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Preparation and interaction mechanism analysis of single-chain fragment variables against phenylethanolamine A

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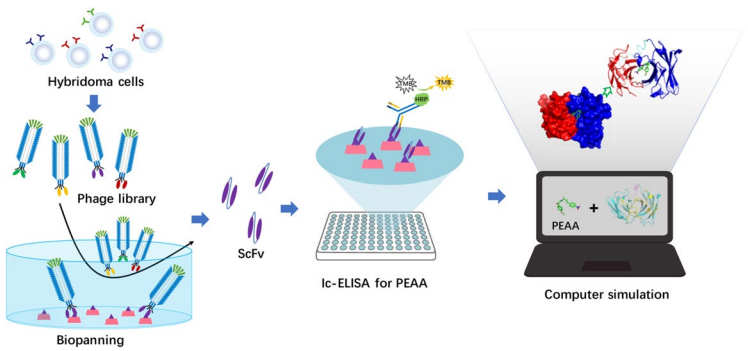
Abstract

This is the first report on the screening, expression, and recognition mechanism analysis of single-chain fragment variable (scFv) against phenylethanolamine A (PEAA), a newly emerged β-adrenergic agonist illegally used as a feed additive for growth promotion. The PEAA-specific scFv scFv, called scFv-32, was screened from hybridoma cell lines by phage display and was found to be optimally expressed in the E. *coli* system. The ic-ELISA results revealed an IC_{50} value of 10.34 μg/L for scFv-32 and no cross-reactivity with other β-adrenergic agonists. Homology modeling and molecular docking revealed the key binding sites VAL178, TYP228, and ASP229. One hydrogen bond, two pi-sigma bonds, and one pi-pi bond maintain the formation of the antibody–drug complex. Alanine scanning mutagenesis of the three predicted key binding sites showed that the mutants completely lost their recognition activity, which confirmed the accuracy of the theoretical analysis. These results are valuable for the preparation of scFvs and the analysis of the molecular recognition mechanism of antigen-antibodies.

Keywords Phenylethanolamine A, ScFv, Recognition mechanism, Homology modeling, Molecular docking

Graphical abstract



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Introduction

Driven by considerable economic benefits, several banned drugs, such as β-agonists, are applied in animal breeding and seriously threaten the environment and human health (Wang et al. 2021). To detect these banned drugs, antibody-based immunoassays are widely applied because they are low-cost, rapid, efficient, and suitable for on-site detection in the production chain (farm or slaughterhouse) (Ouyang et al. 2022). However, traditional polyclonal antibodies and monoclonal antibodies, which are the core reagents for immunoassays, are labor intensive, expensive to produce, limited in recognition performance by animal hosts, and virtually impossible to optimize or evolve once produced (Ahmed et al. 2020). Therefore, it is essential to explore the development of novel antibodies to remedy the deficiencies of traditional antibodies.

With the development of phage display technology, single-chain fragment variable (scFv) has become a promising alternative to traditional antibodies (Basu et al. 2019). The scFv is composed of a variable light chain (VL) and a variable light chain (VH) of traditional antibodies joined by a flexible polypeptide linker and retains the original antigen specificity and affinity (Li et al. 2022). Compared with traditional antibodies, scFv has the characteristics of a simple preparation process, low production cost, and easy performance evolution; thus, it has attracted much attention and has been extensively studied (Peltomaa et al. 2022). Phage display technology involves high-throughput screening by fusing gene-encoded antibodies and capsid proteins on the surface of phages to obtain antibodies that bind to the target antigen (Reader et al. 2019). This technology establishes a direct link between the antibody phenotype and genotype, avoiding the steps of immunization and cell fusion, thus shortening the experimental period, reducing the cost, and increasing stability (Roth et al. 2021). Compared with other scFv screening techniques, such as cell surface display, ribosome display, and yeast display, phage display technology is easier to use and more efficient (Farajnia et al. 2014). Consequently, this approach is widely applied for the preparation of antigen-specific scFvs in the field of food safety detection.

The in vitro evolution of recognition properties by genetic engineering methods (such as error-prone PCR, chain shuffling, and site-directed mutation) is one of the advantages of scFv antibodies (Li et al. 2022). Exploring the mechanism of antibody-antigen interactions is crucial for guiding the evolution of antibodies in vitro. Experimental methods, such as high-resolution X-ray crystallography, can obtain the three-dimensional structure of the scFv but are expensive and labor intensive (Toride and Brooks 2018). Therefore, reliable homology modeling tools, such as SWISS-MODEL software, with more than 400,000 high-quality models have been widely adopted (Bienert et al. 2017). Based on the accurate scFv structural data provided by homology modeling, molecular docking, a bioinformatics prediction method, can be used to search for the optimal binding mode between scFv and antigens through the principles of spatial structure complementarity and energy minimization. Among more than 60 different molecular docking software packages, AutoDock Vina with the PSOVina algorithm and multithreaded acceleration operation have obvious advantages in terms of docking accuracy and speed (Tao et al. 2020). With the help of computer simulation technology, the binding pattern of scFv antigens can be accurately analyzed, key amino acid information can be obtained, and site-directed mutation of these key amino acids can be performed to improve antibody sensitivity simply and quickly. In recent studies, based on the reliable structural insights of different scFvs with fluoroquinolones (Wang et al. 2016), amantadine (Xie et al. 2020), amoxicillin (He et al. 2017), and phenothiazines (Shi et al. 2017) provided by bioinformatics prediction methods, scientists have performed antibody evolution and successfully improved scFv antibody affinity. Therefore, bioinformatics prediction methods provide a new strategy for improving antibody sensitivity.

