Phage displayed domain antibody mimics for pyrethroid and its application in immunoassay

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Abstract: Anti-idiotypic antibodies which can mimic antigens have many potential applications in the immunoassay. This research used the monoclonal antibody of the conserved chemical region of pyrethroids, 3-phenoxybenzoic acid (PBA), and the domain antibody library to develop an environmentally-friendly immunoassay for the detection of pyrethroids. The domain antibodies (A8, B8, and C6) which bound to anti-PBA monoclonal antibody (MAb) were isolated from a naive phage display human domain antibody library. This domain antibody is cloneable, pyrethroid-free, and applicable as a competitive mimetic antigen in the immunoassay. The best immunoassay was achieved using A8, resulting in IC_{50} of 0.714 μg/mL for PBA, 1.775 μg/mL for Cypermethrin, 1.624 μg/mL for β-cypermethrin, 3.675 μg/mL for Fenvalerate, and 4.895 μg/mL for Flucythrinate. This way of selecting anti-idiotypic antibodies to detect pyrethroids could provide potential applications in developing immunoassays for identifying various chemical contaminants in food.

Keywords: domain antibody, enzyme-linked immunosorbent assay, phenoxybenzoic acid, pyrethroid
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1 Introduction

The presence of small molecule chemical contaminants, such as pesticides, mycotoxins, antibiotics and veterinary drugs in foods have attracted great attentions of society since they pose a serious threat to human health. It is necessary to develop a simple and rapid analytical technique for monitoring the residue of small chemicals in foods and feedstuffs[1,2]. Immunoassays are widely used as analytical techniques for such a purpose due to their high sensitivity, specificity, speed, and low cost, easy to operate and high throughput[3-5].

Unlike large molecules, since the small analytes cannot be recognized by two antibodies at the same time, they can not be detected by a sandwich immunoassay. Therefore, a competitive immunoassay is needed, in which the hapten-protein conjugate and the target competitively bind to the antibody. Unfortunately, the conventional competing antigens used for the development of a competitive immunoassay are synthesized by a chemical process. Synthesizing conventional competing antigens are time consuming, complex procedures, and the use of organic solvent. Especially when the hapten is toxic, it may lead a threat to environment and human health[6,7]. Therefore, it is important in developing an environment-friendly method to obtain the competing antigens.

Anti-idiotypic antibodies could be used as competing antigens instead of the conventional hapten-protein conjugates because of its ability of mimicking antigens. Anti-idiotype antibodies against large or small molecules have been developed by monoclonal[8,9] or polyclonal[10,11] techniques. Since phage displayed library firstly reported[12], it has been widely used for including screening antibodies and anti-idiotypic antibodies. The antibodies and anti-idiotypic antibodies screened from phage displayed library could be used effectively for detection of small molecules, such as aflatoxin[13,14], ochratoxin[15], monensin[16], atrazine[17], thiacloprid[18], deoxynivalenol[19], and zearalenone[20]. However, there has not been any reported on select anti-idiotype antibodies against pyrethroids from the naive phage display domain antibodies. However, contaminants which have similar structure usually are present in samples at the same time[21]. Thus, multianalyte immunoassays used to determine the similar contaminants synchronously in a single test have attracted more attention[22]. In this study, we aim to select an anti-idiotypic antibody against pyrethroids from phage display domain antibodies and study its application in multianalyte immunoassay.

Anti-PBA MAb as a model antigen which can recognize several pyrethroids[23] was used for selecting the anti-idiotypic antibody from phage display domain antibodies in this present work. Finally, we developed an immunoassay for detecting pyrethroids based on the anti-idiotypic antibody. The

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immunoassay for pyrethroids was established by using anti-PBA MAb as a coating compound and the phage of positive clones as the competing antigen.

