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Effect of whole and fractionated dietary alfalfa meal on zearalenone toxicosis and metabolism in rats and swine^{1,2,3}

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Experiments were conducted to determine the mechanism by which dietary alfalfa can protect against zearalenone toxicosis. Female weanling rats were fed semipurified diets containing whole alfalfa meal, fractionated alfalfa meal (fiber, solvent extract, and water extract), and purified components of alfalfa (coumestrol, saponin, lignin, coumestrol + lignin, and saponin + lignin) with and without 250 mg zearalenone/kg of diet. All ingredients were provided for 2 weeks at levels corresponding to those found in diets containing 15 and 25% alfalfa. Yorkshire gilts were fed 15 and 25% alfalfa meal with and without 10 mg zearalenone/kg of diet for 4 weeks. The feeding of zearalenone to rats reduced growth and food consumption but this was overcome by 25% alfalfa. Zearalenone also increased the activity of hepatic 3 α -hydroxysteroid dehydrogenase (3 α -HSD), the enzyme believed to metabolize zearalenone to α - and β -zearalenols. Dietary alfalfa did not overcome this effect. Alfalfa fiber was the only fraction to partially overcome the growth-depressing effects of zearalenone while the other fractions had no beneficial effects and 3 α -HSD was not affected by diet. None of the purified components affected growth parameters or 3 α -HSD. The enzyme was also not affected by zearalenone or alfalfa in swine diets. Coumestrol, α -zearalenone, and β -zearalenone were shown to be competitive inhibitors of 3 α -HSD in rat liver. It was concluded that the fiber fraction of alfalfa protects against zearalenone toxicity, and that this effect is not dependent on coumestrol or saponin and is not likely mediated through 3 α -HSD.

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Ces expériences visaient à déterminer par quel mécanisme l'alfalfa alimentaire peut protéger de la toxicose au zéaralénone. Des rats femelles nouvellement sevrés reçurent des diètes semipurifiées contenant un repas complet de luzerne, un repas fractionné de luzerne (fibre, extrait de solvant et extrait d'eau), et des composantes purifiées de luzerne (coumestrol, saponine, lignine, coumestrol + lignine et saponine + lignine) avec et sans diète de 250 mg zéaralénone/kg. Les ingrédients furent ingérés pendant 2 semaines à des taux correspondant à ceux de diètes contenant 15 et 25% d'alfalfa. Des porcs Yorkshire reçurent un repas de 15 et 25% de luzerne, avec et sans 10 mg zéaralénone/kg pendant 4 semaines. L'administration de zéaralénone aux rats réduisit la croissance et la consommation de nourriture, mais ceci fut neutralisé par 25% de luzerne. En outre, zéaralénone augmenta l'activité de 3 α -hydroxystéroïde déshydrogénase (3 α -HSD), l'enzyme censé métaboliser zéaralénone en zéaralénols α et β . La luzerne alimentaire ne renversa pas cet effet. La fibre de luzerne fut la seule fraction capable de neutraliser les effets réducteurs de croissance de zéaralénone, alors que les autres fractions eurent pas d'effets bénéfiques et que la diète n'affecta pas le 3 α -HSD. Aucune des composantes purifiées n'affecta les paramètres de croissance ni le 3 α -HSD. L'enzyme ne fut affectée ni par le zéaralénone ni par la luzerne contenus dans les diètes des porcs. Dans le foie du rat, le coumestrol, les zéaralénols α et β s'avérèrent être des inhibiteurs compétitifs de 3 α -HSD. On a conclu que la fraction fibre de luzerne protège contre la toxicité du zéaralénone, que cet effet n'est fonction ni du coumestrol ni de la saponine et qu'il n'est vraisemblablement pas médié par le 3 α -HSD.

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Introduction

Zearalenone is a uterotrophic mycotoxin that can be produced when cereal grains are stored in the presence of *Fusarium roseum* under conditions of greater than 25% moisture and temperatures of 12-14°C (Caldwell and Tuile 1970). Many different species are susceptible to zearalenone toxicosis, although swine are among the most sensitive. The feeding of zearalenone at levels as low as 1-5 mg/kg diet has been shown to cause hyperestrogenism in prepubertal gilts (Mirocha and Christensen 1974). Mammary gland enlargement, tumefaction of the vulva, and vaginal and rectal prolapse are seen in immature females (Change *et al.* 1979), while immature males can suffer swelling of the prepuce and enlargement of mammary glands (Christensen *et al.* 1972). Zearalenone consumption by

mature female swine can alter reproduction resulting in increased neonatal mortality, and a decrease in fertility, lactation, and litter size (Chang *et al.* 1979).

