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RESEARCH PAPER

An Indirect Enzyme-Linked Immunosorbent Assay for Determination of New Herbicide H-9201 in Water

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Abstract: A sensitive indirect competitive enzyme-linked immunosorbent assay (IC-ELISA) was developed for the detection of new herbicide H-9201, and the performance of ELISA for real sample of environmental water test was evaluated. Starting with phosphorus thiochloride and methanol, an analog with similar structure of hapten was synthesized, and carboxyl group was introduced by reacting with succinic anhydride, then the derivative was conjugated with bovine serum albumin or ovalbumin by means of active ester as immunogen or coating antigen, respectively. IC-ELISA method for H-9201was developed based on the polyclonal antibody generated in rabbits by immunizing with synthesized hapten, with a linear range of 0.05–20 mg L^{-1}. For standard, 50% reduction of the maximum ELISA signal (IC_{50}) was 1.331 mg L^{-1}, whereas the detection limit (IC_{10}) was 0.017 mg L^{-1}. The concentrations of coating solution and primary antibody were optimized to be 1/512 and 1/18000, respectively. No significant cross-reactivity for related compounds, such as DMNT, p-nitrophenol, Methamidophos, Phorate, Chlorpyrifos, and Parathion, was observed. The antibody showed negligible cross-reactivity with *S*-Carboxylethyl-*O*,*O*-Dimethyl-phosphorodithioate, dimethoate, and malathion, but exhibited high cross-reactivity with analogs of hapten. Recovery of H-9201 in environmental water was (80.5% ± 4.5%)–(95.2% ± 3.2%) based on the developed IC-ELISA method. This is the first report for demonstration of new herbicide H-9201 in water by IC-ELISA method.

Key Words: Herbicide; H-9201; Enzyme-linked immunosorbent assay; Polyclonal antibody

1 Introduction

Compound H-9201 (*O*-methyl-*O*-(2,4-dimethyl-6-nitrophenoxy)-*N*-isopropyl phosphoramidothioate) was obtained from a series of synthesized alkyl, aryl, *N*-substituted phosphoramidate whose bioactivity of weed control was predicted and mimicked by QSAR (Quantitative structure-activity relationship)[1,2]. Some researches confirmed that steric and electronic effects of different chemical groups would affect the herbicidal activity[3,4]. Extended field experiments indicated that this compound provided good broad-spectrum weed control in different field conditions (land crop or aquatic crop) against barnyard grass and broadleaf weeds but with less or no side effect and injury to the cultivar. H-9201 has potential to replace some herbicides with side effects to the environment and to be widely used for weed control. The result of supervised field trial showed that H-9201 could be widely used in the fields of paddy, soybean, carrot, foeniculum vulgare, coriander, kidney bean, and pepper for weed control, and it is much safer to maize, cucumber, tomato, green onion, rape, and radish when the chemical is applied after the crop sprouts[5–7].

Many enzyme-linked immunosorbent assay (ELISA) methods have been developed for the determination of

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chemicals with low molecular weight. QIAN *et al*[8] reported an ELISA method for detection of chlorpyrifos-methyl based on the monoclonal antibody, which could be used in real sample analysis, and cross-reactivity with chlorpyrifos was 1.4%. A simple synthetic method for haptens of fenitrothion pesticides with a spacer arm (aminocarboxylic acid) attached at the pesticide's thiophosphate group was developed by CHO Young-Ae[9]; later, ELISA methods(including indirect competitive and direct competitive method) for the detection of fenitrothion were developed, which confirmed negligible cross-reactivity with other OP pesticides except insecticide parathion-methyl. Organic solvent in sample extracts will affect the performance of ELISA. Stöcklein[10] covalently immobilized the monoclonal antibody on the surface of polystyrene microtiter plate and established an ELISA which could be used to detect triazine and phenylurea pesticides in ethanol and hexane.