Phenylethanolamine A (PEAA) is a newly emerged β-adrenergic agonist that is illegally added to animal feed to increase carcass leanness (Bai et al. 2012). However, the residual PEAA in animal-derived foods can potentially threaten the cardiovascular and central nervous systems (Peng et al. 2019). For this reason, the addition of PEAA to animal feed and drinking water is prohibited in various countries (Yan et al. 2014). To detect PEAA, different immunoassays, including enzyme-linked immunosorbent assay (ELISA) (Jiang et al. 2017; Li et al. 2015b; Liu et al. 2022), lateral-flow immunoassay (LFA) (Li et al. 2015a), lateral-flow immunochromatographic assay (LFIA) (Dai et al. 2015), colloidal gold immunoassay (Jiang et al. 2018), immunochromatography assay (Li et al. 2014), and ultrasensitive electrochemiluminescence (ECL) (Tang et al. 2015), have been developed. However, these immunoassays rely on traditional polyclonal and monoclonal antibodies, which are limited by antibody development and production. To overcome these limitations, scFvs are considered reasonable alternative reagents due to their easy preparation, low cost, and ability to undergo in vitro evolution.

Currently, scFv antibodies have been developed against traditional β-adrenergic agonists, such as clenbuterol (CLB) (Lu et al. 2020) and salbutamol (SAL) (Lee et al. 2018). However, the ability of PEAA, a novel β-adrenergic agonist, to produce PEAA-specific scFv antibodies has

not been reported. Therefore, in this study, an anti-PEAA scFv was prepared by phage display technology, and the binding mechanism between scFv and PEAA was explored by homology modeling and molecular docking. These findings are beneficial for the future directed evolution of PEAA-specific scFv antibodies and the development of immunoassays.

Results

Construction and characterization of the scFv library

The hybridoma cell line PEAA-2D8 was collected for RNA extraction, and the quality of the RNA was determined to be excellent by gel electrophoresis (Fig. 1A). After the RNA was reverse transcribed into cDNA, the VL and VH gene fragments of approximately 370 bp in size were amplified from the cDNA using the mouse antibody primer library (Fig. 1B). Then, a scFv approximately 750 bp in length was constructed by splicing the VH-Linker and VL-Linker using SOE-PCR (Fig. 1C). Then, the scFvs digested with SfiI were ligated into pcomb3xss phagemid vectors to generate recombinant pcomb3xss-scFv phagemids. The recombinant phagemids were electroporated into XL1-Blue competent cells, which resulted in the generation of a phage display library with a capacity of 8.0×10^8 CFU/mL. Colony PCR revealed that 83.3% of the initial library contained the full-length scFv (Fig. 1D). The greater the storage capacity and diversity of the initial library are, the more conducive the screening of high-affinity scFv agents is. These results indicated that the constructed initial library had good capacity and diversity and was suitable for subsequent biopanning.

Biopanning of PEAA-specific phage-scFv

Four rounds of biopanning were applied to select scFv phages that specifically recognized PEAA. A gradient reducing the concentration of coating antigen was used to increase the proportion of high-affinity antibodies in the output pool. The results showed that the abundance of anti-PEAA phage scFv particles was significantly enriched 200-fold after four rounds of biopanning (Table S2). Moreover, compared with that of the third round, the recovery rate of the fourth round did not significantly increase, indicating that the specific phages were enriched enough and that no additional biopanning was needed. As shown in Fig. 2A, the polyclonal phage ELISA demonstrated a 4-fold increase in the affinity of the selected phage pool for PEAA. To further identify which clones in the four-round output pool were specific for PEAA, 48 single clones were randomly selected for phage ELISA identification. As shown in Fig. 2B, a total of 10 clones exhibited recognition activity for PEAA ($P/N>2.1$).

Based on the results of phage ELISA, the clone scFv-32 with the highest affinity was selected for nucleotide sequencing, and the sequence was submitted to the NCBI IGBLAST database for homology analysis. As shown in Fig. 2C, VL is composed of 113 amino acids and shares 94.6% homology with IGKV1-117*01. As shown in Fig. 2D, VH is composed of 116 amino acids and shares 82.8% homology with IGHV2-3*01. Homology analysis confirmed that scFv-32 was derived from the mouse germline gene, and the sequence variation was especially concentrated in the frame region. This phenomenon may result from somatic mutations that contribute to the increased affinity of the antibody for PEAA.

Expression and purification of soluble scFv

The phagemid pcomb3xss bears an HA tag for detection and a 6×His tag for purification, which allows soluble expression of scFv. The recombinant phagemid obtained from the scFv-32 clone was subsequently transferred into E. *coli* TOP10F', followed by a sufficient expression of soluble scFv in the periplasmicspace of the bacteria with the induction of IPTG in a low-temperature (18°C)

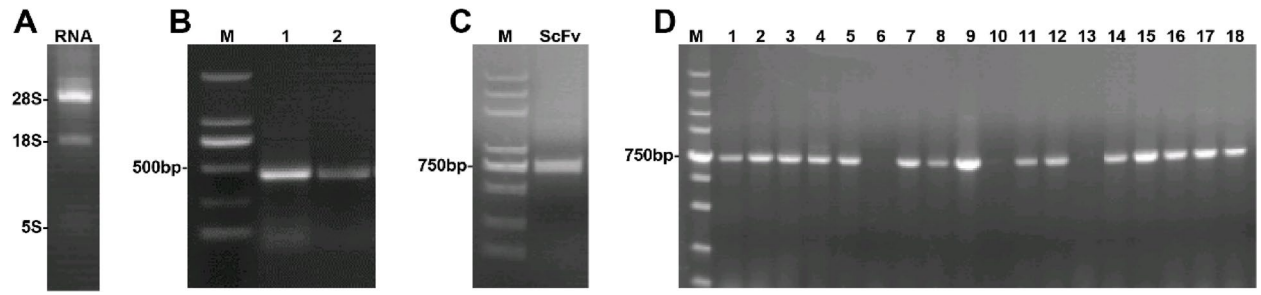


Fig. 1 Construction and characterization of the initial scFv library. **A** Agarose gel (1%) electrophoresis identification of total RNA. **B** Agarose gel (1%) electrophoresis identification of VL and VH. Lane M: marker DL2000 bp; lane 1: VL; lane 2: VH. **C** Agarose gel (1%) electrophoresis identification of the assembled full-length scFv. Lane M: DL5000 bp marker. **D** Colony PCR of 18 clones from the initial library. Lane M: marker DL5000 bp; lanes 1–18: random colonies

