Production and Characterization of Monoclonal Antibodies to a Generic Hapten for Class-Specific Determination of Organophosphorus Pesticides

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Monoclonal antibodies have been generated against a generic hapten, *O,O*-diethyl *O*-(5-carboxy-2-fluorophenyl) phosphorothioate, for the determination of organophosphorus (OP) pesticides in a class-specific manner. In an indirect competitive enzyme-linked immunosorbent assay (ELISA) format, employing a heterologous coating antigen, these monoclonal antibodies showed desirable properties for use in the class-specific determination, *i.e.*, broad specificity and high sensitivity. The IC_{50} values of four commonly used *O,O*-diethyl OP pesticides were fairly uniform ranging from 0.1 to 0.3 μg/mL. The IC_{50} values of three *O,O*-dimethyl derivatives were between 0.3 and 1.4 μg/mL. These values, together with the limits of detection (LOD), were better, in terms of the specificity and sensitivity, compared with the values obtained previously with polyclonal antibodies.

Key words : Organophosphorus pesticides, Class-specific determination, Enzyme-linked immunosorbent assay, ELISA, Monoclonal antibodies

Introduction

Organophosphorus pesticides are widely used in agricultural and domestic settings.[1,2] Although they are known to degrade relatively rapidly, their acute toxicity necessitates more prudent monitoring of their residues in crops and the environment.

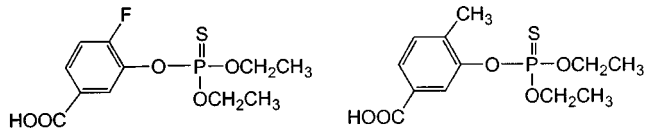
Several methods have been reported for the determination of OP pesticides using a variety of techniques, including gas chromatography and high-performance liquid chromatography.[3] Although these traditional methods are sensitive and reliable, they suffer from some critical drawbacks: high cost, labor intensive and lengthy sample preparation, and inconvenience for use in the field. Therefore, there is growing demand for more rapid and economical methods for determining pesticide residues. Immunoassays that can meet such demands have recently emerged as an alternative to the traditional methods. Immunochemical techniques began recently to gain acceptance as a fast, sensitive, and cost-effective tool for detecting trace amounts of chemicals such as pesticides.[4]

Most of the immunochemical assays developed for the determination of pesticides are aimed at detecting individual pesticides. Class-specific determination of pesticides before chromatographic determination could be an attractive approach for broader pesticide monitoring. If the total quantity of a class of pesticides in a sample can be determined and the quantity is less than the maximum residue limits of certain pesticides in the class, the sample can be eliminated from further inspection for those pesticides. Since the vast majority of food and environmental samples turn out to be under maximum residue limits of pesticides, the time and cost saved by this approach may be enormous. The savings would be greater in the case of major class pesticides, such as organophosphorus and carbamate pesticides. Only a few attempts have been made to develop a class-specific immunoassay for pesticides. An earlier attempt by Banks *et al.*,[5] using conserved structures of OP pesticides as haptens, produced polyclonal antibodies with broad specificities, but lacked sensitivity. Later attempts by Johnson *et al.*[6] and Alcocer *et al.*[7] yielded significant improvements in sensitivity, but lacked uniform response to analytes.

In an attempt to resolve these problems, we tried to develop an immunoassay for class-specific determination of OP pesticides using monoclonal antibodies (mAb) against a generic hapten that has a *O,O*-dialkyl thiophosphate group common in OP pesticides. To test the suitability of the mAb against the hapten for a class-specific immunoassay, the mAb generated were characterized by an indirect competitive ELISA in which an analyte competes with the antigen coated on the plate. To the best of our knowledge, this is the first attempt to generate mAb to a generic hapten to develop a class-specific immunoassay for OP pesticides.