As an experimental chemical, H-9201 provided good broad-spectrum weed control bioactivity, which could be applied in different field conditions. So far, no ELISA method for the determination of H-9201 was reported; our study, for the first time, reported the development of ELISA for H-9201, which could be used in environmental water analysis.

2 Experimental

2.1 Apparatus and reagents

Thiophosphoryl chloride, methanol (HPLC grade), triethylamine, 2,4-dimethyl-6-nitrophenol (DMNT), succinic anhydride, bovine serum albumin (BSA), ovalbumin (OVA), complete freund's adjuvant, and incomplete freund's adjuvant (Sigma, USA); N-hydroxysuccinimide (NHS) and *N,N*'-dicyclohexylcarbodiimide (DCC) (Mercker, USA); *S*-Carboxylethyl-*O*,*O*-Dimethyl-phosphorodithioate (synthesized in our lab.); horseradish peroxidase-labeled goat anti-rabbit IgG (Beijing Biosythesis biotechnology Co., Ltd, Beijing, China); 96-well microtiter plates (Costar, USA); microplate reader (thermo 354, Thermo, USA); UV-visible spectrophotometer (UV-1700); heated magnetic stirrer (Music Instrument Co., Ltd. Shanghai Division); LC/MS (Agilent, USA); nuclear magnetic resonance spectrometer AVANCE AV-500 (BRUKER, Switzerland).

2.2 Hapten synthesis and verification

2.2.1 Hapten synthesis

The hapten synthetic routes are illustrated in Fig.1.

Synthesis of compound I: methanol was slowly added dropwise to the precooled solvent of triethylamine at molar ratio of 1:5 under constant stirring and then stirred for another 15 min below –5 ºC. The mixture was washed by cold water (0 ºC) and then partitioned, and the lower layer of the mixture containing the product of methoxy-thiophosphoryl dichloride was collected and stored at –20 ºC.

Synthesis of compound II: The mixture of compound I and DMNT was dissolved in acetone at molar rate of 1:1 and cooled to 5–10 ºC. The reaction was catalyzed by triethylamine in an ice bath, and the pH value of mixture was ~6. To the stirred solution, 20% NaOH solution (compound (1): DMNT:NaOH is 1:1:1.1, molar ratio) was added, and the pH value was adjusted to 12. The resultant liquor was filtrated, and the filtrate containing compound II was collected.

Synthesis of compound III: compound II in acetone was precooled to 5–10 ºC, then ammonia was added, and the pH value of mixture was adjusted to 8.40%. NaOH solution was added dropwise until the pH value reached 10. Qualitative thin-layer chromatography (TLC) analysis was employed to follow discern the output of the reaction using mixed solvent of petroleum ether/ethyl acetate (4:1, *V/V*) as the mobile phase. After evaporation of acetone, 30 mL ether was used twice for the partition, and the organic phase was collected, followed by desiccating with anhydrous magnesium sulfate and evaporating under reduced pressure. The residue was loaded on a silica gel column and eluted with petroleum ether/ethyl acetate (4:1, *V/V*) to obtain the compound III, which was yellow-brown thick oil with the TLC Rf value of 0.4. The structure of compound III was confirmed by mass spectrometry (MS) and Proton Nuclear Magnetic Resonance (1HNMR). The synthesis rate of compound III was around 70%.