environment. A high concentration of IPTG inhibited the growth of the bacteria, so the optimal induction concentration of IPTG was explored. As shown in Fig. 3A, IPTG was necessary for the expression of scFv-32, but there was no significant difference in scFv expression between different IPTG concentrations. The soluble scFv-32 was purified by Ni-IDA agarose column chromatography and targeted against the 6×His tag. The optimal elution concentrations of imidazole was at 100 and 200 mmol/L determined by SDS–PAGE (Fig. 3B). Western blot analysis of scFv-32 demostrated a single band of approximately 32 kDa, indicating that scFv-32 was successfully expressed (Fig. 3C).

Functional characterization of soluble scFv by ic-ELISA

The ic-ELISA was applied to evaluate the affinity and specificity of the purified scFv-32. The optimal ic-ELISA conditions were determined by checkerboard titration: 12.5 μg/mL coating antigen (PEAA-NH_2-OVA) and 1:8 dilution of scFv-32. As shown in Table 1, purified scFv-32 was inhibited by free PEAA, and the ic-ELISA method based on scFv-32 exhibited an IC_{50} of 10.34 μg/L toward PEAA (average of three independent experiments). This result suggested that the affinity of scFv-32 for PEAA was not sufficient to support its application in sample detection and it is necessary to further enhance the affinity by molecular evolution in vitro. The cross-reactivity (CR) data indicated that scFv had no reactivity toward ractopamine, clenbuterol or salbutamol, which was consistent with what was observed with monoclonal antibodies.

Homology modeling and molecular docking

In the present study, the amino acid sequence of scFv-32 was uploaded to the SWISS-MODEL website for homology modeling (Fig. 4A). The GMQE value of the model was 0.69, and the QMEAN value was -1.17, which preliminarily proved that the quality of the model was optimal. Further evaluation tools, such as VERIFY, ERRAT, and Ramachandran plot, on the official website of the UCLA-DOE LAB-Laboratory Services were applied to evaluate the quality of the model. The model's ERRAT score of 95.045 and VERIFY score of 95.08 are above the standard values of 85 and 80, respectively. A ramachandran plot indicated that approximately 99.5% of the amino acid residues were distributed in the allowed region and that only 0.5% were distributed in the disallowed regions (Fig. 4B). These results showed that the scFv model was suitable for further molecular docking.

AutoDock Vina software automatically docks the PEAA into an active pocket surrounded by the six CDRs of scFv-32. As shown in Fig. 4C, the PEAA-scFv complex, which exhibited the lowest energy docking (-5.7 kcal/mol), was selected as the docking model from the 9 generated docking models. According to the docking results, a total of 13 amino acids were directly involved in the formation of the active pocket (Fig. 4D). The key contact amino acids ASP229, VAL178, TYR228 and PEAA form one hydrogen bond, two pi-sigma bonds, and one pi-pi

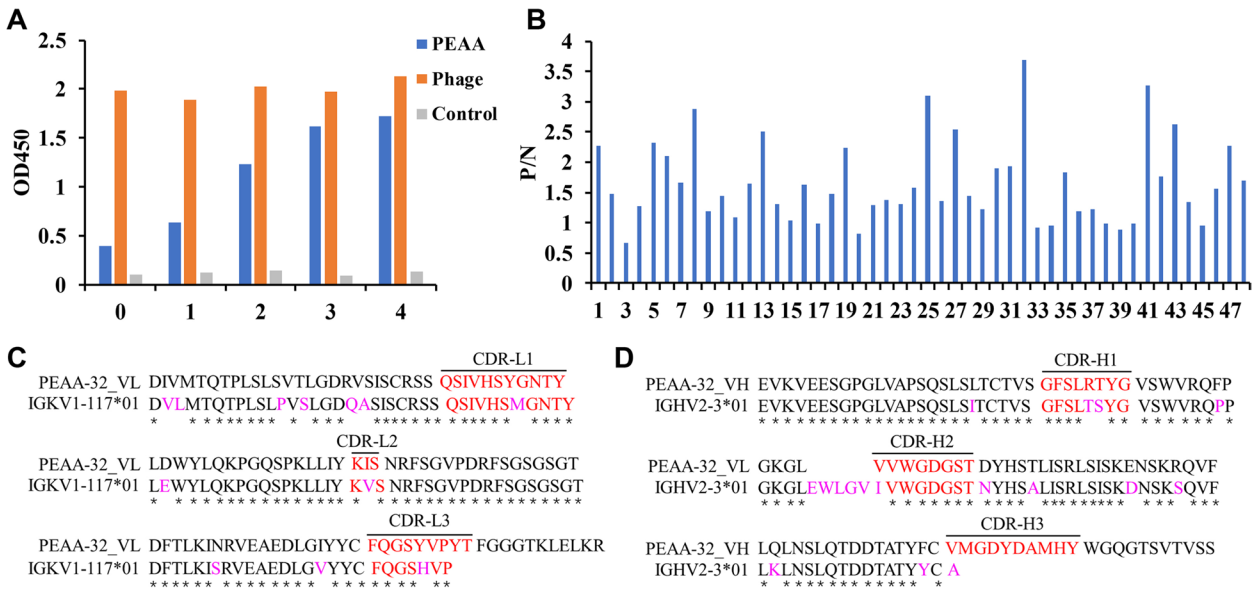


Fig. 2 Biopanning and sequence homology analysis of the anti-PEAA scFv antibody. **A** Results of polyclonal phage ELISA for each round of the output library. **B** Monoclonal phage ELISA result from the fourth round of the output library. P/N: OD_{450}(phage-scFv)/OD_{450}(OVA). **C** Homology analysis of the light-chain amino acid sequence of scFv-32. **D** Homology analysis of the heavy chain amino acid sequence of scFv-32

