Production and Characterization of a Specific Monoclonal Antibody against Mycotoxin Zearalenone

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A monoclonal antibody specific for zearalenone (ZEN) was produced by fusion of mouse myeloma cells (NS-1) and splenocytes isolated from BALB/c mice that had been immunized with a novel type of immunogen. The immunogen was prepared by coupling 5-aminozearalenone, which was synthesized from ZEN in two steps, with bovine serum albumin through the amino group at the C-5 position of the compound. The anti-ZEN monoclonal antibody (Ab 7-1-144) thus obtained belonged to the IgG_1 subclass with λ light chain. The association constant of the antibody for ZEN was 1.1×10^8 L/mol. Cross-reaction studies showed that the antibody was highly specific for ZEN. The cross-reactivities of this antibody for zearalanone, ZEN 4-methyl ether, α-zearalenol, and ZEN 2,4-dimethyl ether were 4.0, 2.5, 0.9, and 0.3%, respectively, of that found for ZEN. Practically no cross-reactivity was observed with β-zearalenol, α- and β-zearalanols, and trichothecene mycotoxins including T-2 toxin, nivalenol, and deoxynivalenol. An indirect enzyme-linked immunosorbent assay (ELISA) based on the competitive binding principle was developed for the detection of ZEN using Ab 7-1-144. The response range for ZEN in the present study was between 0.3 and 100 ng/mL (15 and 5000 picograms per assay). The binding inhibition by ZEN was nearly linear in this range.

INTRODUCTION

Zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone] (ZEN) is a secondary metabolite produced by several species of the genus *Fusarium* and is often detected along with nivalenol and deoxynivalenol in barley, wheat, and other cereal grains (Mirocha et al., 1977; Tanaka et al., 1985; Engelhardt et al., 1986; Lee et al., 1986; Golinski et al., 1988). It was first isolated from the metabolites of *F. graminearum* (*Gibberella zeae*) as an estrogenic mycotoxin (Stob et al., 1962). When fed to animals, the compound causes hyperestrogenism including enlargement of the uterus and infertility (Hidy et al., 1977). Because of these estrogenic effects, there is a need for routine screening for this mycotoxin in food for humans and in feeds for animals.

Current methods for analysis of ZEN in food and feeds include thin-layer chromatography (TLC) (Kamimura et al., 1981; Ichinoe et al., 1983), gas–liquid chromatography (Scott et al., 1978), and high-performance liquid chromatography (Bennett et al., 1985). These methods require complicated sample cleanup and technical skill, which hinder their use for routine screening of large numbers of samples for ZEN.

Immunoassays have recently been developed as alternatives to the conventional chemical methods for detection of ZEN (Thouvenot and Morfin, 1983; Liu et al., 1985; Warner et al., 1986; Dixon et al., 1987). All of the anti-ZEN antibodies previously used for immunoassays were obtained by immunization with a 6′-(carboxymethyl)-zearalenone oxime–bovine serum albumin conjugate, and they are more or less cross-reactive with ZEN derivatives such as zearalenol. To measure ZEN in various foodstuffs and feeds specifically, there is a need for production of a specific antibody to this mycotoxin. For this purpose, it seemed worthwhile to try a novel type of immunogen in which the position of conjugation of the ZEN molecule to a carrier protein is different from the C6′ carbonyl group.

We describe here the synthesis of a new type of immunogen and preparation of a specific monoclonal antibody to ZEN.

MATERIALS AND METHODS

Chemicals. ZEN was purchased from Makor Chemical Co. (Jerusalem, Israel). Reduction of ZEN with potassium borohydride led to zearalenol epimers (Shipchandler, 1975), which were separated by preparative TLC (Golinski et al., 1988). The α- and β-epimers of zearalenol were then separately crystallized in ethyl acetate–hexane. Zearalanone was obtained by the hydrogenation of ZEN (Peters and Hurd, 1975) with 10% palladium charcoal. Reduction of zearalanone with potassium borohydride led to a mixture of zearalanol epimers. The isomers were separated by preparative TLC as above. ZEN 4-methyl ether and ZEN 2,4-dimethyl ether were prepared according to the method of Urry et al. (1966). Zearalenol, bovine serum albumin (BSA, fraction VI), and ovalbumin (OVA) were purchased from Sigma Chemical Co. (St. Louis, MO). Complete Freund's adjuvant (FCA) was obtained from Difco Laboratories (Detroit, MI). All chemicals and organic solvents were of reagent grade or better.