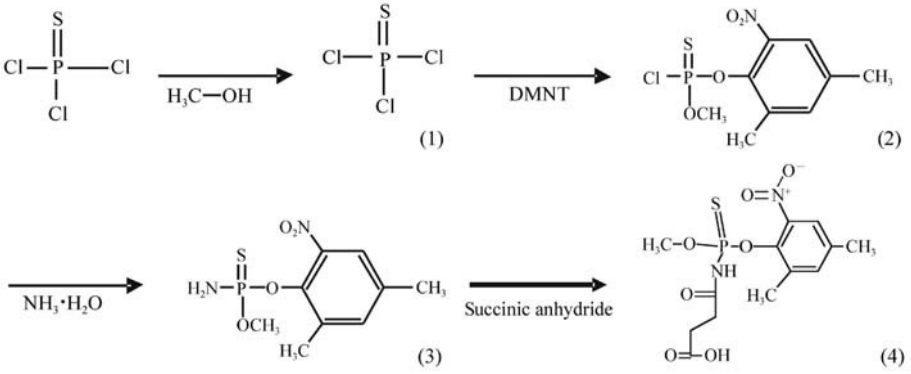


Fig.1 Synthetic routes of analog of H-9201 using as hapten

Synthesis of hapten: To 0.5 mM compound III and 0.8 mM succinic anhydride in acetonitrile, 0.5 mM DMAP was dropped under stirring at 50 ºC for 2 h. The product was washed with 50 mL distilled water and then extracted with dichloromethane, followed by desiccating with anhydrous magnesium sulfate. The organic solvent was removed by evaporation to obtain the hapten, which was identified by MS.

2.2.2 Preparation of hapten-protein conjugates (by active ester method)

Hapten H-9201 was coupled to BSA or OVA as the immunogen or coating antigen by the active ester method, respectively. 0.3 mM hapten in dichloromethane was added dropwise into the mixture of 0.23 mM NHS, 0.33 mM DCC, and 0.033 mM DMAP in DMF. The reaction mixture was stirred overnight at 0 ºC and then centrifuged at 10000 g for 10 minutes. The supernatant was collected, and the organic solvent was removed by evaporation to obtain the active ester, which was redissolved in DMF and then added dropwise in precooled phosphate-buffered saline containing 40 mg BSA or OVA and stirred overnight at 4 ºC. The obtained immunogens and coating antigens were purified by dialysis in phosphate-buffered saline (PBS, pH 7.4) for 72 h at 4 ºC and then stored at –20 ºC. Ultraviolet-visible spectra were used to confirm the final conjugates. The synthetic route for immunogen and coating antigen is illustrated in Fig.2.

2.3 Production of polyclonal antibody to H-9201

The immunization of three New Zealand white rabbits was carried out according to the methods reported by Dong jian, with immunogen of conjugate hapten-BSA[11]. After 5 times of immunization, antiserum for H-9201 was obtained from the heart of each rabbit, purified by saturated ammonium sulfate precipitation and then characterized for antibody titers and analyte recognition by indirect ELISA.

2.4 Indirect competitive ELISA (IC-ELISA)

The IC-ELISA procedure was established under optimized working concentration of coating antigen and antibody[12]. The standard curves were obtained by plotting absorbance against the logarithm of analyte concentration; the IC_{50} and IC_{10} values were calculated according to the equation: I (%) = $[(OD_{control} - OD_{H\text{-}9201})/OD_{control}] \times 100$.

2.5 Specificity of established IC-ELISA

The specificity of antibody for H-9201 was determined by measuring its cross-reactivity (CR) with the H-9201 analog and other structurally related compounds. The specificity of the antibody was determined and calculated as follows: CR (%) = $[IC_{50}(\text{H-9201})/IC_{50}(\text{analog})] \times 100$.

2.6 Determination of H-9201 in pond water with ELISA

Water samples were collected from field pond and filtered through 0.45 μm microporous membrane. 10 ml of filtrate samples were spiked with different concentrations of H-9201 standard prepared in PBS (containing 10% methanol, *V/V*) to make the final concentration of H-9201 0.1, 1.0 and 5.0 mg L^{-1}. Water samples were extracted three times with 3 mL hexane after salting out with 2.0 g NaCl, and the organic solvent was eliminated by nitrogen evaporation. The extract was redissolved in PBST (containing 0.05% Tween, *V/V*) for ELISA test.