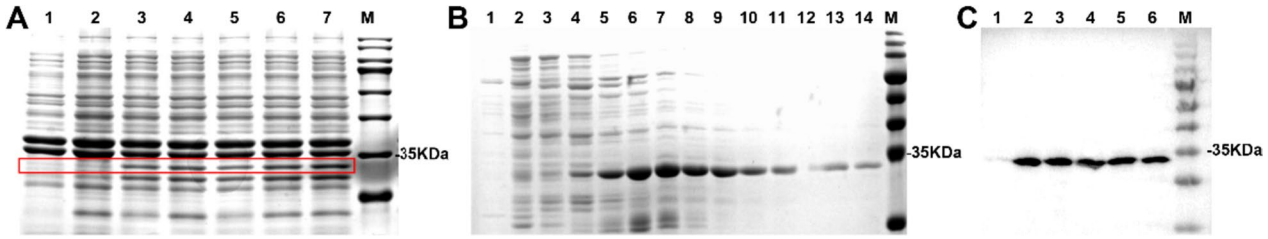
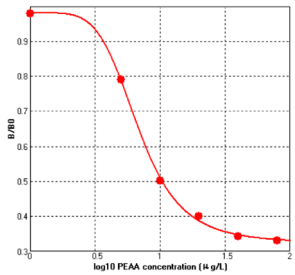


Fig. 3 Soluble expression and purification of scFv-32. **A** SDS–PAGE results of scFv-32 expression at different IPTG concentrations. Lanes 1–7: 0, 0.05, 0.1, 0.2, 0.5, 0.8 and 1.0 mmol/L IPTG; Lane M: 10–180 kDa marker. **B** SDS–PAGE result of scFv-32 antibody purification. Lanes 1–2, lanes 3–4, lanes 5–6, lanes 7–8, lanes 9–10, lanes 11–12, and lanes 13–14: the eluate contained imidazole concentrations of 25, 50, 100, 200, 300, 400 and 500 mmol/L, respectively; Lane M: 10–180 kDa marker. **C** Western blot result of soluble scFv-32. Lane 1: 0 mmol/L IPTG; Lanes 2–6: 1 mmol/L IPTG; Lane M: 10–180 kDa marker

Table 1 Standard curve for the ic-ELISA and cross-reactivity of the scFv-32

The standard curve of ic-ELISA	Analyte	IC_{50} (μg/L)	CR (%)
	Phenylethamine A	10.34	100
	Ractopamine	> 2000	< 1
	Clenbuterol	> 2000	< 1
	Salbutamol	> 2000	< 1

bond, respectively. Since alanine is small in size and has a negligible effect on protein structure, the key amino acid was replaced with alanine to confirm the accuracy of the docking results. As shown in Fig. 4E, three alanine mutants (VAL178ALA, TYR228ALA and ASP229ALA) were successfully expressed. The results of ic-ELISA indicated that all three mutants lost their binding activity to PEAA (Fig. 4F), which supported the accuracy of the recognition mechanism analysis.

Discussion

ScFv antibodies are widely applied in food safety detection because of their ability to bind antigens, low cost of expression in prokaryotic expression systems, and ease of genetic engineering operations (Li et al. 2022). Phage display technology is an effective and low-cost screening technology for scFv antibodies that revolutionizes the traditional monoclonal antibody preparation process (hybridoma technology). A prerequisite for successfully generating scFv antibodies using phage display is the construction of high-quality scFv source libraries. The quality of the scFv source library is primarily evaluated based on the *scFv* gene source, library capacity, and library positivity rate (Xu et al. 2019). In the present study, the *scFv* gene was amplified *via* PCR from PEAA-specific hybridoma cells. Antibody genes in specific hybridoma cells have been selected by antigens in vivo for affinity maturation, which is conducive to improving the affinity and positivity rate of antibodies. After the *scFv* gene was ligated into the pcomb3xss phagemid vector, efficient transformation of the recombinant phagemid vector was achieved via electroporation. Finally, a high-quality scFv source library with a capacity of up to 8.0×10^8 CFU/mL was obtained. This library was confirmed by colony PCR, and the percentage of positive cells was 83.3%. These evaluation criteria demonstrated the successful construction of a high-quality scFv source library.

A suitable biopanning strategy is essential for obtaining high-affinity scFv antibodies *via* phage display technology. Among the various biopanning strategies, solid-phase screening, which involves the use of antigen-coated solid media to adsorb antigen-specific phage-scFv, has been widely applied because of its simplicity and high efficiency (Sompunga et al. 2019). Generally, 3–5 rounds of "adsorption-elution-amplification" screening are necessary. Reducing the antigen concentration and enhancing the elution intensity are beneficial for enriching antibodies with high affinity (He et al. 2019). In this study, a higher antigen concentration (100 μg/mL) was used in the first round of biopanning, followed by decreasing antigen concentrations (50, 25 and 12.5 μg/mL) in subsequent rounds of biopanning. Additionally, incubating the phage library with the carrier protein and blocking solution before each round of biopanning can effectively eliminate interference from nonspecific phage-scFv (Rahbarnia et al. 2016). Washing can affect the biopanning effect to some extent, and increasing the number of washing times with increasing biopanning can effectively eliminate the nonspecific and low binding activity of phage-scFv. The phage