Experimental Section

Reagents and Instruments. Pesticides were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Ovalbumin, Tween 20, Sephadex G-25, Freund's incomplete and complete adjuvant, gelatin, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, HAT and HT cell culture media were purchased from Sigma (St. Louis, USA). RPMI 1640, fetal bovine serum, and penicillin-streptomycin were the products of GibcoBRL (Paisley, U.K) and PEG 1500 and 3,3',5,5'-tetramethylbenzidine (TMB) were the products of Boehringer Mannheim (Mannheim, Germany). Myeloma



Hapten A Hapten B

Figure 1. Structures of immunizing (Hapten A) and coating hapten (Hapten B).

cell line SP 2/0 was provided by Dr. K. J. Hur (Ewha Woman's University, Korea). All the cell culture flasks and plates were obtained from Nunc (Roskilde, Denmark). ELISA plates were washed with a Columbus Plus plate washer from Tecan (Saltzburg, Austria), and well absorbances were read with a Vmax microplate reader from Molecular Devices (Menlo Park, USA). NMR spectra were obtained with a Bruker (Rheinstetten, Germany) ARX spectrometer (300 MHz), using tetramethylsilane as an internal standard.

Hapten Synthesis. Hapten A and B shown in Figure 1 were prepared to be used as immunizing and coating antigen hapten, respectively.

***O,O*-Diethyl *O*-(5-carboxy-2-fluorophenyl) phosphorothioate (Hapten A)**. To a cold (0 °C) mixture of 3-hydroxy-4-fluorobenzoic acid (200 mg, 1.28 mmol) and KOH (85%) (180 mg, 3.2 mmol) in methyl alcohol (10 mL) was added dropwise diethyl chlorothiophosphate (362 mg, 1.92 mmol), and the mixture was heated while stirring for 12 h at 65 °C. The reaction mixture was concentrated, and the residue diluted with ethyl acetate. The acid was extracted into 2 M NaOH solution (10 mL), acidified by adding concentrated HCl, and then extracted into ethyl acetate (3 × 20 mL). The separated organic layer was washed with water and brine, dried over magnesium sulfate, and evaporated under reduced pressure. Flash chromatography of the residue on silica gel (elution with chloroform : ethyl acetate : acetic acid = 30 : 5 : 1) gave a slightly yellow solid (162 mg, 41%). ^{1}H NMR (300 MHz, DMSO-d_6): 7.84 (m, 2H), 7.51 (m, 1H), 4.26 (q, J = 7.0 Hz, 2H), 4.24 (q, J = 7.0 Hz, 2H), 1.3 (t, J = 7.0 Hz, 6H).

***O,O*-Diethyl *O*-(5-carboxy-2-methylphenyl) phosphorothioate (Hapten B)**. To a solution of sodium ethoxide (4.2 mmol) in ethyl alcohol (5 mL) at 0 °C was added dropwise a solution of ethyl 3-hydroxy-4-methylbenzoate (507 mg, 2.8 mmol) in ethanol (3 mL), and the mixture was stirred for 30 min. Diethyl chlorothiophosphate (796 mg, 4.2 mmol) was added and the mixture was stirred for an additional 36 h at room temperature. The reaction mixture was diluted with water (2 mL), and concentrated under reduced pressure. The residue was treated with saturated ammonium chloride (5 mL) and extracted into ethyl acetate (3 × 20 mL). The organic layer was washed with water and brine, dried over magnesium sulfate, and the solvent was evaporated. Chromatography of the residue on silica gel (3% ethyl acetate in hexane) afforded 506 mg (54%) of *O,O*-diethyl *O*-(3-carboethoxy-6-methylphenyl) phosphorothioate as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): 7.87 (s, 1H), 7.77 (m, 1H), 7.28 (s, 1H), 4.27 (q, J = 6.4 Hz, 2H), 4.25 (q, J = 6.4 Hz, 4H), 2.37 (s, 3H), 1.33 (m, 9H). The product (273 mg, 0.89 mmol) dissolved in ethyl alcohol (25 mL) was mixed with 1 M KOH (9 mL) and stirred for 1 h. The reaction mixture was concentrated and diluted with ethyl acetate, and then the acid was extracted into 2 M NaOH solution (10 mL). The aqueous solution was acidified with concentrated HCl, and extracted into ethyl acetate (3 × 20 mL). The organic layer was washed with water and brine, dried over magnesium sulfate, and the solvent was evaporated. Chromatography on silica gel (chloroform : ethyl acetate : acetic acid = 800 : 35 : 1) gave Hapten B as a white solid (261 mg, 96%). ^{1}H NMR (300 MHz, $CDCl_3$): 7.69 (m, 2H), 7.42 (m, 1H), 4.20 (q, J = 6.5 Hz, 2H), 4.18 (q, J = 6.5 Hz, 2H), 2.29 (s, 3H), 1.29 (t, J = 6.5 Hz, 6H).

