Development of an Enzyme-Linked Immunosorbent Assay for Triazole Fungicides

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A competitive enzyme-linked immunosorbent assay (ELISA) for some triazole fungicides was developed using a polyclonal antibody generated against the hemisuccinate of 2-(2,4-dichlorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propanol (DTP) conjugated to bovine serum albumin. In a typical standard curve of DTP the linear range of the test system extended from 10 ± 0.45 to 1200 ± 88.50 ng/mL. The commercially important triazole fungicides, tetraconazole and penconazole, gave cross-reactivities of 150 and 140%, respectively, as compared with that of the standard analyte, showing a linear range for detection from 12 to 850 ng/mL. The reactivities of the antibody produced against other fungicides of the triazole family and fungicides containing either an imidazole, a pyrimidine, or a pyridine group instead of the basic moiety of the triazoles have been tested. The results are discussed by comparing the specificity of the assay with the structural properties of the fungicides tested.

INTRODUCTION

The fungicides of the triazole family represent a class of chemicals known as inhibitors of sterol biosynthesis in phytopathogenic fungi (Scheinpflug and Kuck, 1987; Berg and Plempel, 1988). They are registered for use on several crops, including cereals, in European countries and in the United States.

These commercial fungicides contain as a basic structural moiety 1,2,4-triazole and a phenyl group bearing one or two halogens in positions 2 and 4 connected by a short chain (one to three atoms). A third group (R) bound to this chain characterizes each commercial chemical belonging to this class of fungicides.

The problem of monitoring the residual level of pesticides in foodstuff requires the development of rapid and sensitive procedures to quantify pesticide residues, as they are often present at very low concentrations. The classical methods of pesticide analysis include GLC or HPLC. Since extensive cleanup procedures are needed, these methods are long and tedious (Corley et al., 1974; Monnem and Mumma, 1981) and have limitations in terms of high analytical throughput. The potential of immunoenzymatic assays in pesticide trace analysis is becoming widely appreciated since this analytical technique offers many advantages including sensitivity and speed (Hammock and Mumma, 1980; Van Emon et al., 1989; Jung et al., 1989).

As the commercially important triazole fungicides constitute a class of structurally related compounds, they should be in principle amenable to the determination by immunoassay as a chemical group. To raise an antibody with potential recognition for many triazoles, we have generated an immunogen derived from the basic structural moiety of the triazole chemicals.

The triazole fungicides do not contain any reactive functional groups in a position available for conjugation to carrier proteins. Furthermore, the choice of hapten and the conjugation procedure used affects the ultimate sensitivity and specificity of the immunochemical assay. The importance of the site of conjugation of the hapten to a protein has been extensively demonstrated with pharmaceuticals, insecticides, and herbicides (Flynn and Spector, 1972; Wing et al., 1978, 1980; Goodrow et al., 1990; Harrison et al., 1991).

In the present work, we report the development of an ELISA test for the analysis of triazole fungicides by using a polyclonal antibody generated against the hemisuccinate of 2-(2,4-dichlorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propanol (DTP) conjugated to bovine serum albumin.

MATERIALS AND METHODS

Reagents. Peroxidase-conjugated goat anti rabbit IgG, bovine serum albumin (BSA), hen ovotransferrin (conalbumin, CON), and *o*-phenylenediamine were purchased from Sigma Chemical Co. (St. Louis, MO). All of the chemicals used were of reagent grade from Aldrich, Carlo Erba, and Fluka. HPLC solvents were purchased from Carlo Erba. Tetraconazole and DTP were supplied by Agrimont (Novara, Italy). The other fungicides were supplied by Labservice (Bologna, Italy). BGM-75 was synthesized and tested for fungicidal activity as previously described (Arnoldi et al., 1983).