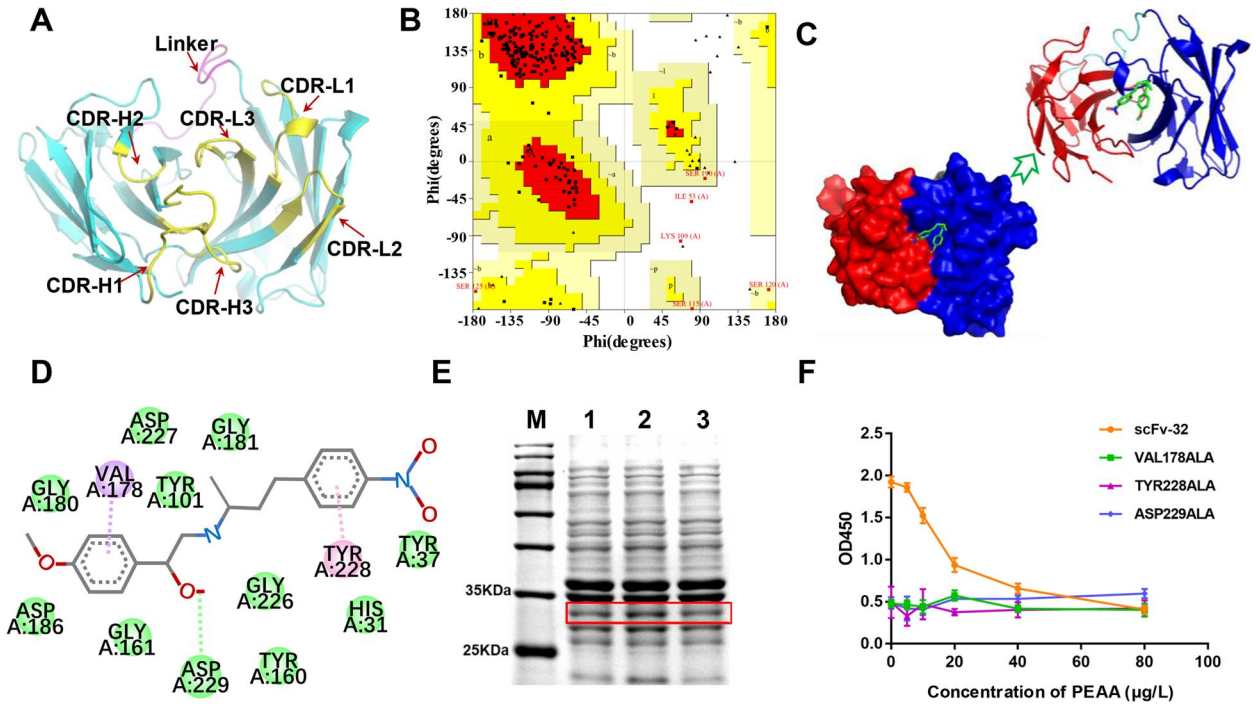


Fig. 4 Homology modeling and molecular docking analysis of scFv-32. **A** 3D conformation of the scFv-32 antibody. **B** Ramachandran plot of the scFv-32 model. **C** Molecular docking of scFv-32 and PEAA. **D** 2D views of the interaction between PEAA and key amino acid residues. **E** SDS–PAGE results of the alanine scanning and scFv-32 mutant fractions. Lane M: 10–180 kDa; Lanes 1–3: TYR228, ASP229 and VAL178 were mutated to alanine; Lane 4: GLY180 was mutated to TRP. **F** Ic-ELISA results for alanine scanning and the scFv-32 mutant

recovery and ELISA results indicated that the biopanning strategy was successful.

E. *coli* is considered to be the most suitable host for soluble scFv due to its simple and inexpensive growth medium, low equipment requirements and fast growth rate (Gupta and Shukla 2017). In the recombinant phagemid vector, a UAG stop codon is present between the antibody gene and gene III. When expressed in the amber mutant strain (XL1-Blue), UAG is read through as Gln, resulting in the expression of a scFv-PIII fusion protein attached to the phage surface. When expressed in nonsuppressor strains, translation is terminated at the UAG, leading to the production of soluble scFv antibodies (Galan et al. 2016). Therefore, the nonsuppressor strain Top10F' was selected as the soluble expression host. Multiple studies have shown that a low-temperature environment and appropriate IPTG concentration are conducive to the correct folding of scFv (Lee et al. 2018; Liu et al. 2021); hence, induction conditions of 18°C and 1 mmol/L IPTG concentration were used in this study.

After soluble expression and purification, a scFv-32 specific to PEAA was prepared with an IC_{50} of 10.34 μg/L. However, the sensitivity of scFv-32 was lower than that of its parent monoclonal antibody (0.63 μg/L). This difference may be due to two reasons: First, scFv retains only the VH and VL of the monoclonal antibody and uses a short peptide chain of 15 amino acids to form a protein with a molecular weight of only one-sixth of that of a complete antibody. This difference in molecular structure may cause a change in the binding characteristics of the scFv. Second, during the amplification and splicing of VH and VL gene fragments, amino acid mutations caused by degenerate primers or mismatches may cause changes in the spatial conformation of the antigen binding site in the scFv, which can impact its affinity and specificity. In the Chinese aquaculture industry, PEAA is explicitly prohibited in animal feed and drinking water, so scFv antibodies need to be as sensitive as possible to recognize PEAA.

