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Development of a One-step Immunoassay for Triazophos Using Camel Single Domain Antibody-Alkaline Phosphatase Fusion Protein

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Abstract

Triazophos is mainly used in Asian and African countries for the control of insects in agricultural production. Camelid variable domains of heavy chain antibodies (VHHs) show great promise in monitoring environmental chemicals such as pesticides. To improve the rate of success in the generation of VHHs against triazophos, genes specifically encoding VHH fragments from the unique allotype IgG3a of an immunized Bactrianus were amplified by using a pair of novel primers and introduced to construct a diverse VHH library. Five out of seven isolated positive clones, including the VHH T1 with the highest affinity to triazophos, were derived from the allotype IgG3a. A one-step enzyme linked immunosorbent assay (ELISA) using VHH T1 genetically fused with alkaline phosphatase (AP) had a half-maximum inhibition concentration of 6.6 ng/mL for triazophos. This assay showed negligible cross-reactivity with a list of important organophosphate pesticides (<0.1%). The average recoveries of triazophos from water, soil and apple samples determined by the one-step ELISA ranged from 83% to 108%, having a good correlation with those by a gas chromatography-mass spectrometry (R^2 = 0.99). The VHH-AP fusion protein shows potential for the analysis of triazophos in various matrices.

Keywords

Variable domains of heavy chain antibody; IgG3a; VHH-AP; One-step ELISA; Triazophos

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Compliance with ethical standards

The animal experiments were approved by the China Agricultural University Animal Care and Use Committee. All procedures performed in this research involving Bactrian camels were in accordance with the ethical standards of the China Agricultural University Animal Care and Use Committee. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

The authors declare that they have no conflict of interest.

Introduction

Since antibodies devoid of light chain were discovered in the serum of the camel (*Camelus dromedarious*) [1] their single domain antigen-binding fragments, known as VHHs or nanobodies, are of increasing interest in a wide variety of applications in life sciences. Compared to conventional antibodies such as polyclonal and monoclonal antibodies (pAbs and mAbs), VHHs have a number of advantages such as small size, high solubility, high thermal stability, refolding capacity and low-cost production.[2–5] VHHs are particularly suitable for genetic manipulation such as construction of a fusion protein with an enzyme or protein to create bifunctional molecules.[6–8] In the last decades, most of VHHs were employed in multiple applications for large molecule (e.g., protein) analysis rather than for small molecules. Intrinsically, there is an uncertainty associated with the generation of VHHs for small molecules because camelids do not always provide high-affinity heavy chain antibodies (HCAbs) needed for sensitive assay development.[9, 10] Although high-affinity VHHs to a diverse group of small compounds have been generated and used to develop highly sensitive immunoassays (ng/mL or pg/mL level), failures in the generation of VHHs of this interest are common.[11, 12] It is necessary to explore new approaches to improve the rate of success in the generation of VHHs with high affinity to small analytes.

The success to obtain a VHH against small molecules is related to chemical structures and properties, proper hapten design, animal species, complete amplification of the genes encoding for any of the VHHs and a valuable biopanning strategy.[11] Hapten design is a critical aspect of both antibody generation and assay development. In general, haptens used to produce conventional antibodies (IgG1) were also suitable for VHH generation. An adequate library of competing ligands could provide various panning patterns for the selection of desired VHHs, as shown in a study that the VHH specific for tetrabromobisphenol-A (TBBPA) selected by using a heterologous coating antigen exhibited about 10-fold higher sensitivity to TBBPA in an immunoassay than that selected by using a homologous coating antigen.[13] For biopanning, the use of either nonspecific elution with triethylamine (pH 11) or specific elution with a gradient of decreasing concentrations of competitive analyte was an efficient strategy to isolate VHHs from phage libraries. Very recently, Pírez-Schirmer *et al.*[14] isolated a VHH with high affinity to microcystin-LR (MC-LR) using an off-rate selection method in which bound phages were incubated overnight with MC-LR and then eluted with trypsin. This method is most probably target specific and can be variable for other analytes.

