measured by other methods. When diluted in wheat flour, ergot sclerotia of wheat could be detected at 0.1% on a dry weight basis, the lowest concentration tested.

In tall fescue seed, no ergot alkaloids were detected in any of the 10 endophyte-free seed lots (Table IV). Fes-

Table III. Measurement of Ergotamine and Related Alkaloids in Various Naturally Occurring Sources Using CI ELISA

source	abs (490 nm)[b]	ergotamine, alkaloids,[a] ng/g
whole wheat flour	1.05	0
wheat ergot sclerotia (*Claviceps purpurea*)	0.21	1.58×10^5
tall fescue ergot sclerotia (*Claviceps purpurea*)	0.43	1.39×10^4
wheat ergot sclerotia diluted in whole wheat flour 0.1% by weight	0.77	2.33×10^2

[a] Calculated by regression. Includes ergotamine, ergostine, ergocristine, and their C_8 epimers. [b] Mean of four wells. Overall mean CV = 8.8%.

Table IV. Measurement of Ergotamine in Tall Fescue Seed by CI ELISA

sample no.	% endophyte[a]	abs (490 nm)[b]	ergotamine alkaloids,[c] ng/g
1	0	1.31	0
2	0	1.26	0
3	0	1.15	0
4	0	1.05	0
5	0	1.33	0
6	0	1.32	0
7	0	1.22	0
8	0	0.99	0
9	0	1.31	0
10	0	1.34	0
11	89	0.94	0
12	81	1.30	0
13	79	1.03	0
14	76	0.82	1.25
15	83	0.93	0
16	73	0.54	4.55
17	98	1.00	0
18	96	0.90	0
19	94	0.41	96
20	98	0.19	865

[a] Determined by microscopy. [b] Means of three wells. Overall mean CV = 10.2%. [c] Calculated from regression. Includes ergotamine, ergostine, ergocristine, and their C_8 epimers.

cue seed infected with the tall fescue endophyte (*A. coenophialum*) varied widely in alkaloid levels (0–865 ng/g) among the 10 seed lots tested, and variability did not correlate with percent endophyte infection. Other undetermined variables, such as fungal biotype, fescue cultivar, age of seed lot, or environmental conditions during the growing season, may have resulted in a shift in the alkaloid profiles of these samples. Yates and Powell (1988) measured an average of 9.5 μg/g total ergot-like alkaloids in one lot of Alabama-grown endophyte-infected fescue seed, of which the majority consisted mostly of ergovaline and its epimer. They also detected varying amounts of ergosine and ergotamine, which they attributed to the presence of ergot sclerotia in their sample. However, there were no detectable ergot sclerotia in our ergotamine-positive endophyte-infected fescue seed (samples 11–20, Table IV), and our data suggest that the endophyte may generate varying levels of ergotamine, ergostine, or ergocristine in fescue seed.

The assay in its present form is a rapid, sensitive, and semiquantitative indicator of the presence of ergotamine and related ergot alkaloids, which provides a practical method of widespread screening of agricultural samples. Our method compares favorably with existing detection methods in terms of speed, sensitivity, cost, equipment, and user training required and could be used to initially screen sample lots and identify those requiring more detailed analytical methods such as HPLC or mass spectrometry. Also, the format of the assay lends itself to modifications that might further enhance the speed and the specificity of the test.

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Expression of Bovine β-Casein in *Saccharomyces cerevisiae* and Characterization of the Protein Produced in Vivo

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Recombinant DNA technology offers numerous opportunities for engineering food proteins and for studying their structure–function relationship. As part of the study of the structure–function of bovine caseins, it is necessary to produce mutant proteins in experimental amounts and correlate their new structure to their physicochemical characteristics. To this end, bovine β-casein was expressed in the yeast *Saccharomyces cerevisiae* by a fusion to the *HXK1* (hexokinase P1) gene. Casein was produced during late exponential/early stationary phase of growth on glucose as would be predicted for a gene under the control of the *HXK1* promoter. Bovine β-casein was posttranslationally modified by yeast. Internal phosphorylated forms were observed as well as a high molecular weight form that appeared to be O-glycosylated and largely localized to the periplasmic space.

Bovine caseins serve as important sources of nutrition in the human diet, and the proteins themselves are used as food additives to enhance the nutritional value and functional characteristics of a variety of products. Proteolytic digestion coupled with decreased solubility of the caseins at low pH is central to the production of cheese and other dairy products. Because of their availability and precipitation at low pH, bovine caseins are used as clarification agents in wine processing. One approach to further elucidate the structural features of caseins important for proper function in micelle formation and functionality in foods is to alter the protein sequence by altering the DNA sequence encoding the protein and to obtain sufficient quantities of the protein for analysis. All of the bovine caseins have been cloned (Bonsing and Mackinlay, 1987), and some have been expressed in *Escherichia coli* (Kang and Richardson, 1988). Bovine β-casein is modified posttranslationally with the addition of five phosphate groups to the protein. Such modification does not occur in *E. coli*. Therefore, we sought to obtain expression of β-casein in the eukaryotic microorganism *Saccharomyces cerevisiae*. Numerous heterologous proteins have been expressed in *Saccharomyces* species (Brake et al., 1984; Hitzeman et al., 1982; Kingsman et al., 1985). Since glycolytic enzymes are normally produced in large quantities in yeast cells (any given enzyme may represent from 1 to 5% of total cellular protein), glycolytic promoters have been exploited in constructs to obtain high yields of foreign proteins. However, the glycolytic enzymes are generally produced constitutively during growth, and such a strategy for expression of a heterologous protein may affect growth efficiency, thereby selecting for variants in the population producing less of the protein product. The hexokinase P1 enzyme encoded by the *HXK1* gene catalyzes the phosphorylation of fructose and glucose at the 6-position. However, the synthesis of hexokinase P1 is glucose repressible, and this isozyme becomes the predominant species only in the absence of glucose or following glucose exhaustion in the medium (Gancedo et al., 1977; Kopperschlager and Hofmann, 1969; Muratsubaki and Katsume, 1979). Thus, the hexokinase P1 gene is a glycolytic gene regulatable by the glucose concentration in the medium. We decided to investigate the utility of the *HXK1* promoter for expression of a heterologous protein, bovine β-casein, in yeast. In addition, we undertook a preliminary characterization of

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