One of the outstanding advantages of scFv is the ease of manipulation at the genetic level to improve its recognition activity. Numerous studies have shown that scFv mutants with improved performance can be obtained through in vitro evolution based on the molecular recognition mechanism between antigens and antibodies (He et al. 2017; Xie et al. 2020; Dong et al. 2020). For example, He et al. analyzed the binding mechanism of scFv and amoxicillin through homology modeling and molecular docking and obtained a scFv mutant with up to sixfold improved sensitivity through directional mutation of key amino acids (He et al. 2017). Another study showed that

the scFv mutant obtained by mutating the amino acid residue GLY107 to PHE had a 3.9-fold increase in affinity for amantadine based on virtual mutation analysis (Xie et al. 2020). Therefore, it is essential to analyze the molecular mechanism through which scFv binds to antigens. In this study, homology modeling and molecular docking were employed to explore the molecular recognition mechanism between scFv-32 and PEAA. The analysis results indicated that three key amino acids, ASP229, VAL178, and TYR228, played major roles in antigen–antibody binding.

Conclusion

In conclusion, an anti-PEAA scFv antibody (scFv-32) was isolated, identified, and prepared from a phage display library in this study. After optimized expression and purification, the ic-ELISA results indicated that scFv-32 specifically recognized PEAA, with an IC_{50} value of 10.34 μg/L. A 3D model of scFv-PEAA was established via computer simulation to explore the molecular recognition mechanism of the antibody-antigen complex. Molecular docking and alanine scanning mutagenesis revealed that TYR37, VAL99, TRP101 and TRP180 are the key contact amino acids. The main interaction forces involved hydrogen bonding, pi-sigma bonding, and pi-pi bonding. This study lays the foundation for further improving scFv affinity through site-directed mutagenesis and supports more efficient PEAA residue monitoring.

Materials and methods

Reagents and materials

Standards, including PEAA, CLB, SAL and RAc, were obtained from the China Institute of Veterinary Drug Control (Beijing, China). Hybridoma cells (2D8), which secrete the monoclonal antibody against PEAA (mAb), were produced at the National Reference Laboratory of Veterinary Drug Residues (HZAU). Anti-HA tag antibody (HRP), anti-M13 antibody (HRP), pcomb3xss, VCSM13 helper phage and E. *coli* XL1-Blue were obtained from Bio-View Shine (Beijing, China). A HiScript II 1st Strand cDNA Synthesis Kit and Phanta Max Super-Fidelity DNA Polymerase were purchased from Vazyme (Nangjing, China). A SteadyPure RNA Extraction Kit, SteadyPure gel DNA Purification Kit and SteadyPure Plasmid DNA Extraction Kit were purchased from Accurate Biotechnology (Hunan, China). SfiI restriction endonuclease and T4 ligase were purchased from NEB (USA). *E. coli* Top 10F' was obtained from TransGen Biotech (Beijing, China). An SDS-PAGE preparation kit was purchased from Sangon Biotech (Shanghai, China).

Construction of the phage-displaying anti-PEAA scFv library

2D8 hybridoma cells (Peng et al. 2019) were resuscitated, after which antibody secretion was detected. Then, the cells were collected for RNA extraction using an RNA Extraction Kit. The cDNA was reverse transcribed and used as a template for PCR amplification of the antibody genes. The specific primers used for the *SfiI* restriction site are listed in Table S1. Amplification of the VH and VL genes was performed using Phanta Max Super-Fidelity DNA Polymerase according to the manufacturer's instructions. Then, equimolar amounts of the VH and VL purified fragments were spliced into scFv genes by SOE-PCR with a linker $(Gly_4Ser)_3$. Subsequently, the scFv gene and phagemid pcomb3xss were digested with the SfiI restriction enzyme. After gel purification, the two purified fragments were ligated by T4 DNA ligase to construct recombinant phagemids. The recombinant phagemid was transformed into competent E. *coli* XL1-Blue (suppressor strain) cells by electroporation under optimized conditions of 25 mF, 1.25 kV and 200 Ω to construct the anti-PEAA phage display library. The electroporated cells were immediately resuspended in SOC medium and incubated at 37°C for 1 h. Then, the transformed cells were gradient diluted and plated on SOB-AG (SOB medium containing ampicillin (100 μg/mL) and glucose (1%) plates at 37°C overnight to calculate the library capacity. The remaining competent cells were added to a final concentration of 15% glycerol and stored at -80°C, which was the initial concentration.

Biopanning of the phage display library

Four rounds of biopanning were performed to screen high-affinity PEAA-specific scFv antibodies from the phage display library. One milliliter of the initial library was inoculated into 250 mL of 2×YT-AG media (2×YT media supplemented with ampicillin (100 μg/mL) and glucose (1%) at 37°C and 250 rpm/min for 5 h. Then, the helper phage (VCSM13) was added to the culture at 37°C and 150 rpm for 1 h. Cultures infected with VCSM13 were centrifuged, and the bacterial precipitates were resuspended in 500 mL of 2×YT-ATK medium (2×YT medium containing ampicillin (100 μg/mL), tetracycline (100 μg/mL) and kanamycin (50 μg/mL)) overnight culture at 37°C. The phages from the culture supernatant were purified with 1/5 (v/v) of 20% PEG8000/2.5 M NaCl on ice for 1 h. The precipitated phages were resuspended in PBS and used directly for biopanning.

PEAA-specific binders can be captured by a positive–negative biopanning strategy with some modifications. Briefly, three 96-well microtiter plates were coated overnight at 4°C with coating antigen (PEAA-NH_2-OVA, Plate 1), carrier protein (1% OVA, Plate 2), or blocking

solution (2% MPBS, Plate 3). The concentrations of the coating antigens used were 100, 50, 25 and 12.5 μg/mL for the first, second, third, and fourth biopanning rounds, respectively. Overnight-coated 96-well plates were blocked with 2% MPBS for 2 h at 37°C. After three washes with PBST, the precipitated phages were incubated on plates 2 and 3 for 1 h at room temperature to prevent interference from nonspecific phages. Unbound phages were then transferred to plate 1 and incubated at 37°C for positive selection. Afterward, plate 1 was washed with PBST. The number of washes needed to remove the unbound phages in the first, second, third and fourth rounds was 3, 6, 15 and 30, respectively. The specific phages were released with glycine–HCl (0.2 M, pH 2.5) for 6 min and neutralized with Tris–HCl (1 M, pH 7.4). E. *coli* XL1-Blue was infected with the neutralized phages and incubated at 37°C and 150 rpm for 1 h. Then, 100 μL of bacterial solution was plated on 2×YT-AG plates for titer determination. The remaining bacterial solution was infected with VCSM13 and used for the next round of biopanning. The recovery rate for each round was calculated according to the following formula: recovery rate = output phages/input phages.