2 Materials and methods

2.1 Materials and Reagents

All the pyrethroids were purchased from Institute for the Control of Agrochemicals. 3-phenoxybenzoic acid (PBA), skim milk (M), bovine serum albumin (BSA), tween 20, 3,3',5,5'-tetramethylbenzidine (TMB), and polyethylene glycol 6000 (PEG 6000) were obtained from Sigma Chemical Co. (New Jersey, USA). The other reagents and anti-M13 horseradish-peroxidase (HRP) conjugate were purchased from GE Healthcare (Beijing, China). The sequencing primers were synthesized by Sangon Biotech (Shanghai, China). The monoclonal antibody of PBA (anti-PBA MAb) was a gift from Dr. Yuan Liu (Institute of Food Quality Safety and Detection Research, Jiangsu Academy of Agricultural Sciences). The human domain antibody library, helper phage KM13 and *Escherichia coli* TG1 were provided by MRC Laboratory of Molecular Biology (Cambridge, England). The antibodies were constructed with CDR1 region, CDR2 region and CDR3 region. All chemical reagents were of analytical grade.

2.2 Biopanning of domain antibodies binding to anti-PBA MAb

A naive phage display domain antibody library with the size of 3×10^9 was used for biopanning of domain antibodies which bind to anti-PBA MAb. The strategy of selection was performed by four rounds selection, and competitive elution was carried out in the biopanning. Briefly, 6-well plate (MaxiSorp, Nunc) was coated with 1 mL anti-PBA MAb overnight at 4 ℃, the well was washed 3 times with PBS and was blocked with 2% MPBS overnight at 4 ℃. After washed with PBS for 3 times, the well coated with anti-PBA MAb was added with 1ml of phage library in 2% MPBS, and was incubated for one hour with gentle shaking. The unbound phages were washed away with PBST and the bound phages were eluted by anti-PBA MAb competitively. Finally, eluted phages were amplified for the following panning.

2.3 Identification of selected domain antibody

The ability of the positive clones to bind with anti-PBA MAb were confirmed by monoclonal phage ELISA. The eluted phages from the last round of elution were used to infect *E. coli* TG1 cells, then *E. coli* TG1 were cultured on TYE plates (containing 100 μg/mL ampicillin and 4% glucose) overnight at 37 ℃. Individual clones were picked randomly and inoculated into the 2×TY medium (containing 100 μg/mL ampicillin and 4% glucose). They were cultured overnight at 37 ℃ with shaking at 250 r/min. 2 μL culture was transferred to 200 μL 2×TY medium (containing 100 μg/mL ampicillin and 4% glucose) and cultured at 37 ℃ with shaking at 250 r/min for about two hours until the $OD_{600\ nm}$=0.5. The helper phage KM13 was added to the culture and incubated for one hour at 37 ℃. The bacteria were collected by centrifugation and re-suspended in 2×TY medium (containing 100 μg/mL ampicillin, 50 μg/mL kanamycin and 0.1% glucose). The culture was incubated overnight at 25 ℃ with shaking at 250 r/min. 100 μL of supernatant were added to 96 wells plates which were coated with 2 μg/mL of anti-PBA MAb and blocked with 2% bovine serum albumin (BSA), at the same time the wells coated with 2 μg/mL of BSA as the negative. Then anti-M13 horseradish-peroxidase (HRP) conjugates were added and incubated in the wells. Finally, peroxidase substrate was added to the wells. The reaction was stopped by adding 2 M H_2SO_4 and the absorbance of 450 nm was read by using a microplate reader. Before each procedure the wells were washed with PBST, except the last one.

Positive clones which were identified by ELISA would be further verified by bacteria PCR, and the primers were DAB-F2 (5'- A G G T G C A G C T G T T G G A G T C T G -3'), and DAB-R2 (5'- T C G A G A C G G T G A C C A G G G T T -3'). The PCR products were analyzed by agarose gel electrophoresis. Finally, the positive clones were sequenced with the primer (5'- C C C T C A T A G T T A G C G T A A C G A -3'). The positive clones were translated from the nucleotide sequences (according to http://web.expasy.org/translate/).