The interrelationships between diet and zearalenone toxicosis and metabolism have been investigated in rats and swine (Smith 1980a, 1980b, 1982; James and Smith 1982). Diets containing dehydrated alfalfa meal have been shown to be among the most effective treatments for zearalenone toxicosis. James and Smith (1982) observed that the inclusion of 25% alfalfa meal in purified rat diets largely overcame growth depression, owing to zearalenone and reduced hepatic residues of zearalenone and α -zearalenone, a major metabolite. The feeding of alfalfa to gilts has been shown to reduce uterine enlargement caused by zearalenone (James and Smith 1982).

The mechanism by which dietary alfalfa protects against zearalenone toxicosis has not been determined. It has been proposed that alfalfa may bind zearalenone in the intestinal lumen to reduce absorption of ingested toxin and also limit reabsorption of toxin excreted in bile (Smith 1982). The adsorptive properties of alfalfa may be related to the presence of surface-active saponins as suggested by Oakenfull and Fenwick (1978). It has also been speculated that some components of

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alfalfa may alter the metabolism of zearalenone by hepatic 3 α -hydroxysteroid dehydrogenase (3 α -HSD), thereby altering its toxicity (Smith 1982). The physiological activity of zearalenone may be influenced by coumestrol, a phytoestrogen present in alfalfa. Coumestrol can cause hyperestrogenism in several species if ingested at appropriately high levels (Newsome and Kitts 1980; Kincaid and Browning 1981).

The current studies were undertaken to determine which components of alfalfa meal can protect against zearalenone toxicosis. The effect of these components on the activity of 3 α -HSD was measured to further determine any role of this enzyme in the alleviation of zearalenone toxicosis by alfalfa.

Materials and methods

Experimental animals and control diets

Female weanling Wistar rats (initial weight of 50–55 g, Woodlyn Labs Ltd., Guelph, Ont.) were used in all rat experiments. They were housed in individual stainless steel cages and fed a commercial stock diet for the first 3 days. A semipurified control diet (Table 1) with various modifications was then fed to the rats for 14 days. Feed consumption and weight gain were measured every 3 days and water was supplied *ad libitum*. At the end of the experimental period all rats were killed and various tissues were excised and stored at -65°C for subsequent assay of 3 α -HSD activity.

Yorkshire gilts (8–10 kg initial weight) were used in swine experiments. Animals were housed in individual concrete pens and fed modifications of a corn-soy control diet (Table 1) for 4 weeks. All animals were then killed and tissues were excised and stored at -65°C for subsequent assay of 3 α -HSD activity.

Rats fed alfalfa meal

A total of 60 rats were fed diets containing 0, 15, or 25% dehydrated alfalfa meal with and without 250 mg zearalenone/kg diet (10 rats/diet). Alfalfa was added to diets at the expense of corn starch, casein, and corn oil to keep all diets isocaloric and isonitrogenous (James and Smith 1982).

Rats fed fractionated alfalfa meal

Rats were separated into 14 groups of 10 each and fed the control diet, alfalfa fiber, alfalfa solvent extract, and alfalfa water extract. The various alfalfa fractions were fed at levels corresponding to those found in diets of 15 and 25% alfalfa meal. All diets were supplied with and without 250 mg zearalenone/kg diet and were again kept isocaloric and isonitrogenous.

Rats fed purified components of alfalfa meal

Six experimental diets were fed with and without 250 mg zearalenone/kg diet to a total of 120 rats (10 per diet). These included the control diet, and at levels corresponding to those found in 25% alfalfa meal diets: saponin (Pederson and Wang 1971) (United States Biochemical Corp., Cleveland, OH), coumestrol (Bickoff *et al.* 1969) (Eastman Kodak Co., Rochester, NY), lignin (Smith 1980a) (Reed Paper Co., Quebec, Que.), saponin + lignin, and coumestrol + lignin. It is recognized that these purified compounds were not necessarily derived from alfalfa and may not exactly duplicate the biological activity of the same compounds found in alfalfa.