Camelids possess a variable percentage of HCAbs (IgG2 and IgG3) in their sera. In Bactrianus and Dromedarius, the content of HCAbs might reach 50–80%, whereas it is only 10–25% in the sera of alpacas and llamas.[15] These data suggest that immunization of true camels might offer advantages over other members of the camelid family including llama and alpaca in generating high levels of IgG2 and IgG3 useful for isolation of desired VHHs. Nonetheless, llamas or alpacas currently are most commonly used to prepare VHHs specific for small molecules, probably due to their ready availability and tractability.[16] In previous studies,[16–18] the primer design was only focused on the VHH fragments from allotypes IgG2b and IgG3b of llamas or alpacas and the constructed library provided limit diversity

for the selection of desired VHHs. On the other hand, the allotypes IgG2a and IgG3a of camelids still remain uninvestigated in the generation of VHHs of any interest. We assumed these allotypes would provide valuable resources for the construction of high diversity of VHH libraries and herein introduced Bactianus IgG3a-derived VHH fragments into a library to explore the generation of novel VHHs with high affinity to small molecules, using the organophosphate insecticide triazophos (*O,O*-diethyl *O*-(1-phenyl-1*H*-1,2,4-triazol-3-yl)phosphorothioate) as a model chemical. Triazophos is widely used to control insects for agricultural production in many Asian and African countries such as China, India, Pakistan, Thailand, Angola and South Africa.[19] The stability and slow degradation of triazophos in the environment give rise to a health threat to human and ecosystems. Triazophos can be absorbed through human skin and respiratory and gastrointestinal tracts. Acute exposure to triazophos may cause poisoning with symptoms such as sweating, headaches, abdominal pain, respiratory depression and chest pain.[20, 21] A rapid, sensitive and cost-effective immunoassay for the detection of triazophos in complicated matrices is always desirable in regions where the pesticide is still in use.

In the present study, we designed a pair of novel primers to amplify the genes encoding VHH fragments from the unique allotypic variant IgG3a of camel and constructed a diverse library containing VHH fragments of allotypes IgG2b, IgG3a and IgG3b. An IgG3a-derived VHH highly selective for triazophos was isolated and genetically fused with alkaline phosphatase (AP) to develop a one-step enzyme linked immunosorbent assay (ELISA) for triazophos in environmental samples. This study may open a new perspective for the generation of high affinity IgG3a-derived VHHs for small molecules.

Materials and methods

Chemicals and reagents

Information about materials, chemicals and reagents is detailed in the Electronic Supplementary Material (ESM).

Animal immunization and lymphocytes isolation

Haptens TR1 and TR2 (see ESM Fig. S1) were synthesized and coupled to the carrier proteins keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) according to the previous report.[22] A three-year old male Bactrian camel was injected subcutaneously with an emulsified mixture of TR1-KLH and Freund's incomplete adjuvant five times biweekly (see ESM Table S1). The blood lymphocytes were isolated by Ficoll gradient centrifugation (Life Technologies, Inc., Grand Island, NY) after the fifth injection according to the method described previously.[23]

Construction of phage displayed VHH library

For library construction, total RNA was extracted from the lymphocytes and the mRNA was reverse transcribed to cDNA.[18] The VHH regions were amplified by nested polymerase chain reaction (PCR) using pairs of primers (see ESM Table S2). The coding sequence of VHH fragments were ligated into the plasmid pComb3X using *Sfi* I restriction sites, and then the ligated material was electroporated into competent cells of *E. coli* ER 2738. The

library size was measured by counting the number of colonies grown on the plates after gradient dilution.

Selection of VHH and fusion of VHH-AP

VHHs specific for triazophos were isolated from the constructed library using a gradient of decreasing concentrations of both coating antigen TR2-BSA and competitive triazophos as detailed in the ESM. One optimal clone, named as T1, showing high binding capacity with triazophos was selected for the fusion of VHH-AP. The gene of VHH T1 was amplified and cloned into the pecan 45 plasmid using *Sfi* I complementary restriction sites. The pComb3x plasmid containing VHH and the pecan 45 plasmid containing VHH-AP were heat shock transformed to *E. coli* TOP 10F' and BL21(DE3)pLysS, respectively. The proteins were expressed following 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) induction and purified with Ni-NTA resin. The size and purity of VHH and VHH-AP were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

ELISA performance

A competitive VHH-based ELISA was carried out according to our previous study.[13] Briefly, A 100-μL solution of TR1-BSA (or TR2-BSA) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) (250 ng/mL) was coated on a 96-well microtiter plate at 4 °C overnight. The plate was blocked with 1% gelatin in phosphate buffered saline (PBS, 7.4) for 1 h at ambient temperature. A serial dilution of triazophos (50 μL/well, 5% methanol in PBS) was added, followed by the addition of 50 μL of VHH (125 ng/mL) in PBS. After incubation at room temperature for 1 h, the plate was washed 5 times with PBST (PBS containing 0.05% Tween-20) and then 100 μL of goat anti-HA tag IgG-horseradish peroxidase (HRP) (diluted at 1:10,000 with PBST) was added. After another incubation and washing, 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) solution was added into the plate and the reaction was stopped in around 10 min by the addition of 50 μL of 2 M H_2SO_4. The absorbance was read at 450 nm and SigmaPlot 10 software was used for curve fitting and data analysis.

