Novel Enzyme Immunoassays for Specific Detection of *Fusarium oxysporum* f. sp. *cucumerinum* and for General Detection of Various *Fusarium* Species

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A part of this work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.
Accepted for publication 11 August 1988.

ABSTRACT

Kitagawa, T., Sakamoto, Y., Furumi, K., and Ogura, H. 1989. Novel enzyme immunoassays for specific detection of *Fusarium oxysporum* f. sp. *cucumerinum* and for general detection of various *Fusarium* species. Phytopathology 79:162-165.

Competitive types of two novel enzyme-linked immunosorbent assays (ELISA) for *Fusarium* species were developed. Antiserum against a strain (F504) of *F. oxysporum* was elicited in rabbits, and a highly specific, sensitive, and accurate ELISA for the homologous strain was developed by using the antiserum with β-D-galactosidase-labeled anti-rabbit IgG as the secondary antibody and cell fragments of the strain attached to Amino-Dylark balls as the solid-phase antigen. All other microorganisms tested, including nine other strains of *Fusarium*, showed little cross-reactivity. When cell fragments of *F. oxysporum* F501 attached to the balls were used as a solid-phase antigen in a heterologous competitive ELISA, the modified system was a general assay for 10 strains of four *Fusarium* species with a high sensitivity. The specificity of the heterologous competitive ELISA was shown to be limited to *Fusarium* species. The reason for developing the improved ELISA is also presented.

Additional keywords: competitive assay, crop plants, enzyme immunoassay, general assay of fusarial species, pathogenic fungi, specific assay.

The genus *Fusarium* includes a tremendous variety of pathogenic fungi that infect a wide range of crop plants and numerous nonpathogenic saprophytes (16). Some are airborne pathogens and others are soilborne. The presence of soilborne *Fusarium* spp. is one of the main impediments to continuous cropping of fields (15). Because no serological method has been successful, *Fusarium* is identified by morphological characteristics. Distinguishing pathogenic and saprophytic isolates of the same species has not been easy. Some *Fusarium* species are divided into *formae specialis*, or races, based on their differential ability to parasitize specific hosts (2). In addition, some *Fusarium* species produce phytotoxins or hormones such as mycotoxins (16), zearalenone (17,25), butenolide (28), fusaric acid (11,26), and gibberelins (27). These contaminants present problems because of their toxicity to animals fed infected crops (18,21). Consequently, a quick diagnostic method for the simple detection of *Fusarium* contaminants in crops to determine the race of the fungus or to measure quantitatively any species of *Fusarium* would be of value to ensure the quality of animal feed and to study the occurrence of *Fusarium* in farm fields.

Progress in the development of enzyme immunoassays (EIA) has been rapid (3,7,9,12). An EIA is a useful means for quick diagnoses of various microorganisms, such as viruses and bacteria, that can be prepared in homogeneous suspensions (4–6,8,10). Few EIA have been developed for fungi because these do not form homogeneous suspensions. However, a novel and rapid diagnostic method for *Fusarium* has been developed in our laboratory. In the present article, we report two highly sensitive and accurate EIAs for *Fusarium* species. One is a specific assay for *F. oxysporum* f. sp. *cucumerinum* F504, and the other assay detected 10 strains of common *Fusarium* species that show specific pathogenicities to different plants.

MATERIALS AND METHODS

Reagents. β-D-Galactosidase (GAL)-labeled goat anti-rabbit IgG was prepared as previously (23). Commercial Amino-Dylark balls (6 mm diameter) (23,24), prepared from ethylenediamine and partial hydrolysate of Dylark (22), the co-polymer of styrene and maleate, were purchased from Sekisui Chem. Ind., Mishima-gun, Osaka, Japan. Other chemicals used were of reagent grade.

Media. Buffers were 0.02 M sodium phosphate-buffered saline (PBS), pH 7.0; buffer A (PBS, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin, and 0.1% NaN_3); buffer B (0.05 M sodium phosphate, pH 7.4, containing 0.01 M ethylenediaminetetraacetic acid and 0.1% bovine serum albumin). For potato-sucrose broth, potatoes were cut into 1-cm cubes, and 200 g of potato cubes was boiled with 1 L of water for 20 min, mashed, and squeezed through a muslin bag. Sucrose (20 g) was dissolved in the above extract, which was made up to 1 L with water, adjusted to pH 5.6, and then autoclaved.