Polyclonal/monoclonal phage ELISA

For polyclonal phage ELISA, 100 μL of PEAA-NH2-OVA (25 μg/μL) was added to the microtiter plates and coated overnight at 4°C. Moreover, the negative control groups were coated with 1% OVA or 2% MPBS. The coated microtitre plates were washed three times with PBST and then blocked at 37°C for 2 h by the addition of 250 μL of 2% MPBS. The input and output phages from each round of biopanning were diluted to 10^{10} cfu/mL, 100 μL of phage supernatant was added to the microtiter plates, the plates were incubated at 37°C for 2 h, and the plates were subsequently washed with PBST 4–6 times. Then, 100 μL of HRP-conjugated anti-M13 antibody was added to the plates, which were incubated at 37°C for 1 h. The plates were washed in the same way as above. After the last wash, TMB substrate was added for colorimetric development at 37°C, the reaction was terminated with H_2SO_4 (2 M), and the absorbance was measured at 450 nm. All the tests were repeated in triplicate, and the given data are presented as the mean values.

For monoclonal phage ELISA, 48 individual clones were randomly picked from the fourth round of the washing sieve bank and cultured in 96-deep-well plates with 500 μL of 2×YT-ATG (2×YT medium containing ampicillin (100 μg/mL), tetracycline (100 μg/mL) and glucose (1%)). After overnight incubation at 37°C, 40 μL of each culture from the plates was added to a new 96-deep-well plate containing 400 μL of 10^10 pfu/mL VCSM13 for 2 h at 37°C. Precipitates from centrifugation in 96-deep-well plates were resuspended in fresh 2YT-ATK medium and incubated for 16–18 h at 37°C. The monoclonal phage-scFv was secreted into the medium. After centrifugation, the supernatant was subjected to ELISA for identification of monoclonal phages, and the method was similar to that used for polyclonal phage ELISA.

Expression and purification of soluble scFv

According to the results of monoclonal phage ELISA, a recombinant phagemid with the highest affinity was extracted and heat-shocked and transformed into E. *coli* TOP 10F' for the production of soluble scFv antibodies. The mixture was spread on a 2×YT-A plate, which was subsequently incubated overnight. The next day, 10 individual colonies were picked randomly into 5 mL of 2×YT-A medium and grown overnight at 37°C. Positive clones carrying the correct plasmid were identified by colony PCR and DNA sequencing. A positive clone was subcultured in 20 mL of 2×YT-A medium with shaking at 37°C until the OD600 was 0.6. It is important to induce antibody expression by optimizing the conditions at an expression temperature of 18°C with various isopropyl β-D-1-thiogalactopyranoside (IPTG) concentrations (0, 0.05, 0.1, 0.2, 0.5, 0.8 and 1.0 mmol/L). After 16–18 h of shock culture, the precipitate after centrifugation of the culture mixture was resuspended in 2 mL of precooled PBS. The resuspended bacteria were disrupted by an ultrasonic cell pulverizer (power 100 W, disrupted for 10 s, intermittent 15S). The mixture was centrifuged at 12,000 r/min and 4°C for 10 min to collect the supernatant. The soluble scFv antibody in the supernatant was detected *via* SDS–PAGE and Western blotting. Finally, the inoculum was enlarged, followed by protein purification via Ni–NTA affinity chromatography.

Functional characterization of soluble scFv

An ic-ELISA was applied to evaluate the affinity and specificity of the purified scFv. The optimal concentration of coating antigen and dilutions of scFv were determined via an ELISA checkerboard. A 96-microtitre plate was coated with 12.5 μg/mL PEAA-NH_2-OVA overnight at 4°C and then blocked with 2% MPBS. After the blocking solution was discarded and the cells were washed three times, 50 μL of purified scFv mixed with 50 μL of various concentrations of PEAA was added to the wells and incubated for 2 h at 37 °C. After being washed, 100 μL of HRP-conjugated anti-HA tag antibody (1:5000 dilution) was added, and the mixture was incubated for 1 h at 37°C. After washing, the bound anti-HA tag antibody was identified by TMB substrate solution.

The cross-reactivity (CR) represents the specificity of the scFv. The CR toward other β-adrenergic agonists was determined in a similar manner by challenging the scFvs

against clenbuterol, ractopamine, and salbutamol. The extent of CR was assessed by comparison of the IC_{50} values. The CR was calculated with the following formula: cross-reactivity = [IC_{50} β-adrenergic agonist/IC_{50} PEAA] × 100%.

Homology modeling and molecular docking

The nucleotide sequence of the scFv identified by monoclonal phage ELISA was obtained by DNA sequencing. Nucleotide sequences were aligned to the mouse IgG database to analyze sequence homology and CDRs (https://www.ncbi.nlm.nih.gov/igblast/). The tertiary structures of the anti-PEAA scFv were modeled by analyzing their amino acid sequences with the SWISS-MODEL service. The compatibility of the 3D structures and the primary sequences of the scFv were validated and evaluated by UCLA-DOE LAB-Laboratory Services (http://services.mbi.ucla.edu/SAVES/).