The performance of the one-step ELISA was similar to the VHH-based ELISA above except VHH was replaced with VHH-AP (100 ng/mL), without the need of goat anti-HA tag IgG-HRP. The AP activity was determined by addition of 100 μL of 1.0 mg/mL *p*-nitrophenyl phosphate (pNPP). The reaction was stopped by addition of 50 μL of 3 M NaOH solution and the absorbance was read at 405 nm. The one-step ELISA was optimized by evaluating the effects of solvent, pH and ionic strength on half-maximum signal inhibition concentration (IC_{50}) and maximal signal (A_0).

Cross-reactivity

The selectivity of the VHH-AP was evaluated by determining the cross-reactivity (CR) of VHH-AP-based ELISA with organophosphate pesticides structurally related to triazophos (see ESM Fig. S2). For these studies CR was calculated as: CR (%)= [IC_{50} (triazophos)/IC_{50} (tested pesticide)] × 100.

Sample preparation and analysis

Soil and apple samples were collected from an orchard and water samples were collected from a paddy field in the suburb of Beijing. For the recover study, triazophos was spiked into water samples at three levels: 5, 20 and 100 ng/mL. After passing through a 0.22 μm filter (Waters Corp., MA), water samples were diluted at least 5-fold before subjecting to one-step ELISA. Each soil or apple sample was homogenized and spiked with triazophos to reach three levels: 20, 100 and 500 ng/g in soil; 100, 200 and 500 ng/g in apple. A 10 g aliquot of air-dried soil or apple sample was extracted with 10 mL of methanol in an ultrasonic water bath for 20 min at room temperature. The mixture was centrifuged at 6000 rpm for 10 min and then the supernatant was filtered through a 0.22 μm organic phase membrane (Waters Corp.). The extract was diluted at least 20-fold prior to ELISA. The resulting ELISA was also applied to analyze triazophos in real world samples including 5 paddy water, 4 soil and 4 apple samples collected from paddy fields or orchards in Beijing area.

The validation of the one-step ELISA with a gas chromatography-mass spectrometry (GC-MS) method is described in the ESM.

Results and discussion

Library construction and characterization

On the strength of the high concentration of IgG2 and IgG3 in sera, camels might become the preferred source for generation of VHHs against triazophos and other analytes. A Bactrian camel was immunized with TR1-BSA since it was successfully used in production of conventional antibodies.[22, 24] In order to obtain genes encoding camel VHHs as completely as possible, the coding sequences of the conservative leader peptide sequence, VHH plus hinge fragments and part of the CH2 domain were amplified by the first round of PCR using a pair of primers (see ESM Table S2). The fragments about 650–750 bp comprising the VHH and part of the CH2 domain with long or short hinge were obtained (data not shown). In the second round of PCR, VHH gene fragments about 350–500 bp (see ESM Fig. S3) were amplified by using a forward primer specific for FRl region of the heavy chain variable genes [16] paired with a set of reverse primers (R1, R2, and R3) specific for either long (IgG2) or short (IgG3) hinge regions (see ESM Table S2). R1 and R2 were generally used for the amplification of VHH fragments from allotypes IgG3b and IgG2b, respectively.[25] To improve the library diversity, we designed a novel reverse primer R3 to amplify VHH fragments (around 400 bp) (see ESM Fig. S3) from the allotype IgG3a. This might be the first study on the use of IgG3a-derived fragments for the construction of a diverse VHH library.

The constructed library comprised VHH fragments from allotypes IgG2b, IgG3a and IgG3b and its capacity was estimated to be 1.8×10^8 colony forming units (cfu). In order to evaluate the diversity of the VHH library, 30 individual clones were randomly picked from the plate for sequencing and different VHH sequences were observed (data not shown), indicating the high diversity of the constructed library. According to the hinge sequences of the 30 clones, the percentage of VHH fragments derived from IgG2b, IgG3a and IgG3b was approximately 40%, 17% and 43%, respectively.