2.4 Production of selected domain antibody

The confirmed positive clones were cultured to amplify the phages. The clones were cultured overnight at 37 ℃ with shaking at 250 r/min. A sample of 5 mL culture was transferred into 500 mL 2×TY medium (containing 100 μg/mL ampicillin and 4% glucose) and cultured at 37 ℃ with shaking at 250 r/min until the OD_{600nm}=0.5. The 10^{11} helper phages KM13 were added and incubated for one hour at 37 ℃. The bacteria were collected by centrifuging (3200g for 10 min) at 4 ℃ and re-suspended with 500 mL 2×TY medium (containing 100 μg/mL ampicillin, 50 μg/mL kanamycin and 0.1% glucose). The culture was incubated overnight at 25 ℃ with shaking at 250 r/min. After centrifugation of overnight culture (3200g for 30 min), 100 mL PEG/NaCl (20% polyethylene glycol, 2.5M NaCl) was added to the supernatant. The mixture was incubated on ice for one hour and then spun (3200g for 30 min) once more. The pellets were re-suspended in PBS and the mixture was precipitated by PEG/NaCl for one more time. Finally, the pellets were re-suspended in PBS and spun (3200 g for 30 min) at 4 ℃ again to remove any bacterial debris. The titer of the phages was determined and the phages were stored at -80 ℃ after adding glycerol.

2.5 Domain antibody-based competitive immunoassay for pyrethroids

To obtain the optimized dilution of domain antibody and anti-PBA MAb, a checkerboard titration analysis was performed by using a different dilution of them in advance. Serial dilutions of phage were added to the wells coated with different concentrations of anti-PBA MAb (2, 1, 0.5, 0.25, and 0 μg/mL).

The wells of a 96-well plate were coated with anti-PBA MAb overnight at 4 ℃. The wells were blocked with 2% bovine serum albumin (BSA). 50 μL of pyrethroid standards at concentrations from 0.078125 to 20 μg/mL (or sample extracts) and 50 μL domain antibody were added. The mixtures were incubated for one hour at 37 ℃. 100 μL of the anti-M13 horseradish-peroxidase (HRP) conjugate (1:5000 diluted in PBS) was added to the wells and incubated at 37 ℃ for one hour. 100 μL TMB substrate was used. After 15 min, the reaction was stopped by adding 50 μL of 2M H_2SO_4. Before each procedure the wells were washed with PBST, except the last one. The absorbance of the wells was detected at 450nm by a microplate reader. Results were shown as IC_{50}.

2.6 Matrix effect and samples analysis

Chinese cabbage was chosen to study matrix effect on the domain antibody-based competitive immunoassay. 1 μg/mL and 2 μg/mL of pyrethroids were added to the pyrethroids free Chinese cabbage matrices, 2 times matrices dilution, and 4 times matrices dilution, respectively. The mixtures were detected by the ELISA developed with domain antibody. To evaluate the recovery of domain antibody-based immunoassay, 20 g pyrethroid free samples

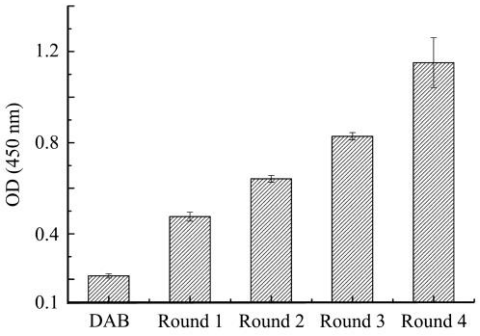
verified by GC were spiked with pyrethroids at the concentrations of 2.0 μg/mg and 3.0 μg/mg, respectively. After 30 min, 20 mL acetonitrile, 8.0 g Na_2SO_4 and 3.0 g NaCl were added. The mixture was strongly shaken for one minute and spun at 4000 r/min for 30 min. The supernatants were dried by nitrogen evaporator and re-dissolved in 10% methanol-PBS, and the solutions were used as sample extract. The ELISA assay was carried out as well as 2.5.

