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RESEARCH PAPER

Preparation of Anti-idiotype Antibodies of *O,O*-Dimethyl Organophosphorus Pesticides by Phage Display Technology

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Abstract: Anti-idiotype antibodies (AId or Ab2) can be used to develop a noncompetitive immunoassay for hapten molecules. In this work, generic hapten, S-carboxyethyl *O,O*-dimethyl phosphorodithioate (CMP), was immobilized on sepharose and then used for purification the class-specific antibodies of organophosphorus pesticides. Affinity-purified positive and negative primary antibodies were used for biopanning of AIds from phage display antibody library. After three rounds of panning, 3 α-AId and 9 β-AId positive clones were obtained. Clones D11 and B9, identified as β-AId and α-AId respectively, were selected to develop a noncompetitive immunoassay system. The IC_{50} value and detection limit of this method for malathion were calculated to be (113.7 ± 34.18) μg L^{-1} and 10.54 μg L^{-1}, respectively.

Key Words: Anti-idiotype antibody; Noncompetitive immunoassay; Organophosphorus pesticides; Phage display

1 Introduction

The current trend of pesticide immunoassay is to develop class-specific immunoassay or multianalytes assay, in which more than one target can be measured in a single test[1]. One valid strategy for class-specific immunoassay is to design and synthesize generic hapten and then prepare a class-specific antibody. Although the generality of the antibody can be improved by this method, its affinity to a given molecule is inferior to those single molecule-specific antibodies, and the sensitivity of such an assay is usually low[2−4].

Theoretical study of Jackson and Ekins has demonstrated that noncompetitive immunoassays are potentially superior to competitive immunoassays in terms of sensitivity, precision, kinetics, and working range of analytes[5]. Sandwich enzyme-linked immunosorbent assay (ELISA) is a widely used noncompetitive immunoassay to determine antigen concentration. However, it has a fundamental limitation that the antigen to be measured must be large enough to have at least two epitopes to be captured; thus it cannot be used to measure low molecular weight compounds, such as pesticides. In order to overcome these drawbacks, a novel immunoassay approach, called "idiometric assay" (Fig.1), was proposed by Bamard and coworkers[6]. Briefly, this assay utilizes α-type and β-type anti-idiotype antibodies (AId or Ab2) that recognize different sites on the anti-hapten antibody (Ab1). The β-AId recognizes an epitope at the unoccupied analyte-binding site, which is masked in the presence of the analyte. Although α-AId recognizes an epitope close to the binding site and is unaffected by the presence or absence of the analyte, it is sterically hindered from binding to the Ab1 in the presence of the β-AId. A number of haptens, such as estradiol[6−8], progesterone[9], estrone-3 glucuronide[10], UDCA 7-NAG (a bile acid metabolite),[11] and 11-deoxycortisol[12], have been determined by this type of assay. And more recently, Niwa *et al*[13] have established a noncompetitive-type ELISA for cortisol based on idiometric assay model, which showed good specificity and approximately threefold higher

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sensitivity (detection limit: 90 pg = 248 fmol cortisol) than a competitive EILSA using the same anti-cortisol antibody, and could be used for the determination of urinary cortisol levels.

However, the difficulties inherent in AId generation, especially in producing an array of different AIds, limit the wide application of idiometric assay[14]. For circumventing this problem, enormous work have been done by researchers, and the preparation of AIds by using phage display technology may be a solution[14−16]. In this study, based on the *O*,*O*-dimethyl OP pesticides class-specific antibody previously developed in our group[3,4], we prepared both α-AId and β-AId of primary antibodies by the phage display technology and developed a noncompetitive-type idiometric immunoassay system for *O,O*-dimethyl OP pesticides.

2 Experimental

2.1 Apparatus and reagents

The generic hapten (*S*-Carboxyethyl *O*,*O*-dimethyl phosphorodithioate) and class-specific antibody of *O*,*O*-dimethyl organophosphorus pesticides were prepared as described in our previous work[3,4]. Human single fold scFv libraries I + J (Tomlinson I + J) were kindly provided by MRC Centre for Protein Engineering (Cambridge, UK). EAH Sepharose 4B, HiTrap Protein G HP (1 mL), and HisTrap FF crude (1 mL) were from GE Healthcare Biosciences. EDC (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride) was from Peptide Institute (Osaka, Japan). HRP-anti-M13 and HRP-anti-HIS_6 secondary antibodies were from Amersham and Genscript, respectively. HRP and TMB (the substrate of HRP) were from Sigma. All other unspecified reagents are analytically pure.