Swine fed alfalfa meal

Swine were fed diets containing 0, 15, or 25% alfalfa meal with and without 10 mg zearalenone/kg diet. Forty-eight swine were used, eight per diet. Alfalfa was added to the diets at the expense of corn and soybean meal to ensure that all diets were isocaloric and isonitrogenous.

Characterization of 3 α -HSD

The activity of 3 α -HSD was measured in liver, uterus, kidney, and leg muscle of both rats and swine. Initial characterization of 3 α -HSD was conducted on pooled samples of tissue obtained from two groups of 10 rats fed either the control diet or the control diet plus zearalenone. A similar procedure was used for swine. Subsequent

TABLE 1. Composition of control diets (%)

Ingredient	Rats	Swine
Casein (vitamin free) ^a	16.3	—
Corn oil ^a	6.0	—
Corn starch ^b	67.8	—
Mineral mixture	3.5 ^c	0.1 ^d
Vitamin mixture	1.0 ^c	0.25 ^d
Cellulose ^e	5.2	—
DL-Methionine	0.2	—
Corn	—	64.05
Soybean meal (49%)	—	33.0
Iodized salt	—	0.5
Limestone	—	0.9
Dicalcium phosphate	—	1.2

^aTeklad Test Diets, Madison, WI.

^bSt. Lawrence Starch Co., Port Credit, Ont.

^cSmith 1981.

^dSmith 1984.

^eCelufil, United States Biochemical Corp., Cleveland, OH.

assays were conducted on samples from individual animals. Approximately 2 g of tissue was homogenized for 30 s at 0°C in 30 mL of 0.1 M potassium phosphate buffer. The homogenate was then centrifuged at $25\,000 \times g$ for 15 min at 0°C . The supernatant, which contained both soluble and microsomal enzymes, was stored on crushed ice prior to assay. The spectrophotometric method of Koide (1969) was used to determine pH optimum, optimum substrate concentration, cofactor dependency, substrate specificity, and kinetic parameters for the enzyme.

A modification of the radioisotopic assay of Thouvenot and Morfin (1980) was employed to determine the activity of 3 α -HSD in rat liver in the presence of various potential inhibitors. The reaction mixture included potassium phosphate buffer (660 μL of 0.1 M, pH 8.0), 34 μL 0.1 M NAD in buffer, 10 μL ($2-5 \times 10^5$ disintegration per minute) [$1,2-^3\text{H}$]androsterone (New England Nuclear, Boston, MA), 10 μL androsterone in methanol (at various concentrations), 10 μL inhibitor in methanol, and an additional 40 μL methanol to ensure steroid solubility. All reaction mixtures were incubated for 5 min at 37°C and the reactions were then initiated by the addition of 34 μL of supernatants. The reactions were terminated after 15 min by the addition of 5 mL ethyl acetate. The phases of the incubation mixture were allowed to separate over 12 h and the ethyl acetate layer was removed. The aqueous phase was extracted twice more with 5 mL ethyl acetate and the extracts were combined and evaporated to dryness under nitrogen. The residues were dissolved in methanol and aliquots were applied to chromatographic plates coated with silica gel G (Analaek Uniplate, Fisher Scientific Ltd., Toronto, Ont.). Plates were developed in benzene:ethyl acetate (3:1). The positions of the substrate ([$1,2-^3\text{H}$]androsterone) and the product ([$1,2-^3\text{H}$]androsterone) were determined by spraying the plates with anisaldehyde: H_2SO_4 : glacial acetic acid (1:2:100) and heating at 100°C for 10 min. Identification of spots was made by comparison with known standards. Spots corresponding to the product were scraped from the plate and radioactivity was quantified by liquid scintillation spectrophotometry.

Substances tested as inhibitors included coumestrol and α - and β -zearalenone. The latter two compounds were purified from a racemic mixture as described by James *et al.* (1982).

Fractionation of alfalfa meal

Approximately 1 kg of alfalfa meal was extracted with acetone by refluxing for 72 h in a soxhlet apparatus. Acetone was separated from the solvent-extract by distillation. The residue remaining after the acetone extraction was further refluxed for 24 h with boiling water. The residue was then centrifuged at $8000 \times g$ for 5 min and dried. This yielded the alfalfa fiber fraction. A water extract was obtained by refluxing whole alfalfa meal for 24 h with boiling water as described