Hapten Synthesis. Succinic anhydride (0.94 g, 9.4 mmol) was added in small portions to a mixture of DTP (2 g, 7.8 mmol) and 4-pyrrolidinopyridine (62 mg, 0.04 mmol) in anhydrous pyridine (10 mL) at 67 °C in 1 h. The mixture was heated at 95 °C for 4 h, and then the solvent was removed in vacuo. The oily residue was dissolved in dichloromethane (40 mL). The organic layer was washed with 1 N HCl (3 × 20 mL) and water (1 × 20 mL), dried, and concentrated in vacuo. The oily residue was crystallized twice from ethyl ether (50 mL) giving 2.14 g (74% yield) of the hemisuccinate of DTP (FF18) as a white solid: mp 106–108 °C; NMR ($CDCl_3/D_2O$) δ 2.65 (4 H, s, $COCH_2CH_2CO$), 4.0–4.3 (3 H, m, NCH_2CHAr), 4.4–4.8 (2 H, m, CH_2O), 7.0–7.5 (3 H, arom), 7.88 and 8.05 (2 s, 1,2,4-triazole). Anal. Calcd for $C_{15}H_{15}Cl_2N_3O_4$: C, 48.40; H, 4.06; N, 11.29. Found: C, 48.34; H, 4.10; N, 11.24.

Hapten Coupling. The hapten (FF18), hemisuccinate of 2-(2,4-dichlorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propanol (33 mg, 0.089 mmol), was dissolved in 1 mL of dry dioxane. To this solution were added tri-*n*-butylamine (26 μL, 0.107 mmol) and then isobutyl chloroformate (14 μL, 0.107 mmol). The resulting mixture was stirred at room temperature for 40 min and then added dropwise to a solution of bovine serum albumin (100 mg) in 10 mL of 0.2 M borate buffer, pH 8.7, and gently stirred at the same temperature overnight.

The same procedure was used to conjugate FF18 to conalbumin but with the following reagents: hapten (12 mg, 0.032 mmol), tributylamine (8.8 μL, 0.036 mmol), and isobutyl chloroformate (4.8 μL, 0.036 mmol) in dry dioxane (1 mL).

The conjugates were dialyzed extensively against water and then lyophilized. This procedure resulted in the covalent binding of 23 mol of hapten/mol of BSA and 5 mol of hapten/mol of CON, as judged from the colorimetric determination of free amino groups of the proteins by trinitrobenzenesulfonic acid (Habeeb,

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1966) and from HPLC analysis of DTP after acidic hydrolysis of the conjugates.

Immunization Procedure. Antibodies to FF18 conjugated to BSA (BSA–FF18) were raised in New Zealand rabbit by subcutaneous injection in multiple sites of 1.0 mg of BSA–FF18 dissolved in 1 mL of phosphate-buffered saline (PBS; 15 mM KH_2PO_4, pH 7.4, 0.15 M NaCl) and emulsionated with 1.0 mL of complete Freund's adjuvant (Sigma). Four secondary immunizations were performed with 2 mg of BSA–FF18 in the presence of incomplete Freund's adjuvant at 21-day intervals. The rabbit was bled 10 days after the last injection and blood collected. Coagulum was allowed to form at 4 °C overnight. The serum was recovered by centrifugation at 2500*g* for 30 min. IgG was purified by 50% ammonium sulfate precipitation repeated twice. The pellet was resuspended in the same volume as the original serum (150 mL) in PBS and then extensively dialyzed against the same buffer. The antiserum was divided in aliquots and stored at −20 °C. The same procedure was used to prepare a sample of serum from blood collected 15 days before the first injection with BSA–FF18 (preimmune serum).

Antibody Purification. Two milliliters of anti BSA–FF18 serum (ca. 16 mg of protein) diluted 1:3 (v:v) in 15 mM KH_2PO_4 buffer, pH 7.4, was absorbed on a protein A–Sepharose CL-4B (Sigma) column (1 × 7.5 cm) equilibrated in the dilution buffer. After the column was washed with the same buffer at the flow rate of 0.4 mL/min, until no absorbance at 280 nm was detected, the antibody was eluted with 0.1 M sodium citrate buffer, pH 3.5, at the flow rate of 0.7 mL/min. The eluted fractions (1.5 mL each) were immediately brought to pH 7.4 by addition of 1 M Tris-HCl buffer, pH 8.5 (0.5 mL). The recovered antibodies were chromatographed on a Sephadex G-25 column (2 × 12 cm) equilibrated in PBS, and the eluted fractions were pooled (0.13 mg/mL protein concentration as determined by the absorbance at 280 nm) and stored as aliquots of 5 mL at −20 °C. The antibody titer was determined by using different dilutions of the purified anti BSA–FF18 antibody in a noncompetitive solid-phase immunoassay in which the polystyrene plates were coated with FF18 conjugated to conalbumin (CON–FF18).