3 Results and discussion

3.1 Selection and identification of positive phage displayed domain antibody

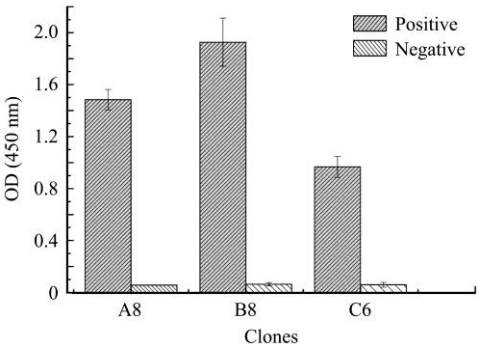
The ability of the library to bind to anti-PBA MAb was detected by polyclonal ELISA. The polyclonal ELISA result of the fourth round was 5 times higher than that of the original library (Figure 1). Positive clones were identified by monoclonal phage ELISA (Figure 2), PCR identification (Figure 3) and DNA sequencing. Then three unique sequences were obtained and presented in Figure 4. Finally, three different phage clones (A8, B8, C6) which was illustrated strong binding capacity to anti-PBA MAb were obtained in present study. This result demonstrated that our strategy was successful in the selection of positive domain antibody clones.

As the DAB library has been described previously[24], the framework regions of the three sequences are highly conserved (Figure 4). There is only one amino acid being identical in the CDR1 region and three amino acids being identical in the CDR2 region while the total numbers of amino acids remain the same. The amino acids present diversification in the CDR3 region, and the number of amino acids are variant.



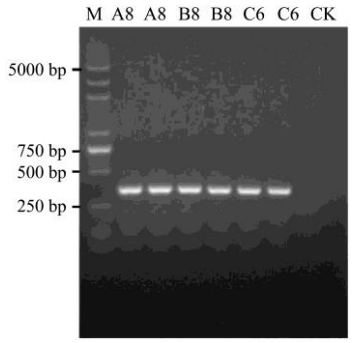
Note: The values of OD were expressed as the mean ± standard deviations (SD) after being subtracted the control value of three measurements.

Figure 1 Polyclonal ELISA was performed using the original DAB library, or the library which was amplified after each round of panning



Note: The values of OD were expressed as the mean ± standard deviations (SD) of three measurements.

Figure 2 Monoclonal ELISA was performed using the clones which were obtained after four round panning



Note: The positive clones with full length gene of domain antibody had stripes at about 360 bp. Lane M: 5000 DNA marker, A8, B8, C6 and CK.

Figure 3 Electropherogram of PCR amplification

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A8          MKKLLFAIPLVVPFYAAQPAMAQVQLLESGGGLVQPGGSL      40
B8          MKKLLFAIPLVVPFYAAQPAMAQVQLLESGGGLVQPGGSL      40
C6          MKKLLFAIPLVVPFYAAQPAMAQVQLLESGGGLVQPGGSL      40
CONSENSUS   MKKLLFAIPLVVPFYAAQPAMAQVQLLESGGGLVQPGGSL
                      CDR-H1                    CDR-H2
A8          RLSCAASGFKINNYDMGWVRQAPGKGLEWVSGIRGPDGST      80
B8          RLSCAASGVSFSNNNMGWVRQAPGKGLEWVSSINMTDGST      80
C6          RLSCAASGVIASHYNMAWVRQAPGKGLEWVSSIIIPDGST      80
CONSENSUS   RLSCAASG        M WVRQAPGKGLEWVS I    DGST

