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Development of a Preliminary Enzyme-Linked Immunosorbent Assay for the Herbicide Trifluralin

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Enzyme-linked immunosorbent assays (ELISA) have been developed for a number of pesticides (Hammock et al. 1987; Jung et al. 1989). Currently, residues of the herbicide trifluralin are analyzed by gas chromatography (Cessna et al. 1985; Downer et al. 1976; Lee and Chau 1983). While accurate, this method can be time consuming and expensive. As a consequence, there is a demand for cost-effective methods which are rapid, dependable, and efficient. This demand could be partially filled by the use of ELISA. This paper describes the steps taken to develop an antibody against trifluralin and reports the results of a preliminary indirect competitive ELISA designed to detect trifluralin residue.

MATERIALS AND METHODS

Reagents and equipment used for the ELISA and buffer solutions are described by Riggle and Dunbar (1990). All stocks and standard solutions were stored at 4°C.

The hapten, 2,6-dinitro-N-propyl-N-(2-carboxyethyl)-4-(trifluoromethyl)benzenamine, (referred to in the text as propionic acid trifluralin) was made in the following manner. First, 3-(propylamino)propionic acid was prepared. This was done by first stirring 1:10 molar amounts of ethyl acrylate and n-propylamine at room temperature for 4 hr. The ethyl-3-(propylamino)propionate was isolated by distillation in an 80% yield. The ester was hydrolyzed by stirring with cold water for 12 hr. The solution was roto-evaporated to remove the water. The solid residue was recrystallized from ethanol and acetone to give a white crystalline product. Next, 4-chlorobenzotrifluoride was nitrated with a mixture of nitric and sulfuric acid at 100 °C for 3 hr to produce 3,5-dinitro-4-chlorobenzotrifluoride. This was recrystallized using ethanol. The material was melted at 58 °C and added to a solution of 3-(propylamino)propionic acid in dry chloroform. The solution was stirred overnight at room temperature. HCl (1 N) was added. The organic phase was separated, washed with distilled water, and then roto-vacuumed to dryness. NaOH (0.2 N) was added. The basic solution was washed with ethyl acetate and then acidified to pH 3 with HCl. The yellow oily material which seperated out was washed with water and then dried over anhydrous sodium sulfate. Attempts to crystallize the material failed. A portion of the yellow oil was analyzed by thin-layer chromatography on silica gel F254 plates which were developed with chloroform:

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methanol: water (24:7:1). A single spot was observed using UV visualization. NMR and IR results were consistent with those expected for the hapten.

The hapten was conjugated to bovine and rabbit serum albumin (BSA and RSA, respectively), using a mixed anhydride method. A portion of the hapten (37.5 mg) (0.1 mmole) was dissolved in dry dimethylformamide (DMF) (1.4 mL) and tributylamine (48 μL) (0.2 mmole). The solution was cooled to 5 °C. Isobutyl chloroformate (13 μL) (0.1 mmole) was added and the reaction was allowed to proceed in an ice bath for 30 min. The resultant mixture was stirred into an ice-cooled solution of BSA or RSA (100 mg), distilled water (1 mL), 1 N NaOH (100 μL) and DMF (2 mL). This mixture was stirred for 30 min, and then 1 N NaOH was added to maintain the pH at 8 throughout the reaction. After standing for 12 h, the mixture was dialyzed, and the pH was adjusted to 4.5. The resulting precipitate was allowed to stand at 4 °C for 4 h and then was separated from the liquid phase by centrifugation. The resulting product was washed with cold acetone, suspended in water and redissolved by addition of 0.1 N NaOH for a final pH of 7.8. The solution was dialyzed for 8 h with running water and then lyophilized for a final yield of approximately 100 mg of product.

Two New Zealand rabbits were inoculated intradermally with 500 μg of the BSA-conjugated hapten and complete Freund's adjuvant, boosted at monthly intervals, and bled on a weekly basis after the second injection. Serum from one rabbit, collected 6 months after inoculation, was used for the assay.