Selection of anti-triazophos VHHs

The phage display VHH library was panned in a heterologous format using TR2-BSA as the coating antigen since this format proved to be an efficient way to select high binding affinity VHH for small molecules in our previous study.[13] The concentrations of both TR2-BSA and triazophos were gradually decreased in an attempt to capture high-affinity binders. Four rounds of panning were carried out and the titer increased each round except the fourth (data not shown). After the fourth round of panning, 31 clones showing strong binding to the coating antigens (OD > 2.0) in the absence of triazophos and inhibition >50% in the presence of 100 ppb triazophos by competitive phage ELISAs were identified as positive clones.

Sequence alignment of isolated VHHs against triazophos

All of the 31 positive clones were sequenced and only 7 clones (T1-T7) with unique VHH sequences were identified (see ESM Fig. S4). The main features of VHHs that distinguish them from VHs of conventional IgG are the occurrence of amino acids, F, E/G, R, and G/P in the FR2 at positions 37, 44, 45, and 47, respectively. The variable domains CDR3 regions possess 13 or 15 amino acids. According to their hinge sequences (see ESM Fig. S4), 5 clones T1-T5 derive from the allotypic variant IgG3a and 2 clones T6 and T7 derive from IgG3b. Among the VHH fragments of three allotypes in the constructed library, IgG3a-derived fragments had the least percentage (about 17%), but most positive clones were found to derive from IgG3a (23/31, about 74%), suggesting the allotype IgG3a showed a stronger immune response to triazophos than IgG2b and IgG3b. These results indicated that the design of the innovative reverse primer (R3) specific for the fragment of IgG3a was a crucial step to obtain a high affinity VHH for triazophos, probably opening a new perspective in generation of camel VHHs for small molecules. Nevertheless, this method may be target specific and more studies on the generation of IgG3a-derived VHHs for other small molecules need to be carried out. Also the response and recognition properties of IgG3a to various antigens should be investigated in the future.

Binding capacity of VHH to triazophos

Seven phagemid vectors containing different positive VHH sequences were used to express and purify corresponding proteins. The size and purity of the VHHs were verified on a 12% SDS-PAGE gel with one major band at MW ~15 kD, shown as VHH T1 (Fig. 1A). The binding capacity of each VHH for triazophos was evaluated by competitive ELISAs using various combination of VHH/coating antigen (Table 1). It is noticeable that all IgG3a-derived VHHs (T1-T5) showed higher sensitivity to triazophos than IgG3b-derived VHHs (T6 and T7) in both homologous and heterologous assays. In general, heterologous formats using TR2-BSA had higher sensitivity than homologous formats using TR1-BSA. The combination of VHH T1 and TR2-BSA gave the highest sensitivity (IC_{50} = 9.3 ng/mL). The variable binding characteristics of VHHs occurring in different assay formats resembled that of some conventional antibodies.[22, 26]] Hapten TR2 has a linker 2-carbon shorter than that of hapten TR1. This heterology allowed T1 to recognize triazophos well with decreased recognition of the linker. Therefore, TR2-BSA was used as coating antigen for the rest of this study.

Binding capacity of the VHH-AP Fusion protein

Because the IgG3a-derived VHH T1 showed the highest sensitivity to triazophos among all the VHHs, the VHH gene of T1 was cloned into pecan 45 and transformed into BL21(DE3)pLysS for the production of VHH-AP. One dominant band around 68 kD was shown on SDS-PAGE analysis for the purified T1-AP fusion (Fig. 1B). The VHH-AP fusion can be readily produced with good yield in *E. coli.* at around 22.4 mg from a 1-L bacterial culture media. It can serve as a bifunctional immunoreagent with combined recognition of triazophos while possessing high enzymatic activity.

Since the bacterial AP used in this study usually exists as a symmetrical dimer, [27, 28] this could lead to unpredictable levels of complexity and aggregation.[29] The dimerization and possible aggregation of the AP fusion protein may alter the binding ability of VHH to antigens.[30–32] Similar to the parental VHH T1, the fusion protein T1-AP showed good binding activity to both coating antigens TR1-BSA and TR2-BSA, but higher sensitivity to triazophos was observed in the one-step ELISA using TR2-BSA rather than using TR1-BSA (see ESM Fig. S5). The binding affinities of both T1 and T1-AP fusion to triazophos were compared by competitive ELISAs based on the same coating antigen TR2-BSA. Equivalent sensitivities were observed (IC_{50} values 8.0 *vs* 8.2 ng/mL), indicating a negligible change for small molecule binding after the dimerization. This result was similar to that of an earlier study in which the fusion of VHH-AP gave rise to a little improvement of VHH binding to TBBPA.[6] Although similar binding capacity was observed between the parental T1 and fusion T1-AP, the one-step ELISA based on the VHH-AP is more attractive than VHH-based ELISA for the detection of triazophos due to its simplicity and cost-effectiveness.