A8          YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAA      120
B8          YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAG      120
C6          YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAT      120
CONSENSUS   YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCA
                    CDR-H3
A8          SDCARNIVTFITKPIGFWGQGTLVTVSSAAAEQKLISEED      160
B8          .RRRRPIQTHMR....YWGQGTLVTVSSAAAEQKLISEED      155
C6          DMWHEGHAALKS.....WGQGTLVTVSSAAAEQKLISEED      155
CONSENSUS                   WGQGTLVTVSSAAAEQKLISEED

A8          LNSAAHYTDIEMNRLGKGAA                          180
B8          LNSAAHYTDIEMNRLGKGAA                          175
C6          LNSAAHYTDIEMNRLGKGAA                          175
CONSENSUS   LNSAAHYTDIEMNRLGKGAA
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Note: The sequences were translated from the nucleotide sequences (http://web.expasy.org/translate/). Positions of the respective complementarity determining regions of the heavy chain (CDR-H1, CDR-H2, CDR-H3) were indicated in boxes (analyzed by http://www.bioinf.org.uk/abs/).

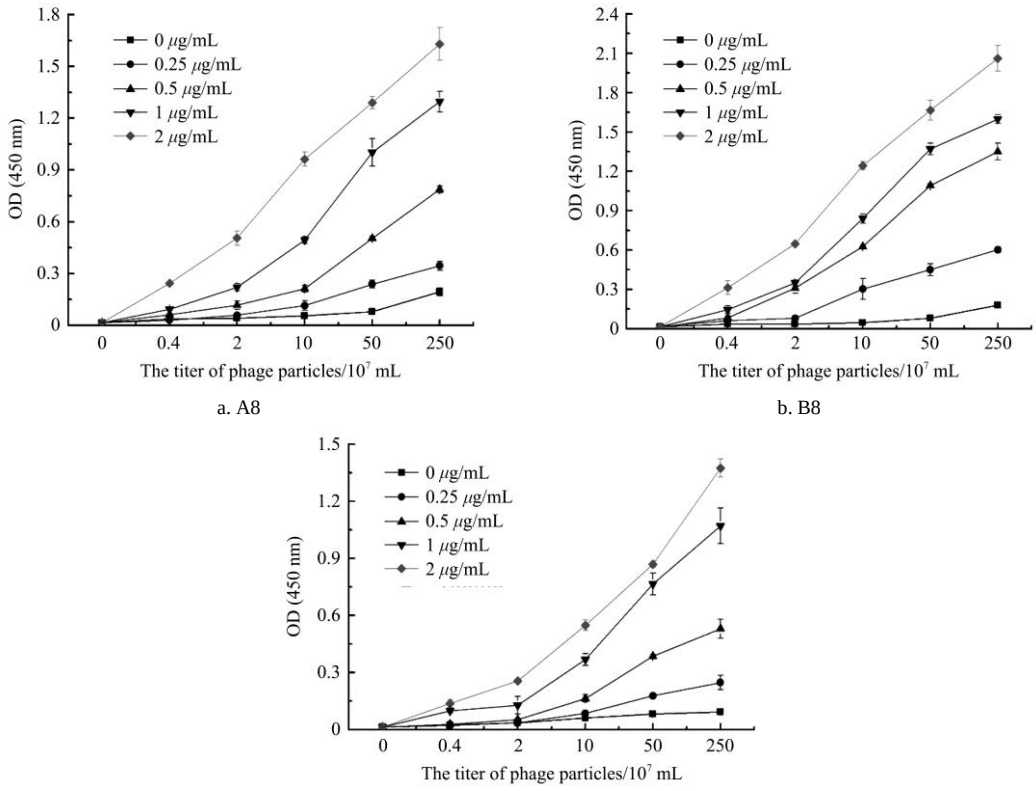
Figure 4 Amino acid sequences of the positive clones