5-Nitrozearalenone. ZEN (40 mg) was dissolved in 0.5 mL of acetic acid. To the solution were added 8 μL of concentrat-

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ed nitric acid, 0.15 mL of water, and 0.8 mg of sodium nitrite. The mixture was stirred at room temperature for 2 days. The reaction mixture was then poured into ice–water, neutralized with saturated sodium bicarbonate solution, and extracted with ethyl acetate. The organic phase was washed with water twice and dried over anhydrous sodium sulfate. The solvent was evaporated in vacuo. The oily residue contained mono- and dinitrozearalenone. 5-Nitrozearalenone (8 mg) was obtained by preparative TLC using *n*-hexane–ethyl acetate (1:1 v/v) as a pale yellow amorphous substance. The R_f value for 5-nitrozearalenone under this condition was 0.44 (cf. R_f value for ZEN was 0.49). This compound had the following property: ^{1}H NMR ($CDCl_3$) δ 1.43 (3 H, d, C-11′), 4.95 (1 H, m, C-10′), 5.37 (1 H, m, C-2′), 6.49 (1 H, s, C-3), 7.06 (1 H, d, C-1′, J = 6 Hz), 8.23 (1 H, s, 4-OH), 11.92 (1 H, s, 2-OH).

5-Aminozearalenone (5-NH_2-ZEN). 5-Nitrozearalenone (5 mg) was dissolved in 0.3 mL of acetone. To the solution were added 0.1 mL of water and 200 mg of sodium hydrosulfite. The mixture was neutralized with saturated sodium bicarbonate solution to pH 7.0. After being stirred at room temperature for 2 h, the reaction mixture was extracted with ethyl acetate. The organic phase was washed with water twice and dried over anhydrous sodium sulfate. The solvent was evaporated in vacuo. 5-NH_2-ZEN was obtained from the oily residue by preparative TLC using *n*-hexane–ethyl acetate (1:1 v/v) as a colorless amorphous substance. The R_f value for 5-NH_2-ZEN under this condition was 0.35. This compound had the following property: ^{1}H NMR ($CDCl_3$) δ 1.38 (3 H, d, C-11′), 5.73 (2 H, s, NH_2), 5.75 (1 H, s, 4-OH), 6.43 (1 H, s, C-3), 6.49 (1 H, d, C-1′, J = 2.5 Hz), 12.07 (1 H, s, 2-OH).

Hapten–Protein Conjugates. 5-NH_2-ZEN was conjugated to BSA for use as an immunogen. Briefly, 5 mg of BSA was dissolved in 420 μL of 0.1 M phosphate buffer (pH 6.8), and 2 mg of 5-NH_2-ZEN in 120 μL of methanol was added gradually to the BSA solution. Twenty microliters of 2% glutaraldehyde solution was then added to the solution, and the mixture was stirred overnight at room temperature. The reaction mixture was dialyzed against saline for 3 days.

ZEN was conjugated to OVA by the Mannich reaction (Frankel–Conrat and Olcott, 1948; Miwa et al., 1977) for use as a solid-phase antigen in an indirect ELISA. Briefly, 100 mg of OVA was dissolved in 0.9 mL of 0.3 M sodium bicarbonate; ZEN (2.5 mg) was dissolved in 100 μL of methanol, and the solution was gradually added to the OVA solution. The mixture was adjusted to pH 6 with 3 M sodium acetate. To this solution was added 1 mL of 7.5% formaldehyde. After the air phase was replaced with nitrogen gas, the reaction tube was tightly closed with a glass stopper. The reaction mixture was allowed to stand for 18 h at room temperature with stirring. The solution was then dialyzed against 10 mM sodium phosphate buffered saline, pH 7.2 (PBS), for 3 days.

The molar ratios of conjugated ZEN or 5-NH_2-ZEN to the carrier proteins were determined spectrophotometrically with 6020 used as the molar extinction coefficient at 315 nm. For 1 mol of BSA and OVA, 25 and 2 mol of ZEN were conjugated, respectively.

Immunization. Five female BALB/c mice, 8–10 weeks of age, were injected at 2–3-week intervals at multiple sites on the back with 100 μg of the immunogen (5-NH_2-ZEN–BSA) in 0.1 mL of sterilized PBS which was emulsified with an equal volume of Freund's complete adjuvant. One week after the third injection, serum was collected from the retrobulbar plexus of each mouse, and titers of antibodies were determined by an indirect ELISA. Two mice with high titers to ZEN received another intraperitoneal booster injection without adjuvant, and spleen cells were prepared 3 days later.