Optimization of the VHH-AP-based ELISA

Because VHH-AP is a bifunctional reagent, in the one-step ELISA, either its binding affinity or enzyme activity could be affected by the physicochemical properties of the assay buffer (e.g., pH and ionic strength) and organic solvents used to stabilize lipophilic compounds. It is essential to optimize the one-step ELISA prior to the application to real samples. Methanol is commonly used because it is applicable to a broad range of sample pretreatments, miscible with water in the assay buffer and compatible with many immunoassays.[33–35] The optimal concentrations of coating antigen TR2-BSA and T1-AP were determined by checkerboard titration. With the increase of methanol in the range of 5–20%, A_0 was typically enhanced from 0.91 to 1.23 A.U. and the IC_{50} values ranged from 7.2 to 28 ng/mL (Fig. 2A). This assay was significantly affected by 40% (v/v) methanol and the IC_{50} value was driven up to 274 ng/mL. The best sensitivity was observed using an assay buffer containing 5% methanol (IC_{50} = 7.2 ng/mL) (Fig. 2A). No obvious shift was observed from the performance of the ELISA at pH 6.0–9.0, because the A_0 and IC_{50} varied slightly in a range of 0.78–0.88 A.U. and 6.5–9.8 ng/mL, respectively (Fig. 2B). A high IC_{50} (20 ng/mL) at pH 5.0 indicated either binding capacity of VHH or AP activity was reduced in the acidic media. Assay sensitivities changed slightly in PBS with addition of NaCl varying in 0–0.8% (w/v), with a shift of A_0 and IC_{50} in 0.83–0.9 A.U. and 6.4–11.4 ng/mL, respectively. Higher concentrations of NaCl (1.6%) apparently increased the IC_{50} to 30 ng/mL and decreased the A_0 to 0.52 A.U. (Fig. 2C). A typical calibration curve of the T1-AP-based ELISA for triazophos was generated under optimized conditions (PBS containing

5% methanol and 0.8% NaCl, pH 7.4) (Fig. 3). This assay had an IC_{50} of 6.6 ng/mL, a limit of detection (LOD, IC_{10}) of 0.6 ng/mL and a linear range of 1.0–40 ng/mL (IC_{20}–IC_{80}).

Although the hapten TR1 used for the generation of VHHs was identical to that of conventional antibodies [16, 18, 23], two types of antibodies exhibited various binding affinity to triazophos. The sensitivity of the VHH-based assay for triazophos is comparable to that of a pAb-based immunoassay (IC_{50} = 5.5 ng/mL) [36] but lower than that from a mAb-based assay (IC_{50} = 0.65 ng/mL) [16]. This mAb was also used to develop a chemiluminescent enzyme immunoassay [24] and a colorimetric immunoassay employing magnetic microspheres and multi-labeled gold nanoparticles, [25] showing high sensitivity and efficiency for the detection of triazophos in agro-products. VHHs generally exhibit lower binding affinity to small molecules than conventional antibodies. It was found that the anti-hapten HCAb titer was much lower than that of the conventional antibody while comparing these antibodies' response to haptens.[9, 10] Conventional antibodies can accommodate haptens in deep pockets built at the interface of the heavy- and light-chain variable domains, providing a high affinity interaction which does not exist in monodomain VHHs. It is not surprising that in some studies low or modest affinity of anti-hapten VHHs were isolated.[4, 37–41] Notwithstanding, several VHHs with high affinity to small molecules, e.g., triclocarban,[25] 3-phenoxybenzoic acid,[18] 2,2′,4,4′-tetrabrominated diphenyl ether [10] and TBBPA,[13] have been generated recently. Also in this assay format amplification using a second antibody system was sacrificed in order to gain speed and sensitivity. In this report, we demonstrate that the T1-AP fusion shows high binding capacity to triazophos and holds good promise for development of selective, sensitive and rapid assays for triazophos.

Cross-reactivity

The selectivity of the T1-AP was evaluated by comparing the IC_{50} value of triazophos with that of 13 other important organophosphate insecticides in the one-step assay (see ESM Fig. S2). VHH-AP fusion was highly selective for triazophos because negligible cross-reactivity to other organophosphate insecticides was observed (<0.1%), consistent with the specificity of conventional antibodies.[22] These results indicate that the selected immunogen KLH-TR1 is appropriate for generating high-affinity VHHs for the highly selective detection of triazphos at low levels.