3.2 Domain antibody-based competitive ELISA for pyrethroids

The optimal concentrations of anti-PBA MAb and the titers of A8, B8, and C6 phage particles were performed by the checkerboard titration analysis and the results were presented in Figure 5. The best property was realized with 1.0 μg/mL of anti-PBA MAb and 5×10^8 of phage for A8; 0.5 μg/mL of anti-PBA MAb and 5×10^8 of phage for B8; 1.0 μg/mL of anti-PBA MAb and 2.5×10^9 of phage for C6. Under the optimal conditions, the domain antibodies (A8, B8 and C6) were assessed for their properties by using a competitive ELISA and the results were shown in Figure 6. The competitive ELISA established with A8 showed the best property. And the IC_{50}s of competitive ELISA established with A8 were 0.714 μg/mL towards PBA, 1.775 μg/mL towards cypermethrin, 1.624 μg/mL towards β-cypermethrin, 3.675 μg/mL towards fenvalerate, 4.895 μg/mL towards flucythrinate, respectively. But pyrethroids without phenoxybenzene group, such as resmethrin, transfluthrin, S-bioallethrin showed no significant competitive capacities. The detection limit and the linear range of the ELISA developed with A8 were shown in Table 1. It showed that the domain antibodies had different competitive capacities with the pyrethroids. The possible explanations were (1) the distinct steric parameters and particle charges of atoms or groups in the pyrethroids[25,26], (2) antibodies acted as a receptor or a detector for the analyte in the immunoassay[27], (3) binding affinity, therefore, the assay sensitivity could increase from hydrogen bonding, hydrophobic

bonding, electrostatic, and van der Waals forces[28].

Using the phage-displayed domain antibody has demonstrated greater advantages compared with soluble expressed domain antibody. Firstly, multiple binding-sites can improve the sensitivity of the developed immunoassay. Secondly, phage displayed domain antibody can be easily and cheaply manufactured. Phage-borne antibody yielded in the culture medium could be precipitated by PEG/NaCl and isolated by centrifugation without using Ni-NTA and the cell lysis which are necessary for the expression of soluble domain antibody.