2.2 Preparation of generic hapten-affinity column

Generic hapten was immobilized on EAH Sepharose 4B using carbodiimide method according to the manufacturer's instruction. Approximately 5 mL of EAH Sepharose 4B preswollen gel was washed with distilled water (adjusted to pH 4.5 with HCl), followed by 0.5 M NaCl. Ten milligrams of hapten was dissolved in 5 mL of coupling solution (50% methanol, pH 4.5). The hapten solution was mixed with the washed gel by gentle shaking on an ice bath. Then, EDC solution (0.5 M) was added in 10 aliquots within the first hour of reaction till the final concentration was 0.1 M. The solution was further incubated on an ice bath overnight, and then the temperature of the solution was increased gradually to room temperature. The total time of the reaction was 24 h. The product was washed in three cycles of alternating pH, with 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl and 0.1 M Tris-HCl Buffer (pH 8.0) containing 0.5 M NaCl. Finally, the product was washed with 50% methanol and then packed into a column (1 mL). The efficiency of the coupling reaction was determined by comparing the amount of hapten in the supernatant of the gel suspension, which was detected by LC-MS under selected ion monitoring (SIM) model, before and after the reaction. To determine the binding ability of the coupling products, the coupling products were incubated with the class-specific antibody for 2 h, and then the unbound antibody in the supernatant was detected by indirect noncompetitive ELISA.

2.3 Purification of class-specific antibody and negative antibody

Hapten-affinity column was equilibrated with 10 mL of binding buffer (20 mM sodium phosphate, pH 7.0). After filtrating through a 0.45-μm membrane, ammonium sulfate-precipitated IgG solution was loaded onto the column, and the column was washed with a binding buffer till no protein was detectable in the eluent. Then, elution buffer (0.1 M glycine-HCl, pH 2.7) was used to elute the bound target antibody. The eluted fractions were collected in tubes containing neutralization buffer (about 100 μL 1.0 M Tris-HCl, pH 9.0 per mL fraction). Finally, the affinity medium was reequilibrated with 10 mL of binding buffer. The negative antibody was affinity purified by protein G Sepharose medium according to the manufacturer's recommendation. The concentration, purity, and activity of the purified antibody were determined by Bradford assays, SDS-PAGE and ELISA, respectively.

2.4 Biopanning of libraries

Human single fold scFv libraries I + J (Tomlinson I + J) were used as the resources for anti-idiotype antibody selection. Both libraries were grown separately, and their phages were mixed in the ratio 1:1 for anti-idiotype antibody selections. The biopanning process was carried out as described by Raats *et al*[14] and Goletz *et al*[15] with minor modifications. Briefly, before the actual selection, a preadsorption was performed using purified negative antibody from the same rabbit as the class-specific antibody used for actual selection, and three rounds of panning were applied. The concentration of the coated antibody was 50, 20 and 10 μg L^{-1} for the first, second, and third round of selection, respectively. The binding phages were eluted by class-specific antibody (with the same concentration as the coating antibody), and the eluted fraction was treated with trypsin for 30 min. The eluted phages were inoculated in E. coli TG1 culture suspension for infection and phage amplification. The amplified phages were used for further round of biopanning.

After biopanning, 48 clones after the second round and 48 clones after the third round of panning were randomly picked and analyzed by ELISA. Positive clone was defined as the ratio of OD_{450} on class-specific antibody-coated plate to that on negative antibody-coated plate, which was higher than 2.1.

2.5 Type identification of anti-idiotype antibody

Representative positive clones were transfected into E. Coli HB2151 for the production of soluble antibody fragments, and the expression products were further analyzed as described by Barnard *et al*[6] for type identification. If the binding of scFv fragments to immobilized class-specific antibody was inhibited by hapten molecules, it was classified as *β*-AId. If the binding of the hapten noninhibitable scFv fragments was inhibited by *β*-AId fragments, it was classified as *α*-AId.