Anti-trifluralin and pre-immunized (collected prior to inoculation) sera were treated with 90% strength $(NH_4)_2SO_4$. Precipitated material was collected in 20 mL of phosphate buffered solution which was then placed in dialysis tubing (Spectrapor) (m.w. cutoff 12,000 - 14,000) and dialyzed five times in fresh batches of PBS (300 mL). The $(NH_4)_2SO_4$ treatment was used in an attempt to over-come possible attachment problems of the assay as described below.

The ELISA followed the same steps as outlined by Riggle and Dunbar (1990). Cross-reactivity tests were done with trifluralin metabolites (code numbers according to Golab et al. 1979) and associated dinitroaniline herbicides (Table 1) to determine antibody specificity. Compounds were assayed at dilutions of 0.1, 1, 10, 100, 1000, and 10,000 ng/mL (1 ng/mL = 1 ppb). Because of low water solubility properties associated with many of the compounds, solutions were prepared with distilled water and 2% DMF. In addition, cross-reactivity tests were done with atrazine, paraquat, glyphosate, alachlor, and fluazifop (Table 1) at 100 μg/mL (100 ppm). Cross-reactivities were determined as described by Dunbar et al. (1990).

RESULTS AND DISCUSSION

Percent inhibition calculations (for details see Riggle and Dunbar 1990) found that trifluralin at 10 ppm produced from 30 to 40 percent inhibition. The sensitivity of the assay was low when compared to results reported for antisera against other pesticides tested at similar concentrations (Gee et al. 1988; Kelley et al. 1985; Niewola et al. 1986; Riggle and Dunbar 1990).

Cross-Reactivity Results. RSA and blocking gel were not cross-reactive (data not presented) while BSA was cross-reactive (data not presented). This was an expected result since a hapten/BSA conjugate was used to immunize the rabbit. The metabolite

TR-13 had the highest cross-reactivity (less than 50%) (Table 2) while TR-2 and TR-40 had cross-reactivities of greater than 15% but less than 30%. TR-15 and TR-3 had cross-reactivities of less than 10% while TR-6, TR-9, TR-21, TR-28, TR-32 (TR 28 and 32 not shown in Table 2), and TR-36M were not cross-reactive. Benefin and ethalfluralin were one third and one quarter cross-reactive while oryzalin and pendimethalin were not cross-reactive. Atrazine, paraquat, glyphosate, alachlor, and fluazifop were not cross-reactive. Absorbance readings for tested compounds which were cross-reactive had coefficients of variation that ranged from 10 to 20%.

Table 1. List of the tested compounds including common names or codes and chemical names.

Common Name or Code Number	Chemical Name
trifluralin	2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)benzenamine
benefin	2,6-dinitro-N-butyl-N-ethyl-4-(trifluoromethyl)benzenamine
ethalfluralin	2,6-dinitro-N-ethyl-N-(2-methyl-2-propenyl)-4-(trifluoromethyl)benzenamine
oryzalin	3,5-dinitro-4-(dipropylamino)benzenesulfonamide
pendimethalin	2,6-dinitro-N-(1-ethylpropyl)-3,4-dimethylbenzenamine
TR-2	2,6-dinitro-N-propyl-4-(trifluoromethyl)benzenamine
TR-3	2,6-dinitro-4-(trifluoromethyl)benzenamine
TR-6	3-nitro-5-(trifluoromethyl)-1,2-benzenediamine
TR-9	5-(trifluoromethyl)-1,2,3-benzenetriamine
TR-13	2-ethyl-7-nitro-1-propyl-5-(trifluoromethyl)-1H-benzimidazole
TR-15	2-ethyl-4-nitro-6-(trifluoromethyl)-1H-benzimidazole
TR-21	4-(dipropylamino)-3,5-dinitrobenzoic acid
TR-28	2,2'-azoxybis-(6-nitro-N-propyl-4-trifluoromethyl)benzenamine
TR-32	2,2'-azobis-(6-nitro-N-propyl-4-trifluoromethyl)benzenamine
TR-36M	3-methoxy-2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)-benzenamine
TR-40	N-(2,6-dinitro-4-(trifluoromethyl)phenyl)-N-propylpropanamide
atrazine	6-chloro-N-ethyl-N-(1-methylethyl)-1,3,5-triazine-2,4-diamine
paraquat	1,1'-dimethyl-4,4'-bipyridinium ion
glyphosate	N-(phosphonomethyl)glycine
alachlor	2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl) acetamine
fluazifop	(±)-2-[4-[(5-trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic acid

The detection limit varied from 0.1 to 1.0 ppm trifluralin. This was high when compared to ELISA detection limits of 0.2 ppb for alachlor (Feng et al. 1990), 0.1 ppb for atrazine (Dunbar et al. 1990), 0.1 ppb for chlorsulfuron (Kelley et al. 1985), and 1.0 ppb for norflurazon (Riggle and Dunbar 1990).