3 Results and discussion

3.1 Synthesis and identification of hapten

The analog of hapten (compound III) was synthesized, and the structure of hapten was clarified by MS and ^{1}H NMR. The results of ^{1}H NMR are described as follows: ^{1}H NMR ($CDCl_3$) d: 1.25 (t, J = 7.2, 3H, OCH_2CH_3), 2.04 (s, 3H, $COCH_3$), 2.34 (s, 3H, $PhCH_3$), 2.44 (s, 3H, $PhCH_3$), 3.70 (bd, 2H, NH_2), 3.77, 3.80 (ss, 3H, OCH_3), 4.09 (q, J = 7.1, 2H, OCH_2CH_3), 7.27 (s, 1H, PhH), and 7.51 (s, 1H, PhH). The synthesis rate of compound III was around 78%. The ^{1}H NMR spectrum of compound III showed two peaks at a ratio around 1:1, which was ascribed to OCH3. This occurs because of the existence of R/S isomers due to chiral phosphorus. The result of MS spectrum (+M) (ESI) *m/z* 299 (M + 23) and MS/MS spectrum also confirmed that the structure of the compound was in accordance with that of designed hapten (compound III).

To a solution of compound III in acetonitrile, succinic anhydride was added, and the carboxyl was induced to the derivative. The result of MS spectrum LC/MS(-M) (ESI) *m/z*: 375 (M – H) and MS/MS spectrum showed the desired hapten was obtained. All the MS spectra are shown in Fig.3 and Fig.4.

3.2 Identification of antigen synthesized by active ester method

Figures 5 and 6 show the UV-vis spectra of hapten,

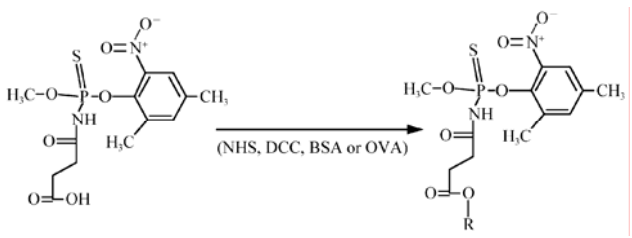


Fig.2 Synthetic route of immunogen and coating agent by active ester method

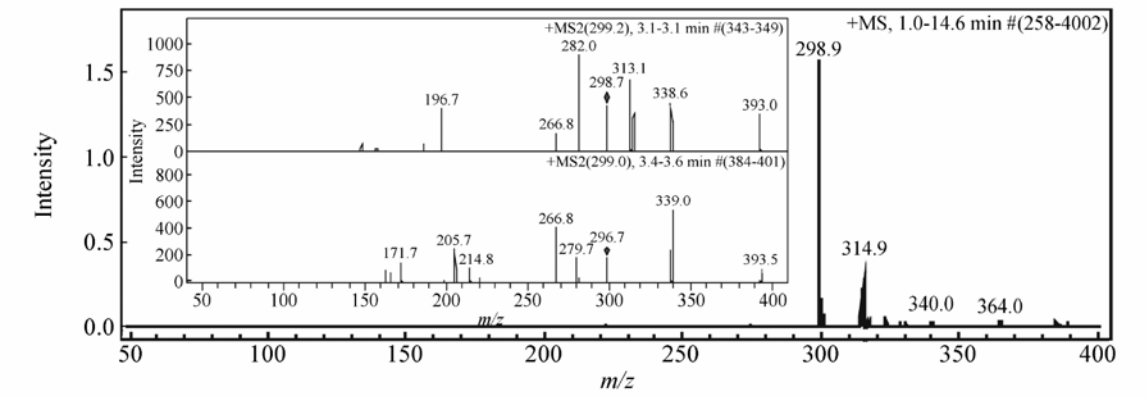


Fig.3 MS spectrum of compound III, inset shows the MS/MS spectrum of ions detected at *m/z* 299 by LC/MS (iron trap)