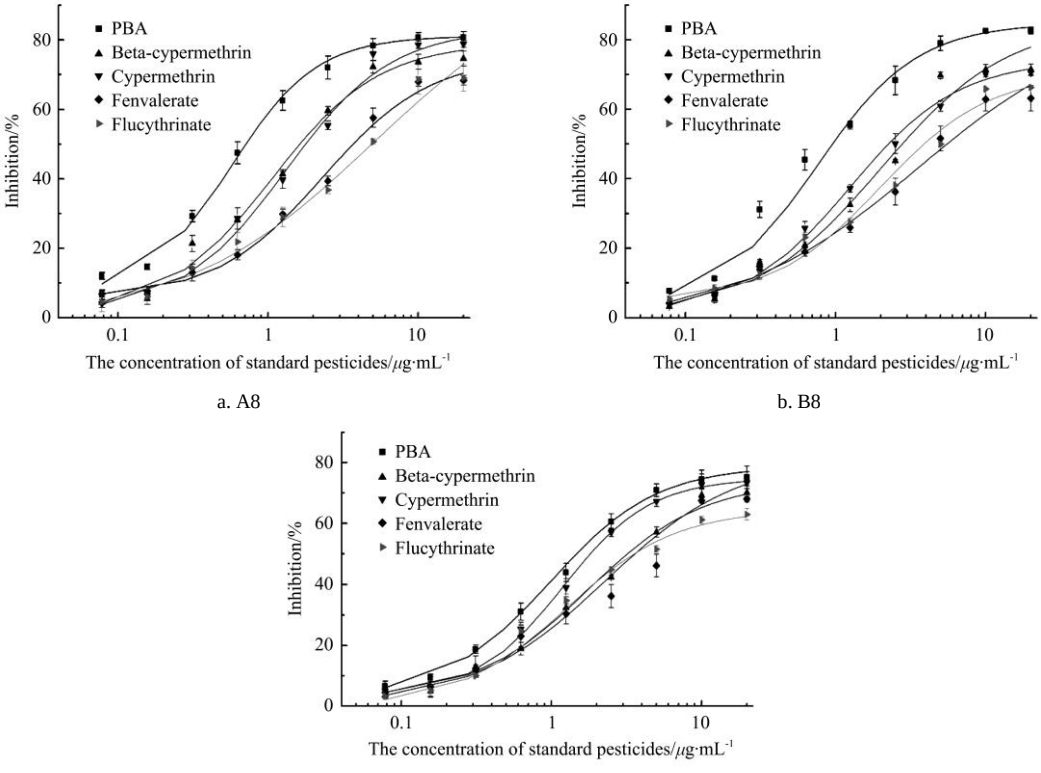


a. A8 b. B8

c. C6

Note: The values of OD were expressed as the mean ± standard deviations (SD) of three measurements.

Figure 5 Results of checkerboard analysis



a. A8 b. B8

c. C6

Note: The values of OD were expressed as the mean ± standard deviations (SD) of three measurements.

Figure 6 Inhibition curves of phage-based ELISA

Table 1 Results of IC_{50}, detection limit and the linear range of PBA and pyrethroids (A8)

Pesticides	IC_{50} /μg mL^{-1}	Detection limit /μg mL^{-1}	Linear range /μg mL^{-1}
PBA	0.714	0.0566	0.256-1.388
Cypermethrin	1.775	0.229	0.422-4.011
β-cypermethrin	1.624	0.197	0.341-3.315
Fenvalerate	3.675	0.241	0.732-7.247
Flucythrinate	4.895	0.220	0.658-37.689
Fenpropathrin	>20	—[a]	—
Deltamethrin	>20	—	—
Permethrin	>20	—	—

Note: [a] The value was not found.

3.3 Matrix effect and samples analysis

The Chinese cabbage matrices were selected for the analysis of matrix effects.The results are shown in Table 2, suggesting that there is no remarkable matrix effect in Chinese cabbage matrices.

To evaluate the effectiveness of the assay, the developed ELISA was performed to detect the recovery. The samples spiked with 2 μg/mL and 3 μg/mL of pyrethroids showed the recoveries ranging from 87.4% to 90.2% (Table 2). The result proved that the domain antibody-based competitive ELISA could be used to monitor pyrethroids in Chinese cabbage.

Table 2 Matrix effect of sample on the phage ELISA and recovery results

	Spiked levels /μg mL^{-1}	Recovery[a] /%		
		Cypermethrin	Beta-cypermethrin	Fenvalerate
Matrix buffer	1.0	81.1±1.2	76.8±1.5	80.8±1.1
	2.0	80.6±2.7	85.6±1.2	86.7±1.2
2 times dilution of matrix	1.0	84.3±6.8	82.6±3.8	84.0±3.0
	2.0	89.6±0.8	87.5±3.5	89.8±1.8
4 times dilution of matrix	1.0	84.8±1.3	88.6±2.4	84.1±2.6
	2.0	92.6±2.9	89.4±2.5	88.6±1.6
Spiked pyrethroids samples	2.0	88.1±1.4	88.1±1.2	87.4±1.3
	3.0	89.0±1.3	90.2±0.5	89.9±2.1

Note: [a] The values were expressed as the mean ± standard deviations (SD) of three measurements.

4 Conclusions

In this study, the domain antibody A8 which bound to anti-PBA MAb was selected by the competitive biopanning strategies from a naive phage displayed domain antibody library. The developed immunoassay using A8 showed high sensitivity to pyrethroids. The mimetic antigen can be produced in a relatively short period in large-scale production, and it is also pyrethroid-free, low-cost and environment-friendly. These results indicated that selecting anti-idiotypic from the phage display domain antibody library would be a useful technology to develop immunoassays for the detection of various food chemical contaminants.

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