2.6 Anti-idiotype antibodies-based noncompetitive immunoassay of organophosphorus pesticides

The idiometric assay format was designed as described[17], and the principle of this noncompetitive immunoassay was outlined in Fig.1. As malathion was most similar to the generic hapten in structure, it was selected for further assay development. The assay conditions such as cosolvent, pH, and ionic strength were selected according to our previous studies[3,4], while conditions such as the amount of immobilized class-specific antibody, the amount of *α*-AId and *β*-AId, and the incubation times were optimized in our experiment.

3 Results and discussion

3.1 Preparation of generic hapten-affinity column

An affinity column was prepared by immobilizing the generic hapten to EAH Sepharose 4B for class-specific antibody purification. The amount of hapten in the supernatant of gel suspension before and after coupling reaction was measured using LC-MS method to monitor the efficiency of coupling. After coupling reaction, the amount of unbound hapten was decreased by 61.6%, while there was no obvious change in control reaction. To verify binding ability of the coupling products toward class-specific antibody, the coupling products were incubated with antiserum for 2 h, centrifugated, and the antibody binding activity of supernatant before and after incubation was determined using indirect noncompetitive ELISA. The results indicated that the positive coupling product could bind the class-specific antibody effectively, while the negative products could not. These results suggested that the hapten molecules were successfully linked to sepharose, and the coupling products could be used for class-specific antibody purification. Then, the sepharose immobilized with generic hapten was loaded onto a column of 1 mL volume for class-specific antibody purification.

3.2 Purification of class-specific antibody and negative antibody

The prepared generic hapten-affinity column was used for class-specific antibody purification. The efficiency of purification was monitored using SDS-PAGE, and the binding activity of purified antibody was analyzed by ELISA. The results indicated that the class-specific antibody was purified successfully (Fig.2a) while maintaining its binding activity (Fig.2b). The negative antibody was purified by protein G affinity column, and the efficiency of purification was desirable as the class-specific antibody. The purified class-specific and negative antibodies were used for panning anti-idiotype antibody by phage display technology.

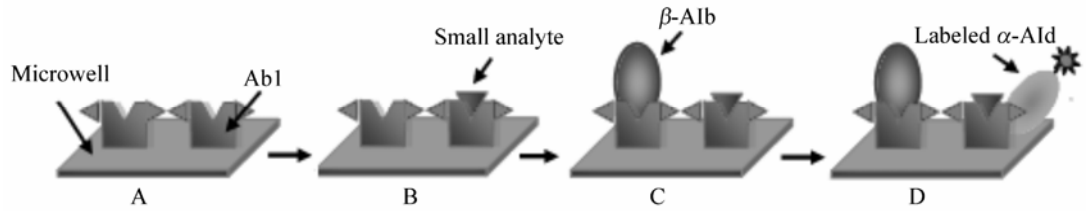

Fig.1 Principle of idiometric assay

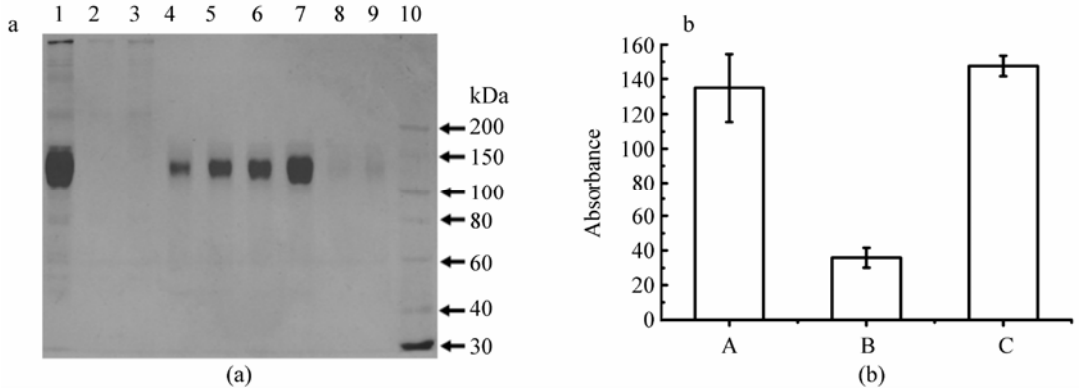

Fig.2 Purification of class-specific antibody

a. Silver-stained 10% SDS-PAGE gel. Lane 1. Ammonium sulfate- precipitated antiserum; Lanes 2 and 3. Flow through (washing); Lanes 4−7. Eluted class-specific antibody; Lane 8 and 9. Flow through (reequilibration); Lane 10. Molecular mass standards. b. Functional activity of purified class-specific antibody. Ammonium sulfate-precipitated antiserum (A), flow through (washing) (B), and purified class-specific antibody (C) were used to carry out ELSA, respectively