Strains tested. The 10 strains of *Fusarium* and 15 strains of other microorganisms used included: *Fusarium oxysporum* f. sp. *cucumerinum* F501, *F. o. cucumerinum* F504, *F. o. melonis* F401, *F. o. lycopersici* F1001, *F. o. batatas* F1201, *F. oxysporum* F105 (saprophyte), *F. solani* f. sp. *pisi* F2001, *F. moniliforme* F2501, *F. roseum* F103, *F. roseum* F3301, *Aspergillus niger, A. flavus, Chaetomium* sp., *Myrothecium* sp., *Microthecium* sp., *Penicillium frequentans, P. charlesii, Trichoderma harzianum* (the stock cultures of Kochi University), *Escherichia coli* K12, *Xanthomonas campestris* pv. *citri, Streptomyces scabies* Obama, *S. scabies* Higashihara, *Pyricularia oryzae* 037 Ken 60-19, *P. oryzae* 001 Kyu 8205A, and *P. oryzae* Ine 72.

Preparation of cell fragments of *Fusarium* species. Each isolate of *Fusarium* was cultured in potato-sucrose broth for 1 wk with shaking and harvested by centrifugation. The pellet was washed twice with water before lyophilization. A suspension of the lyophilized cells of *F. oxysporum* F504 in PBS was placed in a sonic disruptor (Branson Sonifier, model W 185, USA) at 60 W for 15 min in an ice-water bath, and cell wall fragments were collected by centrifugation at 800 *g* for 15 min. The suspension of the pelleted fragments was used for immunization of rabbits as well as for preparing solid-phase antigens.

Immunization. Two rabbits were given subcutaneous and intramuscular injections at 10 points with a 1-ml suspension of the fragments of *F. oxysporum* F504 (1 mg/ml of PBS) emulsified in an equal volume of incomplete Freund's adjuvant. Four booster injections of one half the original dose were given in the same way at biweekly intervals. The rabbits were bled from the ear veins 2 wk

after each injection. The sera were stored at −30 C. Preimmune sera were collected for controls.

Preparation of the solid-phase antigen. Amino-Dylark balls were immersed in 1% glutaraldehyde for 1 hr with mechanical shaking and then washed with PBS. One hundred balls were immersed in a suspension of cell fragments of *F. oxysporum* (3 μg/ml) in PBS at 25 C for 30 min and then incubated at 4 C for 2 hr. After two washes with buffer B, Amino-Dylark balls linked with cell fragments were stored in buffer B at 4 C.

Competitive immunoassay procedures. A suspension of different amounts of the cell fragments of *F. oxysporum* (100 μl) and 100 μl of a 30,000-fold diluted anti-*Fusarium* serum (anti-FO) in buffer B was incubated at 25 C for 1 hr. An Amino-Dylark ball coated with the cell fragments of *F. oxysporum* was added to the tube and then incubated at 25 C for 1 hr with mechanical shaking. After two washes with 1-ml portions of buffer A, the ball was incubated at 25 C for 3 hr with 0.2 ml of buffer A solution of GAL-labeled goat anti-rabbit IgG (200 μU/100 μl) with mechanical shaking. Each ball was washed with 1 ml of buffer A and then transferred to a fresh tube to eliminate nonspecific enzyme activity bound to the wall of the test tube. The GAL activity bound to the ball was assayed.

Measurement of GAL activity. The GAL activity was measured by a modification of published methods (13,23). The Amino-Dylark balls were incubated with 0.2 ml of 0.1 mM 7-β-D-galactopyranosyloxy-4-methylcoumarin in buffer A at 30 C for 30 min to measure the bound-enzyme activity. The reaction was stopped by adding 2 ml of 0.2 M glycine-NaOH buffer, pH 10.6, and the 7-hydroxy-4-methylcoumarin liberated was measured with a fluorometer at 365 and 448 nm as excitation and emission wave lengths, respectively. The amount of GAL-labeled anti-rabbit IgG was expressed in units of GAL activity, and 1 U of the enzyme activity was defined as the amount that hydrolyzes 1 μmol of the substrate per minute.