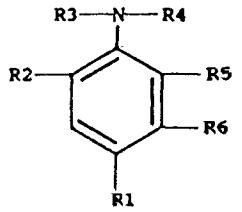

Table 2. List of tested dinitroaniline compounds including compound names, functional groups, and % cross-reactivity values.

compound	R1	R2	R3	R4	R5	R6	% cross-reactive
propionic acid trifluralin	CF_3	NO_2	C_3H_7	C_2H_4COOH	NO_2	--[1]	na[2]
trifluralin	CF_3	NO_2	C_3H_7	C_3H_7	NO_2	--	100
TR-13	CF_3	NO_2	C_3H_7	$C(=R_5)C_2H_5$	$N=R_4$	--	45.1
benefin	CF_3	NO_2	C_2H_5	C_4H_9	NO_2	--	33.3
TR-2	CF_3	NO_2	C_3H_7	H	NO_2	--	29.1
ethalfluralin	CF_3	NO_2	C_2H_5	C_4H_7	NO_2	--	24.8
TR-40	CF_3	NO_2	C_3H_7	COC_2H_5	NO_2	--	17.4
TR-15	CF_3	NO_2	H	$C(=R_5)C_2H_5$	$N=R_4$	--	7.8
TR-3	CF_3	NO_2	H	H	NO_2	--	7.5
TR-6	CF_3	NO_2	H	H	NH_2	--	0
TR-9	CF_3	NH_2	H	H	NH_2	--	0
TR-21	COOH	NO_2	C_3H_7	C_3H_7	NO_2	--	0
TR-36M	CF_3	NO_2	C_3H_7	C_3H_7	NO_2	OCH_3	0
oryzalin	SO_2NH_2	NO_2	C_3H_7	C_3H_7	NO_2	--	0
pendimethalin	CH_3	NO_2	H	C_5H_{11}	NO_2	CH_3	0

[1] --: denotes no functional group present at R6 position.
[2] na: cross-reactivity measurements were not made for the hapten.

Results support that $(NH_4)_2SO_4$ treated antiserum contained antibody against trifluralin. This antibody was cross-reactive to other dinitroanaline herbicides and to some of the associated metabolites of trifluralin. Compounds that were cross-reactive had a CF_3 group (R1) and a NO_2 group at the R2 position. Cross-reactivity was reduced when a H group was substituted at the R3 position. A NO_2 group was not essential at the R5 position; however, a NH_2 group in place of a NO_2 group negated cross-reactivity. Cross-reactivity was also negated if the CF_3 group was absent and if a functional group was present at the R6 position.

High cross-reactivity values of some dinitroaniline compounds (Table 2) may have been partially due to the short connecting bridge of the hapten to protein. The hapten had a C_2H_4COOH group in place of a C_3H_7 group at the R4 position. A longer connecting bridge, such as a $C_5H_{10}COOH$ group, might have resulted in increased specificity to trifluralin and lower cross-reactivity values.

Results of the assay suggest that trifluralin may have attached to either the chemically inert polystyrene and/or to the proteinaceous blocking gel and/or antibodies. This may have contributed to the high CVs and a high detection limit.

Future research is required to determine if a longer connecting bridge on a hapten could improve antibody specificity and if trifluralin attachment affected the assay. If attachment is not a problem or is one that can be rectified, then additional research is required to reduce the variability of the absorbance readings and to improve the detection limit to a level at or near 1 ppb. Sensitivity may be improved by isolating IgG fractions of rabbit antibody by using immunoaffinity columns.

Any reference to trade names or manufacturers within the context of this article is used solely to provide specific information and does not constitute a guarantee or endorsement by the U.S. Department of Agriculture.

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