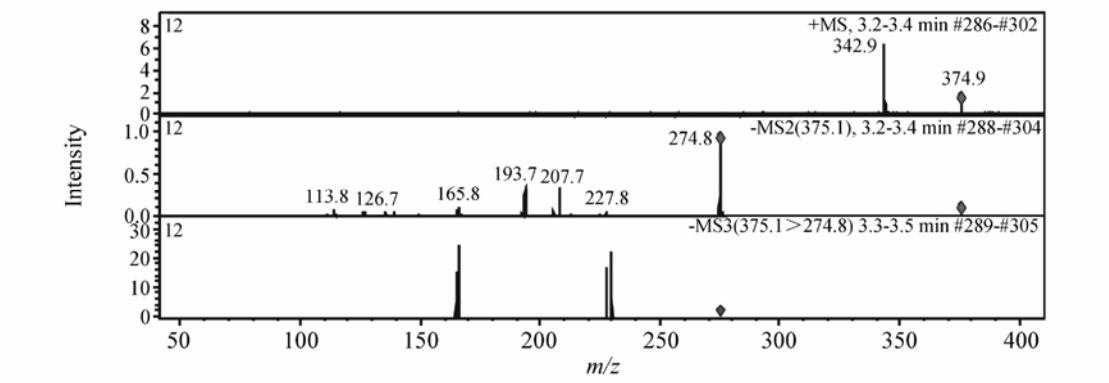


Fig.4 MS spectrum of hapten, follows show MS/MS spectrum of ions detected at *m/z* 375, 275 by LC/MS (iron trap)

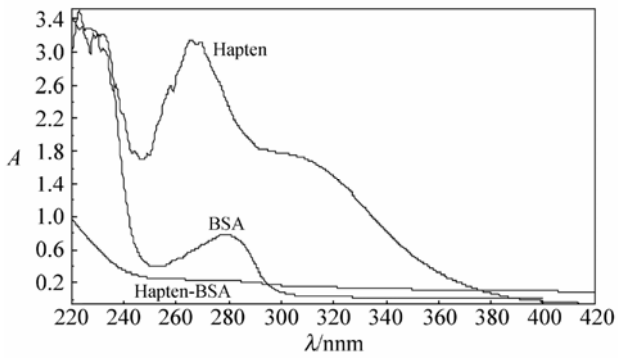


Fig.5 UV spectrum curves of hapten, hapten-carrier protein conjugate, BSA

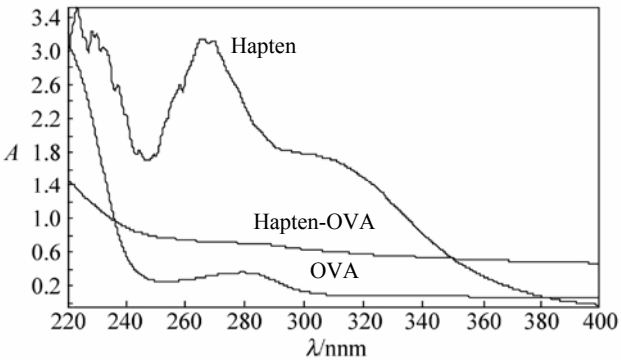


Fig.6 UV spectrum curves of hapten, hapten-carrier protein conjugate, and OVA

conjugates (Hapten-BSA or Hapten-OVA), and carrier proteins. The maximum absorbance wavelength of hapten and carrier proteins (BSA or OVA) is 260 and 280 nm, respectively. Whereas for conjugates, (Hapten-BSA or Hapten-OVA), the maxium absorbance is between 260 and 280 nm, which indicated the successful coupling of hapten to carrier protein.

The conjugation molar ratio of Hapten–BSA and Hapten-OVA is 42.7 and 18.3, respectively, which was assessed by spectrophotometric method. The results of coupling ratio of conjugates are shown in Table 1.

3.3 IC-ELISA

3.3.1 Determinantion of antibody titers and selection of working concentration of coating antigen and antibody

Non-competitive ELISA was used to characterize the reactivity of the antiserum to coating antigen (H-OVA), according to the report of Dong[11].