RESULTS

Antibody response. Antibodies against *F. oxysporum* F504 (anti-FO antibody) were produced in each of two rabbits immunized with F504 cell fragments. The antibodies were trapped by the reaction between the diluted anti-FO antiserum and the immobilized cell fragments of strain F504 of *F. oxysporum* on Amino-Dylark balls. The reaction of the GAL-labeled anti-rabbit IgG tracer with the anti-FO attached to cell fragments on the balls quantitated bound anti-FO antibody. Typical binding curves are shown in Figure 1 for serum from one of the two rabbits.

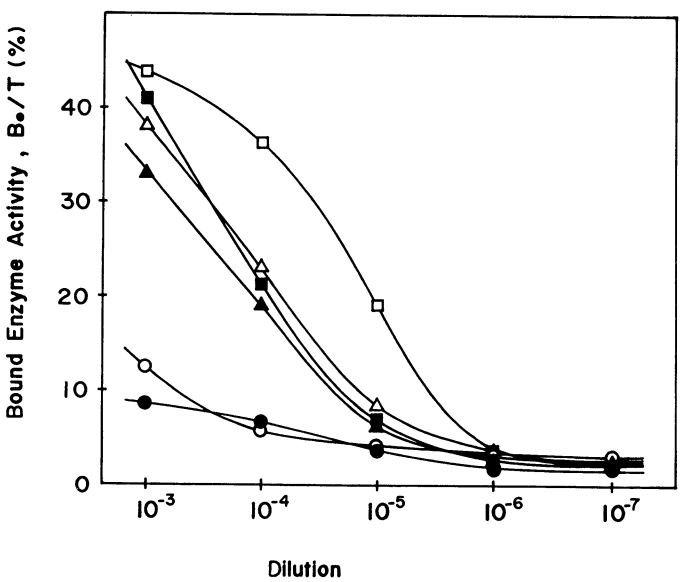

Fig. 1. Quantitative estimation of anti-*Fusarium oxysporum* antibody. Samples of antiserum were collected at 2 (○), 4 (▲), 6 (■), 8 (△), and 10 (□) wk after the initial injection. Closed circles represent bleeding before immunization. The samples of serum were diluted with buffer B and then incubated with Amino-Dylark balls coated with cell fragments. Rabbit antibody bound to the solid-phase antigen was detected by the galactosidase (GAL) activity, with GAL-labeled goat anti-rabbit IgG as the tracer. The curves show the percentage of bound enzyme activities for variously diluted solutions of antisera (B_0) as the ratio of the total enzyme activity of GAL-labeled tracer (T) used.

The antibody titer increased gradually with booster injections and reached a maximum 2 wk after the final injection. An additional booster gave a slight reduction in the titer. Use of preimmune serum showed little nonspecific binding to the cell fragments.

Determination of amount of antigen used for solid-phase antigen and amount of time needed for sonic disruption. Values for nonspecific binding of the diluted solutions of the preimmunation serum to solid-phase antigens (prepared from a 3 μg/ml cell suspension) showed marked differences depending on the time of sonic disintegration of the cells (Table 1). Nonspecific binding was smallest after 15 min of sonic treatment, whereas specific binding of anti-FO antibody to the solid-phase antigens depended on the concentration of lyophilized cells used but not on the duration of sonic disintegration. As a consequence, sonification of a 3 μg/ml fungus hyphae for 15 min was chosen as the standard procedure for preparing the solid-phase antigen (a dilution of 3×10^{-4} of anti-FO was chosen), since this preparation gave sufficient specific binding and relatively small background binding in the EIA (Table 1).

Homologous competitive enzyme-linked immunosorbent assay (ELISA): Specific assay for *F. oxysporum* f. sp. *cucumerinum* F504. A competitive type ELISA for *F. oxysporum* F504 was studied. After extensive trials, an assay procedure involving a three-step process was chosen. Typical dose response curves for ELISA of *F. oxysporum* F504, as well as F501, were determined using the 3 μg/ml suspension of disintegrated cells of strain F504 as the solid-phase antigen; the results are shown in Figure 2. The measurable range for strain F504 was 10–1,000 ng/ml. Although *F. oxysporum* F501 is a strain closely related to strain F504, 100 μg/0.1 ml of strain F501 showed little inhibition of the binding of anti-FO antibody to the solid-phase antigen (Fig. 2). All eight other *Fusarium* species showed little cross-reactivity with this ELISA; their dose response curves were similar to that with strain F501 (data not shown).

