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RESEARCH NOTE

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A Dipstick-Type Enzyme-Linked Immunosorbent Assay for the Detection of the Insecticide Fenitrothion in Food Samples

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Abstract In a previous study, we obtained polyclonal antibodies against the organophosphorus insecticide fenitrothion and developed an enzyme-linked immunosorbent assay (ELISA) for this pesticide. Using these antibodies and an enzyme tracer, a direct competitive ELISA method specific for fenitrothion using a dipstick format was developed. Dipstick ELISA using antibodies to fenitrothion immobilized on an Immunodyne membrane allowed the quick visual detection of fenitrothion at concentrations above 10 μg/L. The IC_{50} value of dipstick ELISA using reflectance detection was 27 μg/L with a detection limit of 2 μg/L. The recovery of fenitrothion from spiked lettuce and rice samples using the dipstick ELISA ranged from 87-107%.

Keywords: fenitrothion, insecticide, enzyme-linked immunosorbent assay, dipstick

Introduction

Quantification of pesticides in food samples has relied exclusively on gas chromatography and high-performance liquid chromatography (1). Although these methods are sensitive and reliable, they are costly, require skilled analysts, and involve time-consuming sample pretreatment (2, 3). Over the past two decades, ELISAs have been developed as a suitable alternative to overcome the shortcomings of traditional methods (2, 3). However, conventional ELISAs allow the quantification of analytes using a photometer combined with computer-automated calculation, and thus are restricted to laboratories. In contrast, dipstick ELISAs, which use an antibody-coated membrane to give quick and inexpensive results, are promising as a qualitative and semi-quantitative on-site test of analytes and have been used as efficient diagnostic tools for detecting pesticides (4-8).

In a previous study, we obtained polyclonal antibodies against the organophosphorus insecticide fenitrothion and developed an antigen-coated and an antibody-coated microtiter plate ELISA for the detection of fenitrothion (9). In this study, using the same antibodies and an enzyme tracer, we developed a competitive antibody-coated ELISA for the detection of fenitrothion in a dipstick format.

Fenitrothion is widely used as a grain protectant and also finds use in horticulture, forestry, and domestic and public health applications (10, 11). Exposure of humans to fenitrothion may result in lethal cholinergic poisoning (11).

Materials and Methods

Reagents and equipments Fenitrothion was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Antibody to horseradish peroxidase (HRP), bovine serum albumin (BSA), gelatin (porcine skin), chicken egg albumin (OVA), HRP conjugated anti-rabbit IgG, casein, protein A, and dioctylsulfosuccinate (DSS) were purchased from Sigma (St. Louis, MO, USA). 3,3',5,5'-Tetramethylbenzidine (TMB) was obtained from Boehringer Mannheim (Mannheim, Germany). Immunodyne ABC membrane was acquired from Pall (Pall Filtrationstechnik GmbH, Dreieich, Germany). Reflectance was measured with an RQflex reflectometer from Merck (Darmstadt, Germany).

Synthesis of haptens and production of antibody The haptens used to prepare the immunogen (Hapten A) and the enzyme tracer (Hapten B) are presented in Fig. 1. The procedures for the preparation of the haptens, enzyme tracer, and antibody are described in our previous papers (9, 12). The antiserum was purified by ammonium sulfate precipitation (13). Protein concentration was determined by the modified Lowry assay (14).

Preparation of test strips ***Method A:*** A section of Immunodyne membrane was cut into square pieces (0.7 × 0.7 cm) and mounted onto a polystyrene plastic strip using double-sided adhesive tape. The diluted antibody (2 μL) was spotted on the membrane. The antibody-coated membrane was dried for 30 min, and then the residual binding sites of the membrane were blocked with a 2% casein solution for 1 hr. After being washed two times with PBST (10 mM phosphate-buffered saline with 0.05%

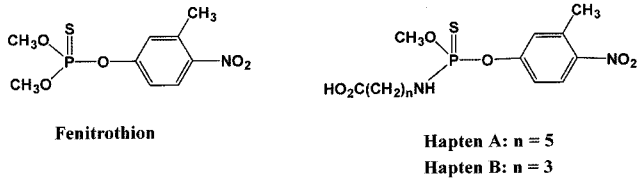

Fig. 1. Structures of the haptens of fenitrothion used as the immunogen (Hapten A) and the enzyme tracer (Hapten B).

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Tween 20), the membrane was dried for 30 min. For the detection using a negative control (4), antibody solution (2 μL) was spotted on the lower part of 0.7×1.5 cm pieces of membrane (reaction zone) while anti-HRP antibody was spotted on the upper part of the membrane (negative control zone). The dilution of anti-HRP antibody was adjusted such that color development in the specific reaction zone and the negative control were similar.

Method B: This method was used for the quantitative color detection using a reflectometer. A section of membrane cut into 10×10 cm square pieces was incubated with diluted antibody for 2 hr at room temperature on a horizontal shaker with gentle agitation. After being washed with PBST, the membrane was blocked with 2% casein solution for 1 hr. After being dried for 30 min, the membrane was cut into 0.7×0.7 cm square pieces, which were then mounted onto a polystyrene strip.

Assay protocol The antibody-coated strip was incubated with a mixture of 200 μL of the standard or sample and 200 μL of the enzyme tracer for 20 min at room temperature. After being washed three times with PBST, the strip was incubated with 400 μL of TMB-DSS solution (a mixture of 400 μL of 0.6% TMB-DMSO solution, 50 μL of 1% H_2O_2 and 1 mL of 0.8% DSS-methanol diluted with 3.6 mL of citrate-acetate buffer, pH 5.5) for 5 min.