3.3 Biopanning of libraries

Human single fold scFv libraries I + J (Tomlinson I + J) were used for anti-idiotype antibody selection. Three rounds of panning were conducted on disposable cell culture bottles. After each round of panning, the titer of the eluted scFv-phage was measured to monitor the efficiency of the selection process. Compared with the second round, the amount of scFv-phages eluted after the third round of panning did not increase (Fig.3a), indicating that the library was enriched with anti-idiotype antibody. After panning, 96 clones were randomly picked and assessed for their binding ability to the class-specific antibody (Ab1) using ELISA, which showed that 15 positive clones (the ratio of OD_{450} between class-specific antibody and negative antibody as coated antibody, respectively, was higher than 2.1) were obtained (Fig 3b). The result of gene sequencing indicated that these 15 clones presented full length V_H and V_L insertion, and the coding sequences of H7 and C8, D10 and C12, and D1 and E6 were identical, respectively. Then C8, C12 and E6 were disregarded, and the other 12 positive clones were chosen for further analysis.

3.4 Type identification of anti-idiotype antibody

The 12 representative positive clones were selected to produce soluble antibody fragments by transfection into E. Coli HB2151, and the soluble products were identified using ELISA. The results indicated that the Ab1 binding activity of clones D6, H7 and B9 was not inhibited by hapten molecules, while all the other clones were hapten inhibitable (Fig.4a). According to the definition of AId, those hapten- inhibitable clones belong to β-AId. When 1 mg L^{-1} of hapten was used for inhibition, the clones D11 and E12 showed the highest inhibition rate (62.0% and 65.7%, respectively). Considering its higher response value, D11 was selected for the following study.Those hapten-uninhibitable clones D6, H7 and B9 were further analyzed to find whether their binding to the class-specific antibody (Ab1) could be inhibited by β-AId (clone D11). The ELISA results (Fig.4b) suggested that binding of clones D6 and B9 was obviously inhibited by β-AId, while the binding ability of H7 was unaffected by β-AId. So, it could be concluded that D6 and B9 belonged to α-AId, and B9 was selected for the following study because of its higher inhibition rate (67.6%).

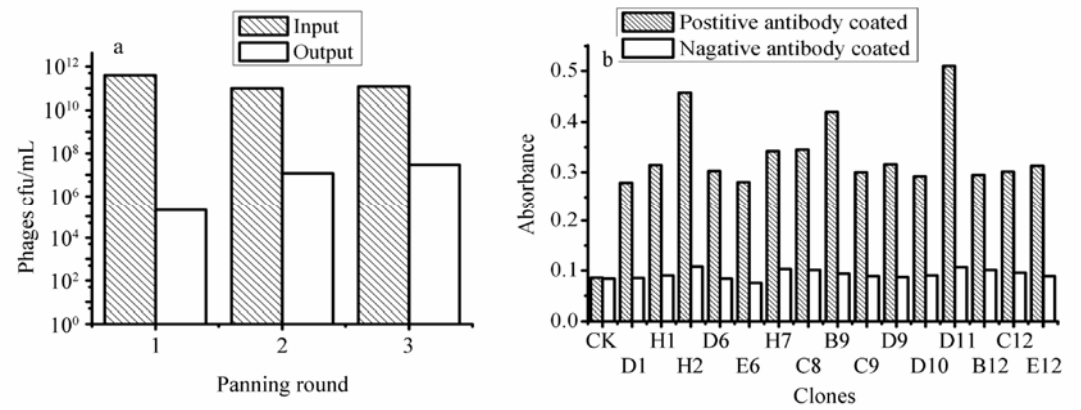

Fig.3 Efficiency of each round panning (a) and reactivity of 15 randomly picked clones (b)