Preparation of Hapten-Carrier Conjugates. Hapten A and B were conjugated to keyhole limpet hemocyanin (KLH) and ovalbumin (OVA), respectively, to be used as an immunogen and a coating antigen. The conjugation method used was the succinimide ester method.[8] The conjugates were purified by gel filtration on Sephadex G-25. The eluted conjugates were dialyzed against water and then freeze-dried before storage at -80 °C.

Immunization. BALB/c female mice (8 weeks old) were immunized subcutaneously with 1 : 1 mixture (v/v, 200 μL) of Hapten A-KLH conjugate (50 μg) in PBS and Freund's complete adjuvant. Two and four weeks after the initial injection, booster injections were given intraperitoneally with the same amount of immunogen emulsified with incomplete Freund's adjuvant. One week after each booster injection, mice were tail-bled and antisera were tested for anti-hapten antibody titer by a noncompetitive indirect ELISA using a homologous coating antigen (Hapten A-OVA). After a resting period of three weeks following the third injection, mice selected as the donors of spleen cells for hybridoma production received a final intraperitoneal injection with the same amount of conjugate in PBS. The mice were sacrificed for cell fusion 3 days after the final injection.

Cell Fusion. SP 2/0 murine myeloma cells were cultured in RPMI 1640 media supplemented with 20% fetal bovine serum and 1% penicillin-streptomycin. Cell fusion procedures were carried out essentially as described by Galfre' and Milstein.[9] Mice splenocytes were mixed with the myeloma cells at the ratio of 5 : 1 and centrifuged. One mL of PEG 1500 at 37 °C was dropped to the cell pellet over 1 min. After addition of 14 mL of noncomplete media (RPMI + penicillin-streptomycin) over 5 min, the cells were left aside for 5 min. The fused cells were then spun down and resuspended at an approximate density of 4×10^5 cells per μL of HAT selection media before they were distributed in a dose of 100 μL per well in 96-well culture plates which were previously coated with feeding cells. The HAT media consisted of RPMI minimal media supplemented with 100 μM hypoxanthine, 0.4 μM aminopterine and 16 μM thymidine. Half of the media in the wells were replaced by fresh HAT media every 3rd day. The HAT media were changed to HT media with no aminopterin, when most of the nonfused cells were eliminated.

Hybridoma Selection and Cloning. Ten days after cell fusion, when the hybridoma cells were grown to approximately 30 to 40% confluent in the well, culture supernatants were collected and screened for the presence of anti-hapten antibodies. Noncompetitive indirect ELISA using Hapten A-OVA as a coating antigen was used for hybridoma screening. Selected hybridomas were cloned by limited dilution and stable antibody-producing clones were expanded. Competitive indirect ELISA using Hapten B-OVA as a coating antigen was then employed to determine if the antibodies from the finally expanded clones could recognize the analyte and the coating antigen on a competitive basis. Selected clones were cryopreserved in liquid nitrogen.

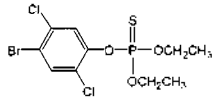
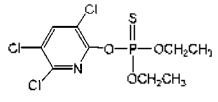
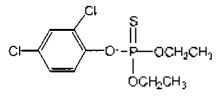
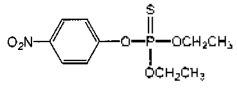
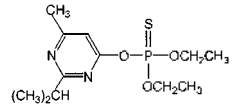
Enzyme-Linked Immunosorbent Assays. In noncompetitive indirect ELISA, all incubations, except for antigen coating, were carried out at room temperature. Microtiter plates (96 well) were coated with Hapten A-OVA (1000 μg/mL, 100 μL/well) in 50 mM carbonate-bicarbonate buffer (pH 9.6) for 16 h at 4 °C. The wells were washed 5 times with PBST solution (10 mM PBS containing 0.05% Tween 20, pH 7.4) and then blocked by incubation with 200 μL/well of 2% gelatin solution. After another washing step, the wells were incubated with 100 μL of serially diluted antibodies in PBST for 1 h. The wells were washed and 100 μL/well of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG diluted 1 : 2000 in 10 mM PBST was added. After incubation for 1 h and washing step, 100 μL/well of a TMB solution (400 μL of 0.6% TMB-DMSO and 100 μL of 1% H_2O_2 diluted with 25 mL of citrate-acetate buffer, pH 5.5) was added to the wells and the reaction was allowed to go for 10 min. The reaction was stopped by addition of 50 μL/well of 2 M sulfuric acid and absorbance was read at 450 nm.