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA was carried out according to the method of Voller et al. (1979), with some modifications. The polystyrene plates (Nunc Immunoplate) were coated by overnight incubation at 4 °C with a solution of FF18 conjugated to conalbumin (CON–FF18) in 0.1 M sodium carbonate buffer, pH 9.6 (0.2 μg/mL, 0.1 mL/well). The coated plates were washed three times with PBS containing Tween 0.2% (PBS–Tween) and then saturated with 3% gelatin in PBS (0.15 mL/well). After incubation for 2 h at 37 °C, the nonbound material was removed by three washes with PBS–Tween, and 50 μL of PBS containing 0.05% Tween and 0.1% gelatin (PBS–Tween–gelatin) was added to each well. DTP or other triazole chemicals (2 μL of solution in ethanol at concentrations ranging from 0.024 to 25 mg/mL) and anti BSA–FF18 antibody (50 μL diluted 1:250 in PBS–Tween–gelatin) were added and allowed to incubate at 37 °C for 4 h. After extensive washing with PBS–Tween, the goat anti rabbit Ig–peroxidase conjugate diluted in PBS–Tween–gelatin (1:10 000; 0.1 mL/well) was added. After three further washes, peroxidase activity was measured following the addition of 0.2 mL of a 0.32 mg/mL solution of *o*-phenylenediamine in 0.1 M phosphate–citrate buffer, pH 5.0, containing 0.03% H_2O_2. The reaction was stopped 5–10 min later by addition of 0.05 mL of 4 M H_2SO_4, and absorbance at 490 nm was measured in a Bio-Rad 3550 microplate reader interfaced with an IBM PC. Background binding was assessed by a blank in which the coating of antigen was omitted.

HPLC Analysis. Standard solutions of tetraconazole in ethanol (Merck, analytical grade) were analyzed by HPLC on an apparatus equipped with a Hewlett-Packard 1050 quaternary pump, a Rheodyne injector, and a Waters Lambda-Max Model 481 UV–vis detector. The analytical conditions were as follows: column Merck Hibar RP18, 5 μm (4 mm × 250 mm), solvent methanol/water 70/30, flow rate 0.7 mL/min. Under these standardized conditions tetraconazole has a retention time of 6.4 min.

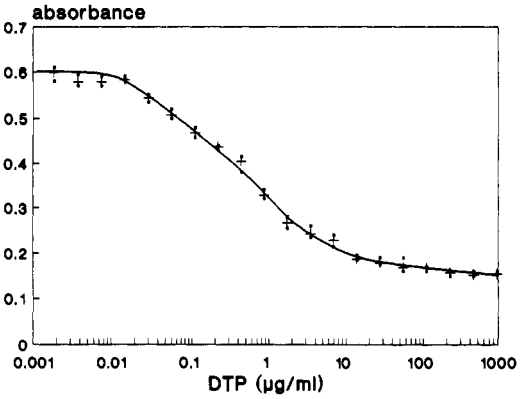


Figure 1. Standard curve for the determination of DTP [hemisuccinate of 2-(2,4-dichlorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propanol] by ELISA. Absorptions are corrected for the specific binding and are the means ± SD of 10 replicates.

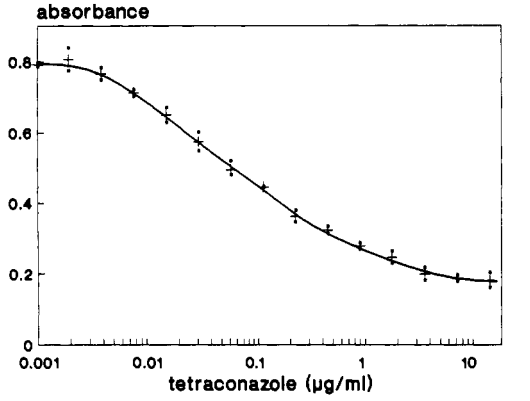


Figure 2. Typical standard curve for tetraconazole. The curve represents the mean and standard deviation of 10-fold determinations.

RESULTS

The optimized ELISA test in the microtiter plate was performed with a working dilution of 1:500 for the anti BSA–FF18 antibody, while the secondary goat anti rabbit Ig–peroxidase-conjugated antibody was diluted 1:10 000. Under these conditions, the absorbance in the standard assays for DTP ranged from 0.150 to 0.600, thus allowing the determination of antigen concentration in a useful working range. Each standard curve was performed in plates after coating with 0.02 μg/well CON–FF18. Blocking with 3% gelatin prevented nonspecific binding of the anti BSA–FF18 antibody.