Monoclonal Antibody Production. Immune spleen cells (1.5×10^8) and myeloma cells (NS-1, 5×10^7) were mixed and fused with 50% polyethylene glycol 4000. After fusion, the cells were selected with HAT (100 μM hypoxanthine/0.4 μM aminopterin/16 μM thymidine) medium in eight 24-well culture plates. Hybridoma cells in one ELISA-positive well were cloned by limiting dilution. Anti-ZEN production in the supernatants of the ELISA-positive cloned cells was confirmed by radioimmunoassay as described below. The hybridoma cells (clone 7-1-144) were grown as ascitic tumors in BALB/c mice injected with Pristane (Edwards, 1981) 7 days before inoculation.

Indirect ELISA. Fifty microliters of ZEN–OVA (3 μg/mL) in 0.15 M sodium carbonate buffer, pH 9.6, was added to each well of a 96-well microtiter plate and incubated overnight at 4 °C. The solution was discarded, and each well was washed four times with 0.2 mL of PBS containing 0.5% Tween 20 (PBS/Tween). For minimizing nonspecific binding by blocking unoccupied solid-phase sites, 200 μL of 0.1% casein in PBS was added and the plates were incubated for 1 h at 25 °C. The casein solution was removed, and each well was washed as above. Fifty microliters of antiserum or culture supernatant (IgG concentration was more than 200 ng/mL) was added to each well, and the plates were incubated for 1 h at 25 °C. The solution was removed, and each well was washed as before. Fifty microliters of a solution of sheep anti-mouse-IgG–β-galactosidase conjugate (10^{-3} dilution in PBS containing 0.1% casein; Amersham International Plc, Amersham, U.K.) was added to each well, and the plates were incubated for 1 h at 25 °C. The antibody–enzyme conjugate solution was removed, and each well was washed as before. The wells were incubated for 1 h at 37 °C with 100 μL of a PBS solution of 0.1 mM 4-methylumbelliferyl β-galactoside (Sigma). The fluorescence intensity of the liberated 4-methylumbelliferone was monitored by a Titertek Fluoroskan reader (Flow Laboratories Inc.).

For isotyping of the antibody by the indirect ELISA, heavy and light chain specific rabbit antisera (Miles, Elkhart, IN) as second antibodies and β-D-galactosidase-conjugated donkey anti-rabbit Ig (Amersham) as the third antibody–enzyme conjugate were used.

Competitive Indirect ELISA. The procedure for competitive indirect ELISA (CI-ELISA) was essentially the same as that for the indirect ELISA described above except that 50 μL of a sample solution of ZEN or its derivatives dissolved in 10% (v/v) methanol in PBS was simultaneously incubated for 1 h at 25 °C with 50 μL of an appropriately diluted solution of monoclonal antibody in PBS containing 0.1% casein. The amount of the antibody bound to the well was determined as described above.

Radioimmunoassay. Radioimmunoassay was carried out by the ammonium sulfate precipitation method. For the titration of antibodies, a mixture of 100 μL of serially diluted culture supernatant and 100 μL of [^{3}H]ZEN (15 000 dpm, labeled by an exchange reaction with tritiated water in the presence of trifluoroacetic acid, 2.0 Ci/mmol) in 100 mM phosphate buffer (pH 7.2) containing 10% normal rabbit serum was incubated in small centrifuge tubes overnight at 4 °C, and then 200 μL of saturated ammonium sulfate solution was added to each tube. After standing 1 h at 25 °C, the tubes were centrifuged at 3000 rpm for 15 min at 4 °C. The supernatants were discarded, and the precipitates were dissolved in 200 μL of 10 mM phosphate buffer (pH 7.2). The radioactivity was measured with a liquid scintillation counter (Aloka LSC 703).

RESULTS

Immunization of Mice. Antibodies against ZEN were raised in five mice immunized with 5-NH_2-ZEN–BSA emulsified with FCA. The antibody titer of each serum was determined 1 week after the third injection. Titration curves are shown in Figure 1. When the titer was defined as the reciprocal of the dilution that produced a 50% decrease in fluorescence intensity by indirect ELISA, two mice (no. 2 and 4) showed titers of 520 and 380, respectively. All other mice had lower titers. CI-ELISA was therefore performed with the two sera with higher titers (mice 2 and 4). ZEN competed effectively with ZEN–OVA used as a solid-phase antigen for antibodies in the sera (Table I). Therefore, we decided to use spleen cells from both mice for the preparation of hybridomas.