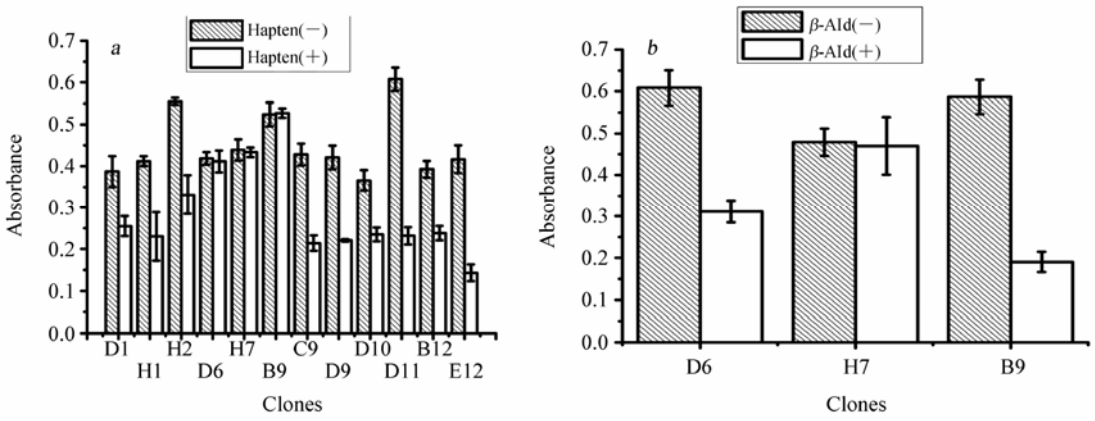

Fig.4 Type identification of the representative positive clones

a. Binding ability of 12 clones to class-specific antibody was analyzed to find whether their binding could be inhibited by hapten molecules. b. Binding ability of those hapten-uninhibitable clones to class-specific antibody was analyzed to find whether their binding could be inhibited by β-AId (D11)

3.5 Anti-idiotype antibodies-based noncompetitive immunoassay of organophosphorus pesticides

Clones D11 and B9, identified as β-AId and α-AId, respectively, were selected for developing noncompetitive immunoassay for OP pesticides. A dose-response curve for malathion was generated under optimized assay conditions (Fig.5), and this dose–response relationship fitted the logistic-log equation (R^2 = 0.9899). The IC_{50} value and detection limit (S/N = 3) of this assay were calculated to be (113.7 ± 34.18) μg L^{-1} and 10.54 μg L^{-1}, respectively. Compared with our previous competitive ELISA using the same class-specific antibody[3,4], the sensitivity of this assay was not improved, but it was higher than what was observed in the work of Garces-Garcia *et al*[18]. This might be due to the low affinity of selected antibodies, which was a common problem in phage display technology. However, the *in vitro* affinity maturation technology can help further improve the affinity of selected scFv[19].

4 Conclusions

In this work, α-AId and β-AId of the *O,O*-dimethyl OP pesticides were successfully selected by phage display technology, and then a noncompetitive immunoassay system was proposed. Compared with our previously reported class-specific competitive ELISA using the same primary antibody, the sensitivity of this assay was not improved, but it was higher than what was observed in the work of Garces-Garcia *et al*[18]. Noncompetitive immunoassay based on anti-idiotype antibodies is still in the primary developing stage, and a lot of work is to be done to explore and assess its potential advantages.

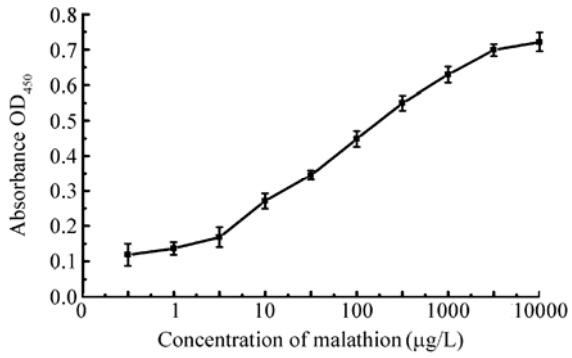

Fig.5 Dose-response curve of malathion

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