A typical standard curve of DTP (standard analyte) is shown in Figure 1. The linear range of the test system extended from 10 ± 0.45 to 1200 ± 88.50 ng/mL (i.e., from 0.01 to 1.2 ppm).

In Figure 2 the curve obtained with the commercial triazole tetraconazole (M-14360) is shown. The curve compared well with that obtained with the standard analyte DTP, but the linear range of detection extended from 9 ± 0.90 to 850 ± 46.0 ng/mL. In Figure 3 are reported the coefficients of variation (CV) that express the standard deviation as a percentage of the mean values of absorbance measured in six ELISA tests, in which two replicates were assessed for each concentration, carried out over 3 months at 15-day intervals. The calculated values were lower than 10% at tetraconazole concentrations ranging from 30 to 1800 ng/mL.

Comparison of ELISA and HPLC methods for measuring tetraconazole in ethanol showed that the detection limit (i.e., the concentration that gave a signal to noise

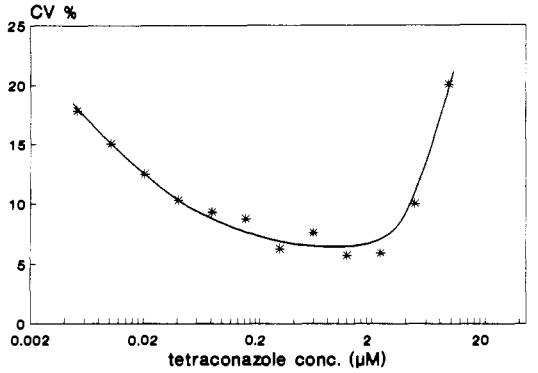


Figure 3. Between-assay CV values of tetraconazole ELISA. The coefficients of variation (CV) here reported express the standard deviation as a percentage of the mean values of absorbance measured in six ELISA tests in which two replicates were assessed for each concentration. The ELISA tests were carried out over 3 months at 15-day intervals by using freshly made tetraconazole stock solutions.

ratio higher than 3) in the conditions used for HPLC analysis was 15 μg/mL, which is more than 1000-fold higher than in ELISA.

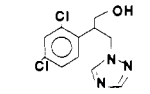
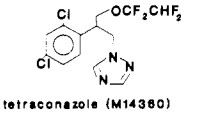
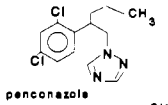
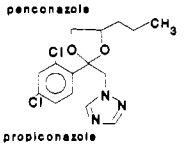
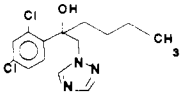
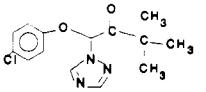
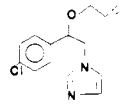
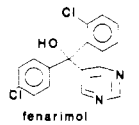
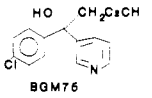
ELISA studies were also performed on other fungicides containing either the 1,2,4-triazole or imidazole, pyridine, or pyrimidine group. The results are shown in Table I and Figure 4. A comparable range of linearity of the ELISA test was found for the triazole chemicals (tetraconazole and penconazole), where a two-carbon-atom chain connects the triazole and the phenyl group and a linear substituent is bound to carbon 2 of the chain. Tetraconazole and penconazole gave a 50% inhibition point (I_{50}), calculated as the mean of 10-fold determinations, at 100 ± 7 and 120 ± 10 ng/mL respectively. Cross-reactivities of 150% for tetraconazole and 140% for penconazole were found, as compared with the 50% inhibition point found for DTP, the standard analyte. Propiconazole and hexaconazole, which bear the same basic moieties (i.e., 1,2,4-triazole and a dihalogenated phenyl group) along with complex substituents, are minimally recognized by the antibody. They showed cross-reactivities of 4.5 and 1.3%, respectively. Analysis of the ELISA curves obtained for the triazole triadimefon, which, along with a complex, large, and bulk substituent on C-1, contains a monohalogenated phenyl group, did not allow a reliable calculation of I_{50}. The range of linearity shown in Table I, however, indicated that triadimefon is poorly recognized by the antibody.