Sample analysis

To evaluate the applicability of the VHH-AP-based ELISA to environmental samples, this one-step assay was used to detect triazophos spiked into water, soil and apple. Matrix effects, inevitable in sample analysis, may cause some false positive/negative results, and a common strategy to overcome this problem is to dilute the extract with assay buffer. Only a small dilution of sample was needed to generate a standard curve similar to that generated in assay buffer, which is an indication of minimal of matrix effects in the samples analysed. In the present study, the filtered paddy water could be subjected to ELISA without detectable matrix effects after a 5-fold dilution by PBS. A 20-fold dilution of soil and apple extracts also minimized the matrix effect. According to the dilution factor of each sample, the linear

range of ELISA for triazophos in paddy water, soil and apple extracts were 5–200, 20–800 and 20–800 ng/mL, respectively, and the LOD values were 3, 12 and 12 ng/mL, respectively.

The levels of triazophos spiked in water and soil (Table 2) were based on the assay ranges abovementioned and in apple were based on the maximum residue limit (MRL) for triazophos (0.2 mg/kg) in the National Food Safety Standard released in China (GB2763–2016).[42, 43] The one-step ELISA for the spiked samples was validated by comparing the results of ELISA with those of a GC-MS method. The average recoveries of triazophos from water, soil, and apple determined by the one-step ELISA were in a range of 94–108%, 89–97%, and 83–93%, respectively, and by GC-MS in a range of 96–107%, 91–101%, and 86–95%, respectively (Table 2). Both methods showed good recoveries and correlated well with each other ($R^2 = 0.99$) (see ESM Fig. S6). The regression equation gave a slope = 0.97 with a small intercept (3.9 ng/mL) indicating the one-step ELISA is a valid method to detect triazophos in environmental and fruit samples. Subsequently, the resulting ELISA was used to detect triazophos from 13 water, soil and apple samples collected from the real world and the pesticide was < LOD in all samples.

ELISAs for small molecules are commonly performed in a two-step competitive protocol which requires the addition of a primary (recognition) antibody followed by a secondary antibody conjugated to HRP or AP. Conventional antibody-based one-step ELISAs for triazophos avoiding the addition of secondary antibodies have been developed by using haptens chemically conjugated with HRP.[18, 23, 30] In this protocol, the molecular ratio of such conjugate is hard to control and costly reagents are required. Furthermore, high concentration of conventional antibodies coated on the microtiter plate are required. As described in the studies by Chen *et al.* [30] and Liang *et al.* [18], 2 and 8 μg/mL (100 μL/well) mAbs were used to develop one-step ELISAs, respectively, while only 100 ng/mL (50 μL/well) VHH-AP was needed in the present study. Moreover, advances in recombinant DNA technology enabled the easy construction of a VHH and AP fusion protein which can be produced in bacteria with a high yield (around 22 mg/L). Although the sensitivity of VHH-AP-based ELISA is lower than those of conventional antibody-based ELISAs, the former assay is good enough to detect low levels of triazophos in the environment and in food below the MRLs. The one-step ELISA showed great promise for rapid analysis of triazophos in various samples, especially in the case of a large screening campaign, reducing the analysis time and the cost of analysis.

Conclusion

This study demonstrated an original approach to generate camel IgG3a-derived VHHs specific for triazophos and the application of the isolated VHH T1 in a one-step ELISA for triazophos in environmental and fruit samples. A Bactrianus was selected for the generation of VHHs against triazophos because a higher percentage of HCAbs (IgG2 and IgG3) are found in camel sera compared to alpaca or llama sera. A pair of innovative primers specific for the VHH fragments of the unique allotype IgG3a from camels were designed to construct a high diversity of VHH library, from which five positive IgG3a-derived VHHs were isolated. This strategy paves a way for the generation of VHHs for small molecules in general by using an alternative resource (IgG3a) in Bactrian camels beside IgG2b and