The results for repeatability of the ELISA for the strain F504 are summarized in Table 2. Good recoveries (98–106%) were obtained for samples from five different dose levels of *F. oxysporum* F504, with coefficients of variation being less than 24% for within- and between-day assays (deviation among multiple assays of the same sample measured the same day and on different days, respectively).

Heterologous competitive ELISA: General assay of 10 *Fusarium* species. Disintegrated fragments of the heterologous strain F501 cells, immobilized on the Amino-Dylark balls, bound

TABLE 1. Bound galactosidase (GAL)-activities measured with reaction of two dilutions of anti-FO or normal rabbit serum with the solid-phase cell wall fragments of *F. oxysporum* F504 after sonication three times, with GAL-labeled goat anti-rabbit IgG as the second antibody

Concentrations (μg/ml cells)	Sonification (min)	GAL activity: Anti-FO dilution (10^{-4})	(3×10^{-4})	Preimmune dilution[a] (10^{-4})	(3×10^{-4})
1	15	170	99	NT[b]	
	20	164	106	NT	
	30	170	111	NT	
3	15	231	130	33	21
	20	223	134	50	42
	30	219	123	89	60
10	15	247	152	NT	
	20	251	137	NT	
	30	226	138	NT	

[a] Preimmunation serum of the rabbit was used instead of anti-FO.
[b] NT = not tested.

sufficient anti-FO antibody for detection. The dose-response curves for the competitive ELISA of three *Fusarium* species with F501 cell fragments as the solid-phase antigen are shown in Figure 3. The measurable range of 10 ng to 10 μg/ml for the homologous strain F504 is wider than that of the original assay. The heterologous ELISA, however, provides a way to assay all 10 strains of the fusarial species and *forma specialis*. Cross-reactivity values at B/B_0 50% value, a common index used for cross-reactivity (1), as well as the measurable ranges of antigen concentration for ELISA, are summarized in Table 3.

The results of the repeatability tests using strain F501 are summarized in Table 4. Because the dose response curve is gently sloped, the measuring range is wide and the obtained recoveries are between 95 and 116% for nine samples of *F. oxysporum* F501, with coefficients of variation being less than 71.8% for within-day assay.

Specificity of the modified ELISA. Dose response curves for the 10 strains of *Fusarium* species measured by the heterologous competitive assay were similar within the same measuring range (Table 3). This assay is specific for *Fusarium* species. Other microorganisms such as *E. coli, S. scabies, X. campestris* pv. *citri*, and 11 strains of fungi of nine species; three strains of *Pyricularia oryzae*, two species of *Aspergillus, Chaetomium* sp., *Myrothecium* sp., *Microthecium* sp., two species of *Penicillium*, and *T. harzianum*, showed extremely low cross reactivities (Table 3).

DISCUSSION

Successful immunoassays have so far been limited to applications involving water-soluble or homogeneous suspensions of antigens or antibodies. Although most bacteria are insoluble in water, they make homogeneous suspensions with extremely fine particles, which allow direct assay by an ELISA method (4–6,8,10,14,19). Fungal species consist of insoluble large mycelia, which are difficult to convert into a homogeneous suspension. It is difficult to effectively use the mycelia directly for ELISA.

An ELISA of *F. oxysporum* F504 was developed by means of a competitive-type ELISA. This assay allowed us to use a highly diluted solution of anti-FO as an immune reagent, which may have been responsible for the low background value in the assay.