To get some insight into the specificity of the antibody produced, we have tested its reactivity against fungicides containing either the imidazole, pyrimidine, or pyridine group instead of the basic moiety of the triazoles. Fenarimol and BGM-75, which have a pyrimidine and a pyridine group, respectively, showed no cross-reactivity. Imazalyl, bearing the imidazole group along with a simple substituent containing an unsaturated bond, has very low cross-reactivity.

CONCLUSIONS

The hapten we have used for conjugation with BSA contained the basic structural moiety of compounds of the triazole family (i.e., 1,2,4-triazole and dihalogenated phenyl group) connected by a two-carbon chain bearing a succinoyloxymethyl group as the handle. The results of the present work indicate that the ELISA we developed cannot be considered a class-specific assay, since the assay detection limits for some commercial triazoles are too low for their routine analysis. The different sensitivities found

Table I. Cross-Reaction of Some Triazoles and Related Compounds in ELISA with Regard to DTP

Fungicide formula	Range of linearity μg/ml	Cross reactivity [a] %
 standard analyte (DTP)	0.010-1.20	100
 tetraconazole (M14360)	0.009-0.86	150
 penconazole	0.013-0.88	140
 propiconazole	0.60-16.80	4.5
 hexaconazole	2.16-139	1.3
 triadimefon	7.6->440	n. d. [b]
 imazalyl	5.94-105	0.5
 fenarimol		0
 BGM75		0

[a] Cross-reactivity is expressed as that concentration of DTP causing a 50% inhibition of binding × 100 divided by the concentration of the compound tested that caused 50% inhibition of binding. [b] nd, not detected.

in ELISA with the various triazole chemicals tested likely arise from the chemical nature of the chain connecting the basic structural moieties. The low cross-reactivity found for propiconazole and hexaconazole, however, also suggests a role of the substituents present on the chain (X, Y, and Z of Figure 5) in determining the antibody/antigen complex formation. In propiconazole and hexaconazole, C-2 is quaternary because neither X nor Y is a hydrogen atom. In the case of triadimefon, the poor reactivity with the antibody likely arises from the bulk substituent (Z) bound to C-1, as well as from the lack of a C-2 carbon and of a second halogen in the phenyl group. On the whole, the antibody appears to be very sensitive to the steric hindrance of the chain.

No cross-reactivity was found for the non-triazole chemicals fenarimol and BGM-75, and negligible cross-reactivity was found for imazalyl.

The present study has allowed the development of a competitive ELISA for the registered fungicide tetracon-

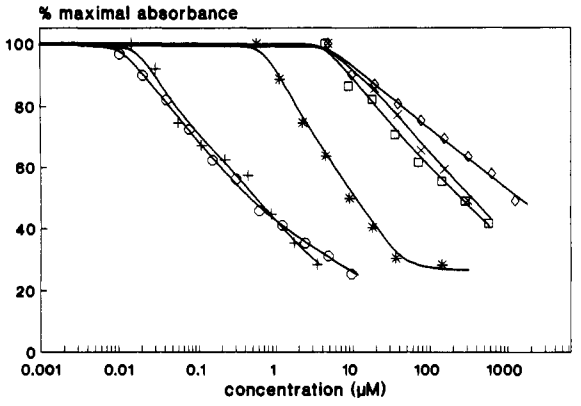


Figure 4. ELISA curves for penconazole (+), propiconazole (*), hexaconazole (□), triadimefon (◇), and imazalyl (×) in comparison with tetraconazole (○).

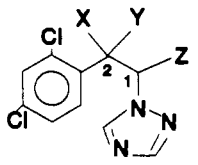


Figure 5. Basic structural moieties recognized by the antibody.

azole with high reproducibility of the assay, as demonstrated by between-assay CV values.

Since ELISA allows a rapid throughput of samples, it offers a powerful tool for the determination of the commercial triazoles tetraconazole and penconazole for which the antibody produced shows high cross-reactivity. The sensitivity of the assay (12–13 ppb detection limit) is significantly higher than the detection limit found in the determination of tetraconazole by the HPLC method. Enhanced sensitivity, however, would be desirable for routine analysis of triazole residue in foodstuff. We are selecting a different ELISA format and alternate hapten coating with the aim of developing an assay suitable for reliable and easy quantification of tetraconazole and penconazole traces in foodstuff.

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