Establishment of Hybridoma. Spleen cells from immunized mice were fused with NS-1 myeloma cells at a ratio of 3:1, and hybridomas were selected in HAT

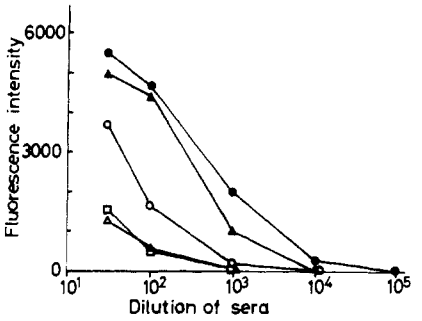


Figure 1. Titration curve of sera of mice immunized with 5-NH_2-ZEN-BSA. The titer of each serum was determined by indirect ELISA. The solid-phase antigen was zearalenone–OVA prepared by the Mannich reaction. Titers of the sera are as follows: 1 (○), 90; 2 (●), 520; 3 (□), 70; 4 (▲), 380; 5 (△), 95.

Table I. Percent Inhibition by Zearalenone of Mouse Antibody Binding to Zearalenone on the Solid-Phase Antigen[a]

	% inhibition			
mouse	1000 ng/mL	100 ng/mL	10 ng/mL	1 ng/mL
2	51.8	20.7	3.5	1.6
4	56.0	40.0	29.0	18.0

[a] Antisera were obtained from mice following three subcutaneous inoculations of 5-NH_2-ZEN-BSA. Determination was carried out by competitive indirect ELISA.

medium. Among 192 cultures seeded with fused cells, 188 yielded hybridoma clones (98%). Culture supernatants were removed and screened for antibodies against ZEN by indirect ELISA. Only one supernatant was ELISA positive.

Hybridoma cells in this culture were expanded and cloned by limiting dilution. Since all ELISA-positive clones thus obtained produced antibodies with essentially the same cross-reactivity, we selected one clone for recloning. From the recloned hybridomas, we selected one clone that secreted a satisfactory amount of antibody and designated it clone 7-1-144.

Characterization of Monoclonal Antibody. The monoclonal antibody (Ab 7-1-144) secreted by clone 7-1-144 was characterized by using a culture supernatant of this clone. The subclass of the antibody was identified as IgG_1 and the light chain as λ chain.

The association constant of Ab 7-1-144 to ZEN was 1.1×10^8 L/mol, as calculated by Scatchard analysis (Scatchard, 1949) after the radioimmunoassay. Ab 7-1-144 was also obtained from the ascites fluid of mice injected with clone 7-1-144 cells. The titer of the ascites fluid was more than 140 000.

The standard curve of the antibody by the CI-ELISA is shown in Figure 2. The detection limit of ZEN was estimated to be 0.3 ng/mL (15 pg/assay). Binding inhibition by ZEN was nearly linear in a range of 1–100 ng/mL, and the upper limit of the measurement was 100 ng/mL.

Cross-reactivity of Ab 7-1-144 was also determined by CI-ELISA. Structures of ZEN and its derivatives used as inhibitors are presented in Figure 3. As shown in Table II, this antibody is highly specific for ZEN and only very weakly cross-reactive with ZEN 4-methyl ether, zearalanone, α-zearalenol, and ZEN 2,4-dimethyl ether. The results obtained with ZEN and α- and β-zearalenol showed that the antibody is able to discriminate functional carbonyl and hydroxy groups at the C6′ position. The low cross-reactivity (4.0%) with zearalanone indicates that the presence of a double bond between C1′ and C2′ is essential

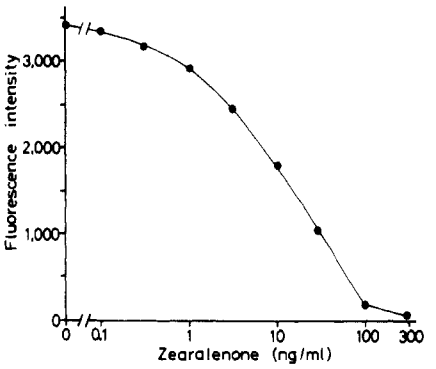
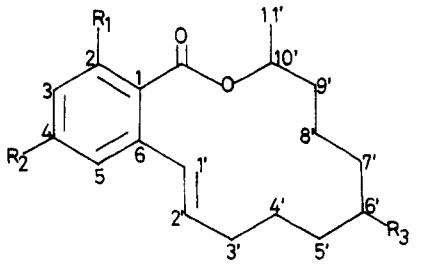


Figure 2. Competitive indirect ELISA standard curve for zearalenone with monoclonal anti-zearalenone antibody (Ab 7-1-144). The solid-phase antigen was zearalenone–OVA. Each point represents duplicate determinations in a single microtiter plate.