IgG3b. The VHH T1 with high affinity to triazophos was genetically fused with AP, demonstrating an integrated triazophos-binding capacity and enzymatic activity. The equivalent sensitivity between the T1 and the T1-AP-based assays for triazophos illustrated genetic fusion with AP did not negatively impact the binding activity of the VHH. The VHH-AP-based one-step ELISA showed a good correlation with GC-MS for the detection of triazophos spiked in water, soil, and apple samples. In comparison with the conventional antibody-coated ELISA, less immunoreagents were required in the assay protocol (VHH-AP). This assay is promising for determining triazophos at low levels in complicated environmental and food samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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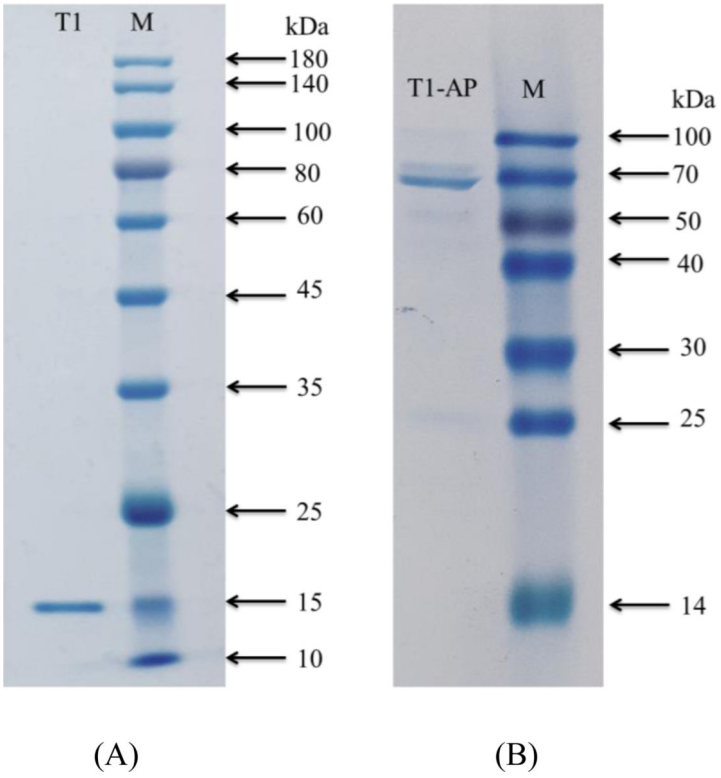

Fig. 1.

SDS-PAGE gel image of purified VHH T1 (A) and T1-AP fusion (B) under reduced conditions. Molecular weight markers (M) are shown and their sizes are indicated.

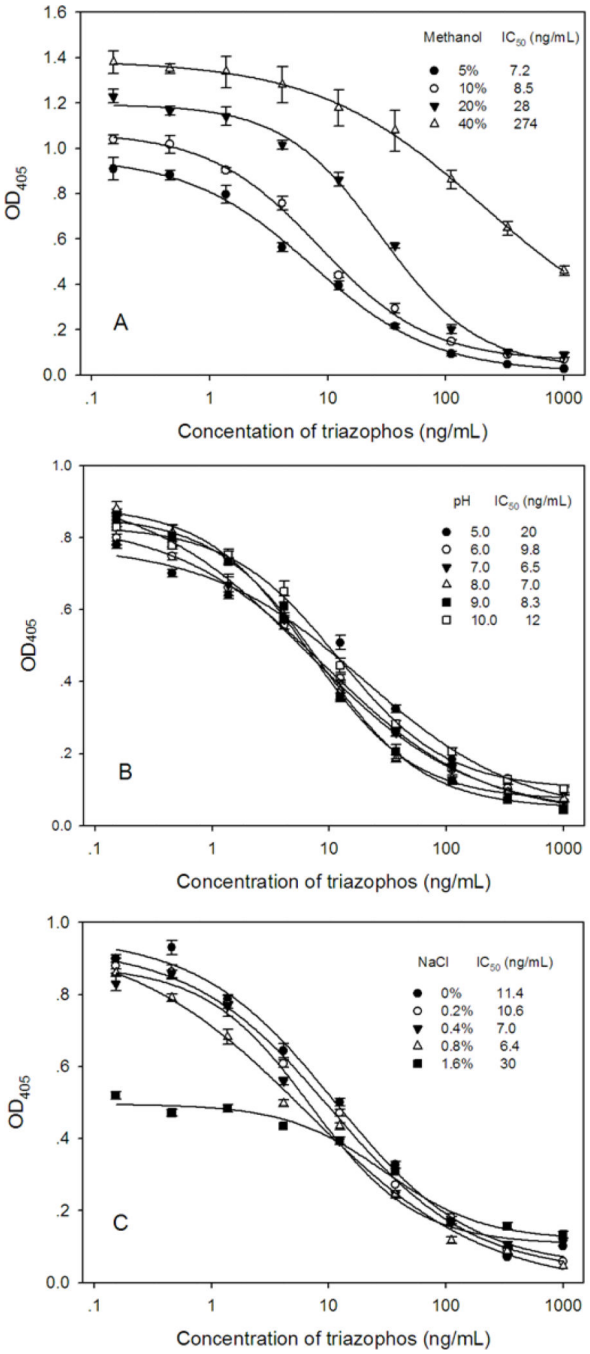

Fig. 2.