Checkerboard assays, in which several dilutions of antibodies were titrated against varying amounts of the coating antigen (Hapten B-OVA), were used to select the most suitable antibody and to have a rough estimate of appropriate antigen coating and antibody concentrations for competitive assays. Competitive assays were performed as follows. To microtiter plates coated and blocked as described above, 50 μL/well of OP pesticide standards dissolved in 10 mM PBS containing 10% methanol and 50 μL/well of antibodies diluted with PBST were added. The subsequent steps were the same as those in noncompetitive assays. Competition curves were obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoidal curves were fitted to a four-parameter logistic equation,[10] from which IC_{50} values (concentration at which binding of the antibody to the coating antigen is inhibited by 50%) were determined.

Results and Discussion

Synthesis of Immunizing and Coating Antigen Haptens. The purpose of this study is to develop an immunoassay that can determine organophosphorus pesticides in a class-specific manner, not individually. Since most of the commonly used OP pesticides have an aromatic ring and thiophosphate moieties, we decided to synthesize a generic immunizing hapten having both moieties to generate antibodies with a high affinity to the pesticides. However, we tried to eliminate any bias toward a particular structure existent in commonly used pesticides in order to generate antibodies with relatively uniform affinities to the pesticides. The strategy we adopted was to avoid an aromatic ring with a substituent at para position which is present in parathion, and to place instead a substituent on the aromatic ring that is absent in commonly used pesticides. Hapten A designed on this ground gave quite satisfactory results as described below.

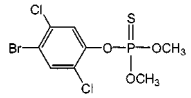
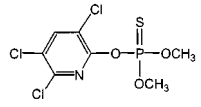
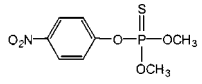
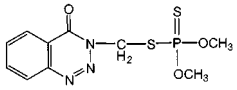
Table 1. IC_{50} and limit of detection values of *O,O*-diethyl OP pesticides using mAbs H-7 and H-9

Pesticides	H-7		H-9	
	IC_{50} (ng/mL)	LOD (ng/mL)	IC_{50} (ng/mL)	LOD (ng/mL)
 Bromophos-ethyl	124	12	121	1.1
 Chlorpyrifos-ethyl	117	8	111	9
 Dichlofenthion	198	10.1	183	18
 Parathion-ethyl	304	0.8	269	7.5
 Diazinon	10,826	150	8,911	480

Immune Response of Mice to the Hapten Conjugate. To see if the immunogen is capable of eliciting the immune response in the mice, antisera from three mice injected with Hapten A-KLH were collected after 2nd and 3rd booster injections and tested for the presence of antibodies recognizing the immunizing hapten by a noncompetitive indirect ELISA using the homologous coating antigen (Hapten A-OVA). All mice sera exhibited high levels of polyclonal antibodies; the titer values after the 3rd injection were 0.25-0.79 at 1/160000 dilution. The titer values after the 3rd injection were 2 to 3 times higher compared with those after the 2nd injection.

Production of Hybridomas and Cloning. Two mice that showed higher titers of polyclonal antisera compared with the third one were used for cell fusion. The spleen cells from these animals were fused with SP 2/0 murine myeloma cells and the resulting fused cells were tested for the presence of

Table 2. IC_{50} and limit of detection values of *O,O*-dimethyl OP pesticides using mAbs H-7 and H-9

Pesticides	H-7		H-9	
	IC_{50} (ng/mL)	LOD (ng/mL)	IC_{50} (ng/mL)	LOD (ng/mL)
 Bromophos-methyl	652	0.9	649	49
 Chlorpyrifos-methyl	329	25	297	9
 Parathion-methyl	1422	75	1062	57
 Azinphos-methyl	99305	n.d.[a]	80651	n.d.[a]

[a]not determined.

antibodies recognizing the immunizing hapten in the same manner as described above for polyclonal antisera. Out of fifty five wells containing hybridoma cells, four wells were found to have cells producing anti-Hapten A antibodies. The cells in the four wells were subsequently subjected to cloning procedures and the clones obtained were screened by a competitive indirect ELISA using parathion and Hapten B-OVA (coating antigen) as competitors. Two clones, designated H-7 and H-9, were finally selected for further characterizations.