Optimization of dipstick ELISA system The concentrations of antibody to be immobilized on the membrane and the enzyme tracer to be added to the assay solution were optimized, as were the times for competition and the concentration of methanol used to dissolve the pesticide in assay solution. For experiments performed to select the most suitable concentrations of antibody and enzyme tracer, color development without pesticide (control) was examined. For the other experiments, color development of both the control and pesticide-containing solution were examined.

Competition curves were obtained from the reflectometric measurement by plotting absorbance against the logarithm of analyte concentration. The reflectance was normalized by transformation to % B/B_0, $[(A-A_{ex})/(A_0-A_{ex})] \times 100$, where A is absorbance of sample, A_0 is absorbance at zero dose of the analyte, and A_{ex} is absorbance with an excess of the analyte. Sigmoidal curves were fitted to a four-parameter logistic equation (15), from which IC_{50} values (concentration at which binding of the antibody to the enzyme tracer is inhibited by 50%) were determined.

Analysis of fenitrothion in food samples One mL of fenitrothion dissolved in a methanol solution was added to 1 g of pesticide-free lettuce leaves or rice grains that had been chopped or ground in fine pieces, respectively. After setting this aside for 24 hr, the leaves or grains were incubated in 5 mL of methanol for 10 min with four vigorous shakes and then filtered through Whatman No. 1 filter paper. The container and the residues were rinsed with 5 mL of methanol, the rinsate was filtered, and the filtrate was combined with the previous filtrate. The methanol was evaporated to dryness under reduced pressure, and the residue was extracted with 10 mL of 30 % methanol-PBS. The extract was analyzed by dipstick ELISA using reflectance detection.

Results and Discussion

Optimization of the dipstick ELISA format Optimization of the concentrations of antibody to be spotted on the membrane and the enzyme tracer added as a competitor was achieved by checkerboard assay using various combinations of tracer and antibody at several concentrations. The optimum concentrations of antibody for the test strip to be prepared by methods A and B were 0.41 and 0.061 mg/L, respectively. The optimum concentrations of tracer for use with test strips prepared by methods A and B were 0.134 and 0.084 μg/L, respectively.

The color development after competition with tracer for 5 and 10 min was much weaker than that seen with longer competition times. Competition times longer than 20 min resulted in higher background noise with no enhancement of color intensity. Therefore, 20 min was chosen as the optimum.

It is necessary to include an organic cosolvent in the standard or sample solution to dissolve nonpolar pesticides. The effect of methanol as the cosolvent on ELISA performance was evaluated by obtaining standard curves at several methanol concentrations (10-100%). The color intensity increased with methanol concentrations up to 30%, and then decreased at higher concentrations. A concentration of 30% was chosen as the optimum based on the color intensity that was the highest.

Performance of dipstick ELISA system Dipstick ELISAs based on visual detection require a negative control to evaluate 'reduced color development' of positive samples. Anti-HRP antibody diluted 1:4,500 was found to be a suitable negative control with the same color intensity as that at the detection zone with no pesticide. Figure 2 presents the results of the assay using the integrative negative control.

Since a fenitrothion concentration of 10 μg/L caused a slight but distinguishable difference compared to the negative control, the detection limit of fenitrothion using visual detection was considered to be 10 μg/L. Figure 3 presents the calibration curve for fenitrothion using reflectance detection. The IC_{50} value for fenitrothion was 27 μg/L with a detection limit of 2 μg/L.

Analysis of fenitrothion in food samples The recovery of fenitrothion added to food samples determined by dipstick ELISA is presented in Table 1. The recovery values

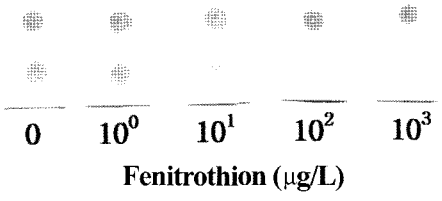

Fig. 2. Results of the dipstick ELISA with the integrative negative control at different fenitrothion concentrations.

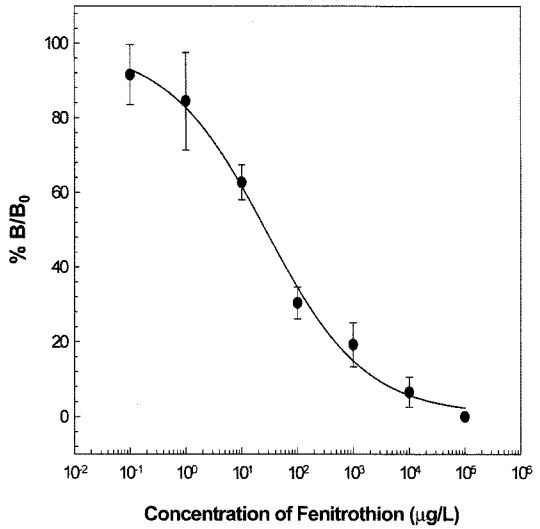

Fig. 3. ELISA competition curve for fenitrothion by dipstick ELISA using reflectometric measurement. Each point represents the mean of 6 determinants. Vertical bars indicate±SD about the mean.

Table 1. Recovery of fenitrothion added to vegetable samples[1)]

Fortified concentration (μg/L)	Rice	Lettuce
1	101	101
10	89	87
100	97	96
1000	97	107

[1)]Data are the mean of triplicate determinations.

were satisfactory, ranging from 87 to 107%. Therefore, it can be stated that there was no significant matrix effect on the detection of fenitrothion in vegetable samples, and that the samples can be analyzed directly by the dipstick ELISA after a brief clean-up procedure. In conclusion, the dipstick ELISA developed in this study can be used as a convenient tool for the rapid preliminary screening of fenitrothion residues in food samples.

Acknowledgments

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