The optimal incubation times for all steps of the competitive ELISA of *F. oxysporum* F504 were studied, with cell fragments of the same strain attached to Amino-Dylark balls. Under optimal conditions, this assay is able to measure 1–100 ng of cell fragments of strain F504 in 0.1 ml of a sample solution. This assay was specific for strain F504 and showed little cross-reactivity with nine other strains of *Fusarium* species, including *F. oxysporum* F501. F501 possesses pathogenicity against cucumber similar to that of strain

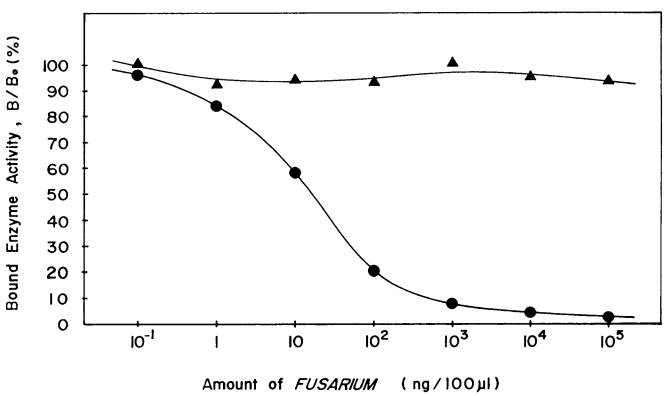

Fig. 2. Dose response curves of the homologous competitive ELISA for strains F501 (▲) and F504 (•) of *Fusarium oxysporum* with suspensions (3 μg/ml) of the disintegrated cells of *F. oxysporum* F504 as the solid-phase antigen. The curves show the amount of bound enzyme activities for various log doses of competing *F. oxysporum* F504 or F501 (B) as a percent of bound enzyme without a competing antigen (B_0).

TABLE 2. Repeatability of within- and between-day assays for homologous enzyme immunoassay (EIA) of *F. oxysporum* F504, with cell fragments of F504 used as the solid-phase antigen

Added amount (ng/tube)[a]	Amount estimated (ng)	
	Within-day assay[b]	Between-day assay[b]
1	1.06(106.0)[c]±0.24[d](24.0)[e]	1.02(102.0)±0.24(24.0)
3	2.99(99.7)±0.24(8.1)	2.98(99.2)±0.54(18.1)
10	9.88(98.8)±0.52(5.2)	10.17(101.7)±1.53(15.0)
30	30.16(100.1)±0.74(2.4)	30.14(100.1)±2.3(7.6)
100	100.3(100.3)±1.83(1.8)	99.4(99.4) ± 5.5(5.5)

[a] A 0.1-ml aliquot of a sample solution was used per tube.
[b] Five experiments were made.
[c] Number in parentheses indicates recovery (%).
[d] Mean ± SD.
[e] Number in parentheses indicates coefficient of variation (%).

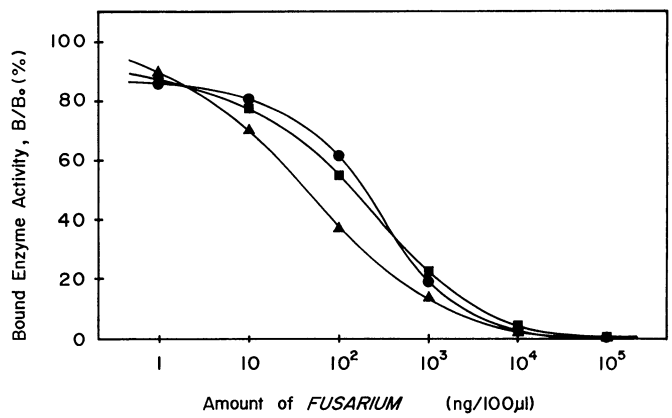

Fig. 3. Typical calibration curves of the heterologous competitive ELISA for three *Fusarium* species with the use of Amino-Dylark balls loaded with cell fragments of *F. oxysporum* F501. Bound enzyme activity is plotted against log doses of *F. oxysporum* f. sp. *cucumerinum* F501 (•) and F504 (■) and of *F. roseum* F3301 (▲). B = activity of competing bound enzymes, B_0 = activity of bound enzyme without a competing antigen.