Compound	R_1	R_2	R_3	$C_{1'}-C_{2'}$
Zearalenone	−OH	−OH	=O	=trans
α-Zearalenol	−OH	−OH	αOH	=trans
β-Zearalenol	−OH	−OH	βOH	=trans
Zearalanone	−OH	−OH	=O	saturated
α-Zearalanol	−OH	−OH	αOH	saturated
β-Zearalanol	−OH	−OH	βOH	saturated
Zearalenone 4-methyl ether	−OH	$-OCH_3$	=O	=trans
Zearalenone 2,4-dimethyl ether	$-OCH_3$	$-OCH_3$	=O	=trans

Figure 3. Structures of zearalenone and other resorcyclic acid lactones tested for cross-reactivity.

Table II. Cross-Reactivity of the Monoclonal Anti-Zearalenone Antibody

compound	IC_{50}, ng/mL	cross-reaction,[a] %
zearalenone	11.2	100
α-zearalenol	1 258.9	0.9
β-zearalenol	>10 000	<0.1
zearalanone	281.8	4.0
α-zearalanol	>10 000	<0.1
β-zearalanol	>10 000	<0.1
zearalenone 4-methyl ether	446.7	2.5
zearalenone 2,4-dimethyl ether	4 466.8	0.3
nivalenol	>10 000	<0.1
deoxynivalenol	>10 000	<0.1
T-2 toxin	>10 000	<0.1

[a] (IC_{50} of zearalenone/IC_{50} of a test compound) × 100.

for this antibody. The very weak cross-reactivities with ZEN 4-methyl ether (2.5%) and ZEN 2,4-dimethyl ether (0.3%) suggest that this monoclonal antibody recognizes the functional hydroxy groups at C2 and C4.

DISCUSSION

Immunoassay procedures for measuring various mycotoxins have recently been developed because of the simplicity of the procedures for cleaning up the sample

extracts and the capability of routine and simultaneous determination of large numbers of samples with high sensitivity (Harder and Chu, 1979; Lee and Chu, 1981; Pestka and Chu, 1984; Xu et al., 1986). One major critical point of the immunoassay is how to prepare antibodies with the desired specificity. Therefore, monoclonal antibodies have been introduced for measuring mycotoxins (Hunter et al., 1985; Casale et al., 1988; Pauly et al., 1988). Another important problem concerning this point seems to be the method of preparing immunogens.

Both polyclonal (Thouvenot and Marfin, 1983; Liu et al., 1985; Warner et al., 1986) and monoclonal (Dixon et al., 1987) antibodies against ZEN have been prepared by using immunogens in which ZEN was conjugated to carrier proteins by taking advantage of the reactivity of the keto group at the C6′ position. These antibodies did not satisfactorily discriminate zearalenol (C6′ OH) from ZEN (C6′ keto), though they could be used for the determination of ZEN.

Therefore, we used 5-aminozearalenone as a hapten. This compound was prepared by the nitration of ZEN followed by its reduction to the amino group.

Although the mice immunized with this immunogen raised antibodies that were not strictly specific for ZEN (data not shown), a hybridoma clone prepared by the fusion of spleen cells from these mice with NS-1 cells produced a monoclonal antibody with high specificity for ZEN. On the basis of the concentration required to give 50% maximal fluorescence intensity in the competitive ELISA, the cross-reactivities of the monoclonal antibody (Ab 7-1-144) for ZEN, ZEN 4-methyl ether, α-zearalenol, and ZEN 2,4-dimethyl ether were 4.0, 2.5, 0.9, and 0.3% of that found for ZEN, respectively. These results suggest that the antibody recognizes hydroxy groups at C2 and C4, the keto group at C6′, and the double bond at C1′ to C2′. The strict specificity of Ab 7-1-144 for ZEN suggests that this antibody could be useful for the determination of ZEN in agricultural commodities. The standard curve of the CI-ELISA with the present antibody (Figure 2) indicates that the sensitivity for ZEN is 0.3 ng/mL. The sensitivity obtained from this assay was more than that described in previous papers about high-performance liquid chromatography (Bennett et al., 1985) or ELISA (Dixon et al., 1987).

When the monoclonal antibody was applied to the competitive indirect ELISA of ZEN in spiked (20–2000 ng/g) barley, the average recovery was 102%. And the levels of ZEN detected in 10 naturally contaminated samples were comparable to those determined by liquid chromatography (unpublished data). The results suggest that this monoclonal antibody is suitable for quantitative screening of ZEN in naturally contaminated barleys.

Our study indicates that this antibody is also applicable to radioimmunoassay and could measure the toxin at concentrations between 0.1 and 100 ng/mL.

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