Table 1 Coupling ratio of conjugates

Items[a]	OD (280 nm)	Molecular weight	Coupling ratio
Hapten	2.364871	375	---
BSA	0.238982	67000	---
OVA	0.358752	45000	---
Hapten-BSA	0.799844	---	42.7
Hapten-OVA	0.713245	---	18.3

Titers of antibodies were determined by two-dimensional titration method in a checkerboard, with various concentrations of the coating antigen and the antiserum. The results showed that rabbit antiserum displayed a titer of 1/102400, and the working concentration was 1/18000 dilution for antiserum and 1/512 dilution for coating antigen.

3.3.2 Inhibition curve

With optimized combination, the IC-ELISA was conducted with a series of concentrations of H-9201 standard prepared in PBS (10% methanol, *V/V*). The inhibition rate displayed a linear relationship with logarithm concentration of H-9201 in the range of 0.05−20.0 mg L^{-1}, and the regression equation was obtained (Fig.7). Under optimized IC-ELISA condition, IC_{50} value was 1.331 mg L^{-1}, and the limit of detection (LOD, IC_{10}) was 0.017 mg L^{-1}. The effects of ionic strength, pH, and organic solvents on ELISA performance were evaluated, and the results showed that the effects would be negligible under neutral pH when the content of methanol was less than 15%, acetone less than 10%, and concentration of Na^+ less than 0.5 M.

3.3.3 Cross-reactivity

Several structure-related pesticides and compounds were tested for their cross-reactivity with antibody for H-9201. As shown in Table 2, no significant cross-reactivity was found for related compounds, such as DMNT, *p*-nitrophenol, Methamidophos, Phorate, Chlorpyrifos, and Parathion. The antibody showed negligible cross-reactivity (less than 20%) with *S*-Carboxylethyl-*O*,*O*-Dimethyl -phosphorodithioate, dimethoate, and malathion, but exihibied high cross-reactivity with analogs of hapten.

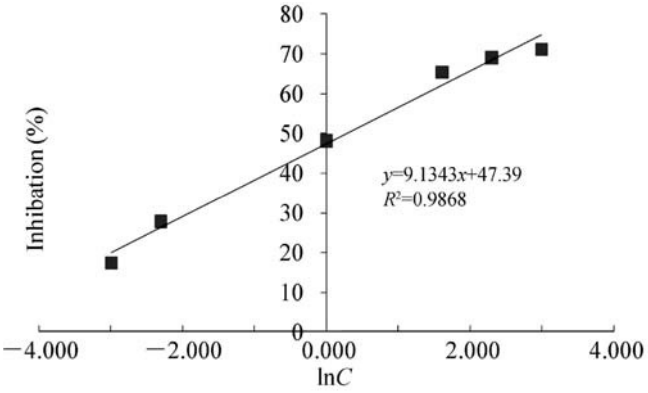


Fig.7 Inhibition curve of antibody for H-9201

Table 2 Cross-reactivity of H-9201-related compounds, and other extensively used pesticides

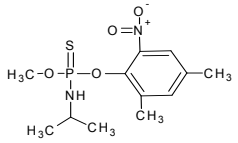
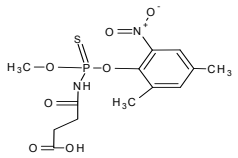
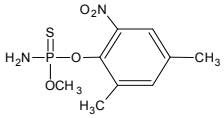
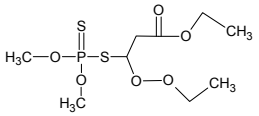
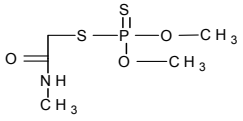
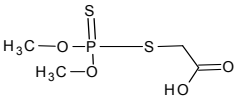
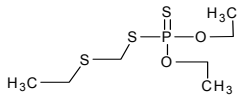
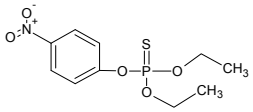
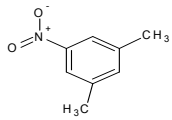
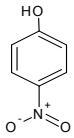
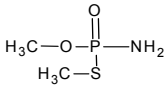
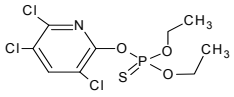
Structure	Compound	IC_{50} (mg L^{-1})	Cross-reactivity (%)
	H-9201	3.514	100
	Compound IV	3.644	96.44
	Compound III	3.557	98.78
	Malathion	17.703	19.85
	Dimethoate	23.06	15.24
	*S*-Carboxylethyl-*O*,*O*-Dimethyl -phosphorodithioate	24.66	14.25