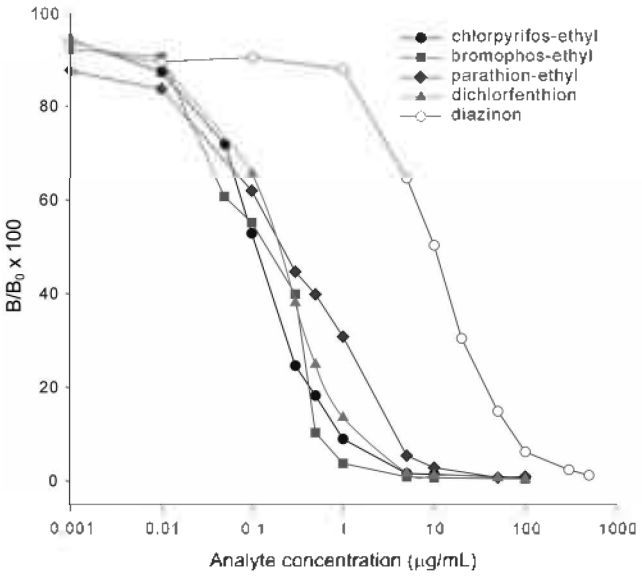


Figure 2. Dose-response curves for *O,O*-diethyl OP pesticides using mAb H-7.

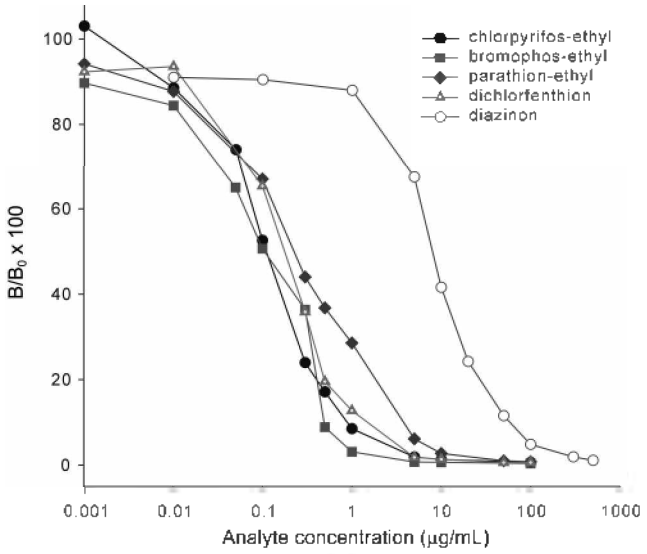


Figure 3. Dose-response curves for *O,O*-diethyl OP pesticides using mAb H-9.

Characterization of the mAbs. The mAbs from the two hybridoma clones were tested for their specificities toward 9 different OP pesticides. Five of them were *O,O*-diethyl OP derivatives and the rest were *O,O*-dimethyl derivatives. As shown in Figure 2, in an indirect competitive ELISA using Hapten B-OVA as a coating antigen, mAb H-7 showed broad specificities toward the ethyl derivatives. The specificities were fairly uniform except with diazinon which was very low. Almost the same pattern was observed with mAb H-9 (Figure 3). In both cases the slopes of the inhibition curves were sharp and the backgrounds were very low. The IC_{50} values of the *O,O*-diethyl OP derivatives excluding diazinon were 0.12-0.30 μg/mL using H-7 and 0.11-0.27 μg/mL using H-9 (Table 1). The reduced affinity of both antibodies toward diazinon could be explained by the unique aromatic

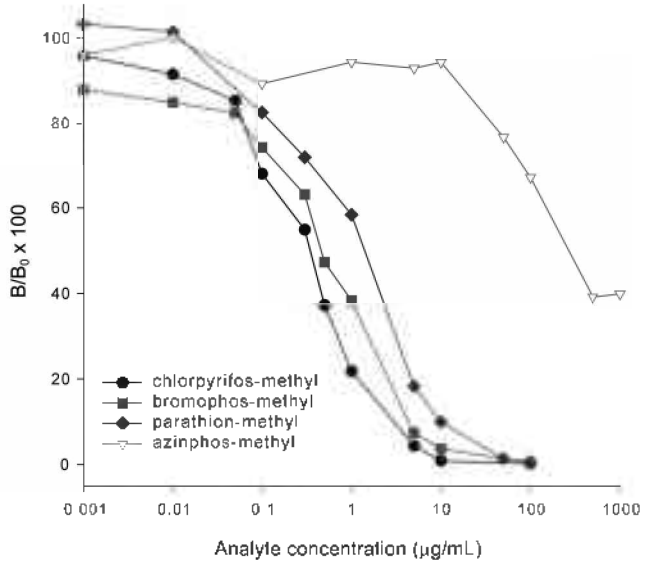


Figure 4. Dose-response curves for *O,O*-dimethyl OP pesticides using mAb H-7.