Effects of methanol percentage (A), pH (B) and NaCl content (C) on the performance of one-step ELISA for triazophos. The data are average values of triplicate samples.

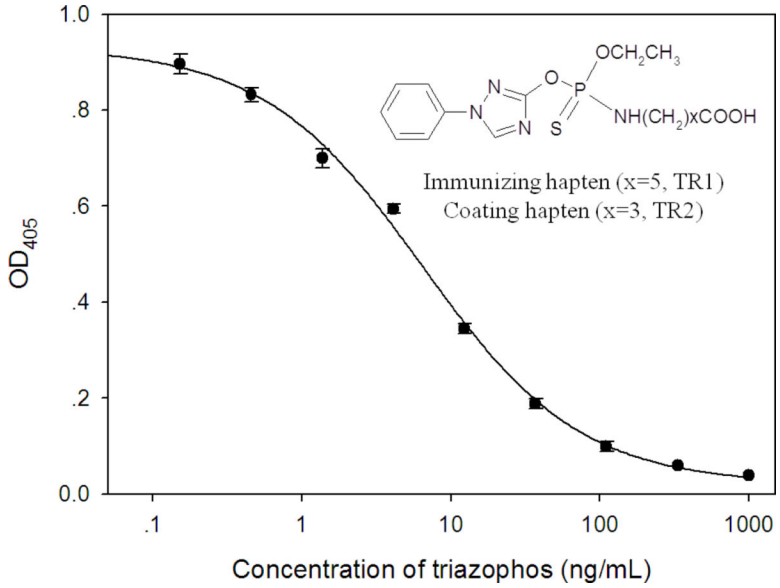

Fig. 3.

Calibration curve of one-step ELISA for triazophos in PBS under optimized conditions (5% methanol, pH 7.4). The data are average values of six replicates performed on different plates.

Table 1.

IC_{50} values of triazophos (ng/mL) by VHH-based ELISA with different combinations of VHH and coating antigen. The value. shown is the average of three replicates and the corresponding standard deviations.

	Coating antigens	
VHH	**TR1-BSA**	**TR2-BSA**
T1	17 ± 0.8	9.3 ± 0.5
T2	25 ± 1.2	22 ± 0.7
T3	70 ± 0.9	61 ± 0.3
T4	95 ± 2.1	78 ± 3.1
T5	108 ± 2.0	82 ± 0.8
T6	135 ± 1.3	131 ± 1.6
T7	134 ± 1.2	156 ± 0.4

Table 2.

Recoveries obtained from spiked samples by one-step ELISA and GC-MS

Sample	Triazophos spiked (ng/mL or ng/g)	ELISA, n = 3		GC-MS, n = 3	
		Mean ± SD[a] (ng/mL or ng/g)	Average recovery (%)	Mean ± SD[a] (ng/mL or ng/g)	Average recovery (%)
Paddy water	0	< LOD[b]		< LOD[c]	
	5	4.7 ± 0.1	94	5.35 ± 0.2	107
	20	21.6 ± 0.4	108	20.6 ± 0.6	103
	100	105 ± 4.2	105	96 ± 2.9	96
Soil	0	< LOD[b]		< LOD[c]	
	20	18.6 ± 0.6	93	20.2 ± 0.6	101
	100	89 ± 2.7	89	91 ± 5.5	91
	500	485 ± 9.7	97	490 ± 24.5	98
Apple	0	< LOD[b]		< LOD[c]	
	100	83 ± 4.2	83	87 ± 2.6	87
	200	170 ± 3.4	85	190 ± 3.8	95
	500	465 ± 18.6	93	430 ± 12.9	86

[a] Standard deviation

[b] LODs of ELISA for triazophos in paddy water, soil and apple samples are 3 ng/mL, 12 ng/g and 12 ng/g, respectively.

[c] LODs of GC-MS for triazophos in paddy water, soil and apple samples are 5 ng/mL, 20 ng/g and 50 ng/g, respectively.