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Development of Monoclonal Antibody-Based Direct Competitive Enzyme Linked Immunosorbent Assayfor the Detection of Parathion- methyl

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1. Introduction

Organophosphorus (OPs) insecticides are widely used, for agricultural and domestic purpose, in the control of insect pests. However, most of the above compounds are highly toxic to non-target organisms, either invertebrates or vertebrates, including humans. The monitoring of pesticide residues in groundwater, soil, food contamination, and other environmental samples has gained increasing importance worldwide. Established procedures for detecting pesticides include high-pressure liquid chromatography (HPLC) and gas chromatography (GC). These require extraction of the samples to concentrate the residues and remove interfering matrix materials. Over the last ten years immunochemical methods such as enzyme-linked immunosorbent assay (ELISA) have been increasingly used for detection of pesticides. ELISA and related methods have several advantages and facilitate analysis of large number of samples. ELISAs are much less expensive to run and their detection limits can be as good or better than those of instrumental methods. The initial screening method should be technically simple, inexpensive and useful for the routine analysis of a large number of samples. Immunological methods are becoming increasingly popular in pesticide residue analysis, because they are rapid and inexpensive and have several advantages over conventional analytical procedures. The high selectivity and sensitivity of the immunoassays make them very useful for detecting a wide variety of pesticides at nano- and pico-mole levels. Immunoassays have been developed for a number of pesticides which include endosulfan, metalaxyl, chlorsulfuron, 2,4-D, diclofop-methyl, molinate, and atrazine. Currently, no immunoassay has been developed for the OPs pesticide, parathion-methyl (PM). The objective of this study was to develop a rapid and simple, but highly sensitive, ELISA for the PM. This study was concentrated on the development of direct competitive enzyme-linked immunosorbent assay (DC-ELISA) for the determination of the organophosphorus insecticide, PM (O,O-dimethyl O-4-nitrophenyl phosphorothioate), in environmental samples.

2. Materials and methods

Monoclonal anti-PM antibodies were developed and used in these techniques. In order to obtain the antibody to PM, hapten synthesis developed in this study, which places aminocorboxylic acid directly at the thiophosphate group of organophosphorous pesticides. Two haptens for immunogens, which differ in the length of the bridge (C3, C5) linked to the thiophosphate group in bridge length, were synthesized and conjugated to BSA and/or KLH and then used as immunogens, and with OVA for coating antigen. These two derivatives were also conjugated with HRP to be used as homologous competitors in DC-ELISA (used as enzyme tracers). Conjugations were accomplished by N-hydroxy-succinimide active ethers method. Ten hybridoma cell lines produced anti-PM-MAb were established by PEG fusion method with immunizing BALB/c mice spleen cells and myeloma cell (P3 63Ag8.V653). The fused cells were screened by indirect noncompetitive ELISA using 500 ng/ml PM-A-OVA and PM-B-OVA as coating antigens. Ten kinds of MAb against PM were obtained. Using these MAb allowed developing ELISA system characterized with high specificity and sensitivity. Conditions of direct competitive ELISA method for PM detection were also optimized, and checked the cross reactivity and recovery ratio of the three kind of water samples. And then application to 20 kinds of environment samples, which collected near by Jinju. The concentration were calculated by MPM software(Bio-rad Co., USA).