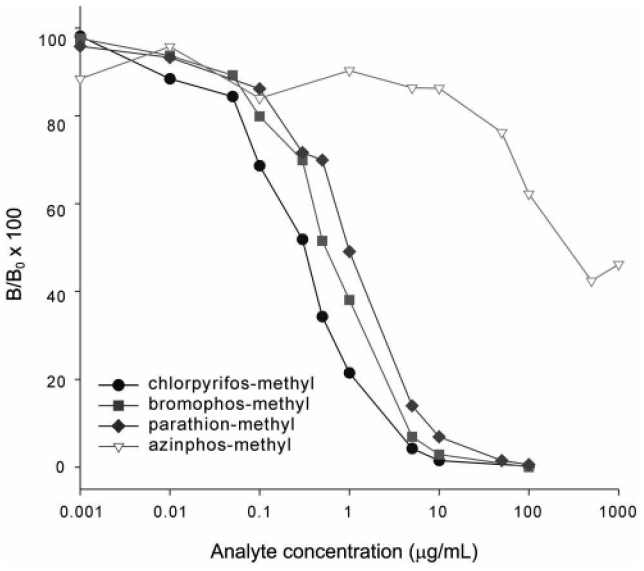


Figure 5. Dose-response curves for *O,O*-dimethyl OP pesticides using mAb H-9.

structure of diazinon (bulky alkyl substituent).

Inhibition curves for four *O,O*-dimethyl OP derivatives, using H-7, is shown in Figure 4. The specificities are fairly uniform, except with azinphos-methyl, which was very low. Almost the same pattern was observed with mAb H-9 (Figure 5). The IC_{50} values of the *O,O*-dimethyl OP derivatives, excluding azinphos-methyl, were somewhat higher than those for the ethyl derivatives: 0.33-1.42 μg/mL using H-7 and 0.30-1.06 μg/mL using H-9 (Table 2). This affinity difference is expected, since the immunizing hapten was an ethyl derivative. These results suggest that both the aromatic ring and the thiophosphate group were involved in inducing antibodies recognizing OP pesticides. Since the two mAbs show almost the same specificities toward the analytes tested, it is quite probable that they were descendants of a single ancestor hybridoma cell.

The ELISA developed by Banks *et al.*[5] for the detection of multiple OPs had a problem of poor sensitivity; the lowest IC_{50} value was *ca.* 5 μg/mL. Therefore, performance of the ELISA we developed is better in terms of sensitivity than that of Banks *et al.*. The ELISA developed by Johnson *et al.*[6] for the detection of multiple OPs had a problem of non-uniform response to the pesticides; the IC_{50} ratio between chlorpyrifos and parathion was *ca.* 1/1000. Therefore, the performance of the ELISA we developed is better in terms of uniformity of response than that of Johnson *et al.*

To confirm that the mAbs were specific only to organophosphorus pesticides, some carbamate pesticides were tested to see if they were recognized by the antibodies. None of them showed any sign of inhibition of antibody binding to the coating antigen (data not shown). Therefore, the mAbs produced from hybridomas H-7 and H-9 were specific only to OP pesticides.

Conclusion

With the aim of developing an ELISA for the detection of organophosphorus pesticides in a class-specific manner, we produced monoclonal antibodies against a generic OP hapten, *O,O*-diethyl-O-(5-carboxy-2-fluorophenyl) phosphorothioate, and examined the characteristics of the antibodies with a competitive indirect ELISA. The antibodies showed a quite uniform specificity to OP pesticides. Although this method does not meet the ideal criteria for uniform response to all OP pesticides, it works well for several commonly used pesticides. Therefore, the present work demonstrates the feasibility of using immunochemical methods for the detection of multiple OP pesticides.

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