TABLE 3. Cross-reactivity values of various microorganisms of the heterologous ELISA with measurable ranges of the assay for various strains of *Fusarium* species[a]

Strain	Cross-reactivity (B/B_0 50%)	Measurable range (ng/tube)
F. oxysporum f. sp. *cucumerinum* F504	100.0	1–100
F. oxysporum f. sp. *cucumerinum* F501	135.7	1–100
F. oxysporum F105	58.6	1–100
F. oxysporum f. sp. *melonis* F401	78.6	1–100
F. oxysporum f. sp. *lycopersici* F1001	100.0	1–100
F. oxysporum f. sp. *batatas* F1201	114.3	1–100
F. solani f. sp. *pisi* 2001	164.3	1–100
F. moniliforme F2501	178.6	0.1–100
F. roseum F103	65.7	1–100
F. roseum F3301	26.4	1–100
Escherichia coli K 12	<0.001	
Xanthomonas campestris pv. *citri*	<0.001	
Streptomyces scabies Obama	<0.001	
S. scabies Higashihara	<0.001	
Pyricularia oryzae 037 Ken 60-19	<0.001	
P. oryzae 001 Kyu 8205A	<0.001	
P. oryzae Ine 72	<0.001	
Aspergillus niger	<0.001	
Aspergillus flavus	<0.001	
Chaetomium sp.	<0.001	
Myrothecium sp.	<0.001	
Microthecium sp.	<0.001	
Penicillium frequentans	<0.001	
Penicillium charlesii	<0.001	
Trichoderma harzianum	<0.001	

[a] The values were determined using anti-FO and Amino-Dylark balls coated with cell fragments of *F. oxysporum* F501.

TABLE 4. Repeatability of within-day assay for heterologous ELISA of *F. oxysporum* F501 with use of the cell fragments of F501 as the solid-phase antigen

Amount added (ng/tube)[a]	Amount estimated (ng)[b]
1	1.16 (116)[c] ± 0.8[d] (71.8)[e]
3	2.90 (96.7) ± 0.95 (38.5)
10	9.91 (99.1) ± 2.9 (29.0)
30	30.7 (102.3) ± 3.3 (10.7)
100	103.2 (103.2) ± 22.3 (21.7)
300	286.9 (95.6) ± 64.6 (22.5)
1,000	1,025 (102.5) ± 123 (12.1)
3,000	3,117 (103.9) ± 521 (16.7)
10,000	10,370 (103.7) ± 2,680 (25.8)

[a]Sample concentration was ng/0.1 ml.
[b]Seven experiments were made.
[c]Number in parenthesis indicates recovery (%).
[d]Mean ± SD.
[e]Number in parentheses indicates coefficient of variation (%).

F504, although slight differences have been observed between F501 and F504 regarding their spore formation and pigment production (20).

Although strain F501 showed little cross-reactivity in the homologous competitive assay, cell fragments of strain F501 absorbed on Amino-Dylark balls possessed sufficient immune activity against anti-FO antibody to use in a heterologous ELISA for general detection of *F. oxysporum* and other fusaria. The dose response curve for the homologous competitive ELISA of *F. oxysporum* F504 with the same strain as the solid-phase antigen was steep, with a narrow measurable antigen range, while that for the heterologous competitive ELISA of the same strain sloped gently over a wide measurable antigen range (10–10,000 ng/ml, Fig. 3). The heterologous assay allowed us to measure all 10 *Fusarium* species with high sensitivity, since dose response curves were similar to the curve for strain F504.

The principle of the immunoassay developed in this study uses the specific binding of rabbit antibody to a solid-phase antigen as the primary immune reaction. An immune equilibrium state established between the antigen to be assayed and the solid-phase antigen against the antibody in an aliquot of antiserum is detected using GAL-labeled goat anti-rabbit IgG antibody. Because the solid-phase antigen and the antigens to be assayed compete for the primary antibodies, use of a heterologous antigen on the solid phase should cause a decrease in the amount of antibody bound to the solid-phase antigen, because more antibody is bound to the assaying antigen in the mixture. These competitive assays were very sensitive to changes in the bound antigen. In fact, selection of an appropriate solid-phase antigen expanded the assay from an assay specific for a single antigen to an assay that reacted with all fusarial isolates.

Because infection of crops by *Fusarium* occurs mainly in soil and the soil contains numerous microorganisms, the specificity of the heterologous competitive ELISA for *Fusarium* species, which may allow direct assay of the soil, would provide a valuable new tool for precise study of infections of various plants caused by *Fusarium* species. Application of this ELISA for study of fusarial infections of crop plants is currently under way, and the results will be reported in the near future.

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