3. Results and Discussion

Haptens : Haptens were synthesized and conformed by TLC and NMR. The cloudy mixture of Hapten A (PM-A) was purified as column chromatography (silica gel, chloroform/ ethyl acetate/ acetic acid=65:35:1 as the elute phase) and gave as an oil (508 mg, 81% yield). The pure product appeared as a single spot on TLC (Rf=0.80, chloroform/ ethyl acetate/ acetic acid = 50: 50: 1 as the mobile phase); ^{1}H NMR ($CDCl_3$) ; 8.24(2H, d, J=8.9, Ar), 7.38(2H, d, J=8.3, Ar), 3.81(3H, d, J=14.1, CH_3OP), 3.47(1H, quin, J=7.4 NH), 3.17(2H, sext, J=6.8 NCH_2), 2.46(2H, t, J=7.0, CH2CO), 1.88(2H, quin, J=7.0, $CH_2C\underline{H_2}CH_2$). ^{13}C NMR (300 MHz, $CDCl_3$) 179.33(COOH), 155.92, 144.49, 125.35, 121.44(Ar), 41.26, 30.92, 26.17(CH_2), 21.27(CH_3). Hapten B (PM-B) was also synthesized as the route of hapten A synthesis method. The resultant oil (552 mg, 88% yield) was analyzed by TLC, only one spot could be seen (Rf=0.81 using chloroform/ ethylacetate/ acetic acid=50:50:1); ^{1}H NMR (300 MHz, CDCl3) ; δ 8.24(2H, d, J=8.9, Ar), 7.37(2H, d, J=9.2 Ar), 3.81(3H, d, J=14.2, CH_3OP), 3.34(1H, quin, J=7.3 NH), 3.09(2H, sext, J=6.9 NCH2), 2.37(2H, t, J=7.3, CH2CO), 1.68(2H, quin, J=7.6, $NHCH_2C\underline{H_2}$), 1.56(2H, m, $C\underline{H_2}CH_2CO$), 1.40(2H, m, $(CH_2)_2C\underline{H_2}(CH_2)_2$) ; ^{13}C NMR(300 MHz, $CDCl_3$), δ 169.31(COO-), 168.40(C=O), 155.96, 144.42, 125.34, 121.41(Ar), 41.36, 33.75, 30.86, 25.84, 24.06(CH_2), 20.75(CH_3).

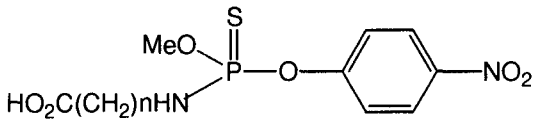


Fig. 1. Structure of parathion-methyl hapten.
(PM-hapten A; n=3, PM-hapten B; n=5),

Production and Characterization of MAbs. Fusions were performed from all of the mice despite the results of some fusion sera, since it has been reported that high-affinity MAbs can be produced form mice with low-affinity antisera. Screenings of fusion cultures were conducted with standard(1 μg/mL) or optimum coating concentrations using the homologous conjugate-coated ELISA format in simultaneous noncompetitive and competitive assays. The standard coating concentration was enough to identify many positive wells. However, the use of optimum coating concentration for each culture supernatant was revealed as essential to find the highest number of competitive wells. From 8 mice fusion, a total of 10 hybridomas were successfully abstained. Their culture supernatant assayed in the homologous conjugate-coated ELISA format to select those MAbs have good titter and competition with PM (0.1 ppm) as competitors. Those MAbs having good competitive for PM (the distance value was cutoff 1.5 between Max. and Min. absorbance and Min. absorbance). Finally 2, 2, and 6 kinds of hybridoma cell line were obtained form PM-A-KLH, PM-B-BSA, and PM-B-KLH immunogens, respectively. All of them were IgG_1 κlight chain isotype. Fig. 2 depicts the inhibition curves obtained with best or specific MAbs for PM by indirect competitive ELISA.