Table 2 (Continued)

Structure	Compound	IC_{50} (mg L^{-1})	Cross-reactivity (%)
	Phorate	>500	< 0.70
	Parathion	> 500	< 0.70
	DMNT	> 500	< 0.70
	*p*-Nitrophenol	> 800	< 0.44
	Methamidophos	> 1000	< 0.35
	Chlorpyrifos	> 1000	< 0.35

3.4 Recovery of spiked water samples

Water sample from field pond, after treatments, that is, precipitation and filtration, was spiked with H-9201 standard. Recovery of H-9201 in environmental water based on the developed IC-ELISA method at the spike levels of 0.1, 1.0 and 5.0 mg L^{-1}, was 80.5% ± 4.5%, 86.8% ± 2.5%, and 95.2% ± 3.2%, respectively.

3.5 Discussion

H-9201 has an O-methyl thiophosphate group and aromatic ring in common with organophosphorus in the structure. Therefore, to achieve a high sensitivity ELISA for this chemical, it was desirable to synthesize conjugates preserving both aromatic ring and thiophosphate group as those in H-9201. Zhang[13] reported that several immunogens had been synthesized with different attachment sites of the analyte moiety to generate antisera against fenthion (containing aromatic ring and thiophosphate group). Their results showed that only those immunogens with partially exposed aromatic or thiophosphate groups seemed to generate antisera with higher titer values, but affinity of antibody was not good, whereas immunogens with well-exposed aromatic and thiophosphate groups generated antibodies with lower titer values but high specificity. Similar results were found in our study, eg. the developed ELISA for the herbicide H-9201 with very low recognition of some organophosphorus containing O-methyl thiophosphate group. Goodrow[14] developed a generalized "size-exclusion" concept for designing immunogen hapten structures, in which the hapten was required to contain appendages smaller than those of the target analyte thereby polyclonal antibodies could be raised that exclude recognition of analytes larger than the target molecule, and also the affinity of generated antibodies was much better than before when the ethyl-isopropyl of simazine was changed to methyl-ethyl[15,16]. In our case, the second hapten containing isopropyl was synthesized by compound II with *α*-aminopropionic acid, the antisera with higher titer values were generated, but with very low recognition ability of H-9201, this phenomenon could be explained by "size-exclusion" concept. 3-(4-Dimethoxyphosphorothioyloxy phenyl) propanoic acid was synthesized as hapten to generate antibody for class-specific determination of *O*,*O*-dimethyl organophosphorus pesticides by Liu[17], and several haptens were conjugated to carrier protein as immunogen to generate

broad specificity polyclonal antibodies for a multiple pesticide immunoassay by Wang[18]. Our study also confirmed that *O*-methyl thiophosphate group as the moiety of immunizing hapten could be used to generate antibody, which could recognize some organophosphorus pesticides. The effect of spacer arm attached at the pesticide's thiophosphate group conjugated to the carrier protein was studied by Kim[19]. Several haptens of fenthion differing in spacer arm length (4–8 carbon atoms) were synthesized, and they were conjugated to carrier protein to be used as immunogens, and the results showed that spacer arm length between 4–8 carbon atoms had no significant effect on the sensitivity of ELISA. So in our case, spacer arm with length equivalent to 4 carbon atoms was used.

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