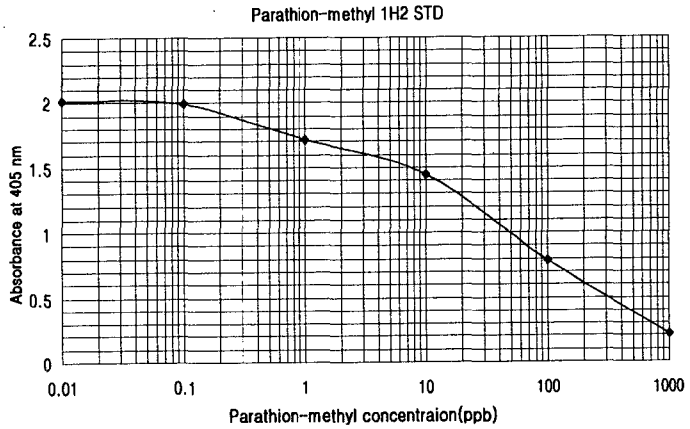
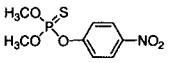
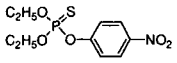
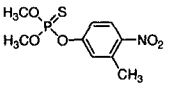
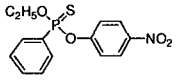
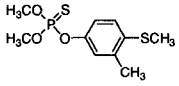
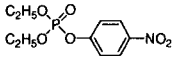
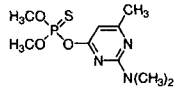
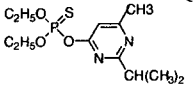
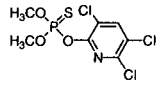
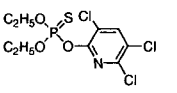
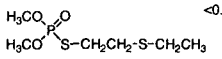
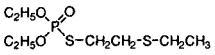
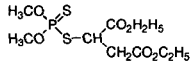
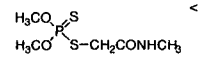
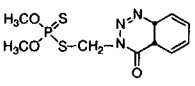
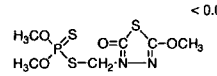
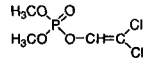
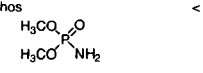
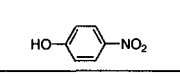
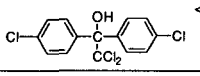


Fig. 2. Standard curve of homologous competitive ELISA and heterogous competitive ELISA for parathion-methyl. The coating antigen was PM-B-OVA at concentration of 25 ug/mL and the antibody was 1H2 MAb at a dilution of 1/5000. Each point is the mean of two well replicates.

Optimization of DC-ELISA. The coating of microtitre plate wells with MAb 1H2 diluted 1:200 with PBS (pH 7.4) followed by competition step between PM standard solutions in distilled water (or samples) and PM-B-HRP conjugate (dilution 1:10000). Detection limit for this technique was 1 ppb, IC_{50} is 20 ppb, linearity range is 10~100 ppb. For the compounds structurally related to PM cross-reactivity was observed for parathion-ethyl (25%), EPN (10%) and fenitrothion (12.5%); for other organophosphorous pesticides it was less than 1%.

Table 1. Cross-reactivity of PM-1H2 to PM and related compounds.

Compound	Chemical structure	CR%	Compound	Chemical structure	CR%
Parathion-methyl		100	Parathion		25
Fenitrothion		12.5	EPN		10
Fenthion		< 0.1	Paraoxon		0.2
Pirimiphos-methyl		< 0.01	Diazinon		< 0.01
Chlorphyrifos-methyl		< 0.01	Chlorpyrifos		< 0.01
Dimeton-s-methyl		<0.1	Demeton-s		< 0.01
Marathion		<0.1	Dimethoate		< 0.01
Azinphos-methyl		<0.1	Methidathion		< 0.01
Dichlorvos		<0.1	Methamidophos		< 0.01
4-nitrophenol		< 0.01	Dicofol		< 0.01

Spiked on tree kinds of waters such as tap water, distilled water, and river water, the recovery averaged between 98 and 110%. The method developed can be used for screening of environs-samples for PM residues without complicated clean up. By application of the method to 20 kinds of environmental water samples, there are not detected PM (less than 1 ppb). The continued worldwide use of PM as an insecticide necessitates the development of new simple and sensitive analytical method for monitoring of agri-food and environmental samples.

4. Acknowledgement

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5. References

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