AutoDock Vina was used to dock PEAA into the active site pocket of the scFv. For docking studies, the 3D structure of the ligand PEAA was searched for and downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/). The scFv and PEAA ligand models were imported into AutoDock Vina for Hydrogens Add and Compute Gasteiger, respectively (Safarpour et al. 2018). Since the CDR region of the antibody is the main region for target recognition, PEAA ligand docking was limited to the CDR region of the scFv (Pierce et al. 2014). The default parameters in the AutoDock Vina software were used. After running the AutoDock Vina docking program, a total of nine possible results were obtained, and the result with the lowest binding energy was further visualized by PyMOL software.

Alanine scanning mutagenesis

To verify the accuracy of the computer simulation, these three amino acids, VAL178, TYR228 and ASP229, which contributed most to the interaction between PEAA and scFv, were mutated to ALA (Li et al. 2020). The steps for site-directed mutagenesis were as follows: First, the required mutant plasmid was amplified *via* PCR using the scFv-32 recombinant plasmid as a template. Second, the mutant plasmid was digested with the restriction enzyme Dpn I and transformed into *E. coli* DH5α cells. Third, the mutant vectors were transformed into TOP 10F competent cells, and the correct sequence was identified by sequencing. Soluble expression of the three scFv mutants was performed under optimal expression conditions, and ic-ELISA was used to evaluate the recognition performance of the scFv mutants.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44149-024-00113-2.

Additional file 1. The two tables list the primer sequences and proportions used for VH and VL gene amplification and enrichment of phage-scFv by biopanning.

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Authors' contributions

All the authors contributed to the study conception and design. Material preparation, experimental design and implementation, data analysis and original manuscript writing were performed by LL and RH. The experimental verification was completed by HML, SYH, JXL and YS. DPP is an instructor and corresponding author and is responsible for the research design and manuscript revision. All the authors read and approved the final manuscript.

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Availability of data and materials

The data used to support the findings of this study are included within the article.

Declarations

Ethics approval and consent to participate

Ethical approval was not needed for this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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References

Ahmed, S., J. Ning, D. Peng, T. Chen, I. Ahmad, A. Ali, Z. Lei, bakr Shabbir M. Abu, G. Cheng, and Z. Yuan. 2020. Current advances in immunoassays for the detection of antibiotics residues: a review. *Food and Agricultural Immunology* 31 (1): 268–290. https://doi.org/10.1080/09540105.2019.1707171.

Bai, Y., Z. Liu, Y. Bi, X. Wang, Y. Jin, L. Sun, H. Wang, C. Zhang, and S. Xu. 2012. Preparation of polyclonal antibodies and development of a direct competitive enzyme-linked immunosorbent assay to detect residues of phenylethanolamine A in urine samples. *Journal of Agricultural and Food Chemistry* 60 (46): 11618–11624. https://doi.org/10.1021/jf3036066.

Basu, K., E.M. Green, Y. Cheng, and C.S. Craik. 2019. Why recombinant antibodies-benefits and applications. *Current Opinion Biotechnology* 60: 153–158. https://doi.org/10.1016/j.copbio.2019.01.012.

Bienert, S., A. Waterhouse, T.A. de Beer, G. Tauriello, G. Studer, L. Bordoli, and T. Schwede. 2017. The SWISS-MODEL Repository-new features and

functionality. *Nucleic Acids Research* 45 (D1): D313–D319. https://doi.org/10.1093/nar/gkw1132.

Dai, M., Y. Gong, A. Liu, L. Zhang, J. Lin, M. Zhang, and X. Yu. 2015. Development of a colloidal gold-based lateral-flow immunoassay for the rapid detection of phenylethanolamine A in swine urine. *Analytical Methods* 7 (10): 4130–4137. https://doi.org/10.1039/C5AY00641D.

Dong, S., M. Gao, Z. Bo, L. Guan, X. Hu, H. Zhang, B. Liu, P. Li, K. He, X. Liu, and C. Zhang. 2020. Production and characterization of a single-chain variable fragment antibody from a site-saturation mutagenesis library derived from the anti-Cry1A monoclonal antibody. *International Journal of Biological Macromol* 149: 60–69. https://doi.org/10.1016/j.ijbiomac.2020.01.152.

Farajnia, S., V. Ahmadzadeh, A. Tanomand, K. Veisi, S.A. Khosroshahi, and L. Rahbarnia. 2014. Development trends for generation of single-chain antibody fragments. *Immunopharmacology and Immunotoxicology* 36 (5): 297–308. https://doi.org/10.3109/08923973.2014.945126.

Galan, A., L. Comor, A. Horvatic, J. Kules, N. Guillemin, V. Mrljak, and M. Bhide. 2016. Library-based display technologies: Where do we stand? *Molecular Biosystems* 12 (8): 2342–2358. https://doi.org/10.1039/c6mb00219f.

Gupta, S.K., and P. Shukla. 2017. Microbial platform technology for recombinant antibody fragment production: A review. *Critical Reviews in Microbiology* 43 (1): 31–42. https://doi.org/10.3109/1040841X.2016.1150959.

He, X., C.F. Duan, Y.H. Qi, J. Dong, G.N. Wang, G.X. Zhao, J.P. Wang, and J. Liu. 2017. Virtual mutation and directional evolution of anti-amoxicillin ScFv antibody for immunoassay of penicillins in milk. *Analytical Biochemistry* 517: 9–17. https://doi.org/10.1016/j.ab.2016.10.020.

He, J., X. Tao, K. Wang, G. Ding, J. Li, Q.X. Li, S.J. Gee, B.D. Hammock, and T. Xu. 2019. One-step immunoassay for the insecticide carbaryl using a chicken single-chain variable fragment (scFv) fused to alkaline phosphatase. *Analytical Biochemistry* 572: 9–15. https://doi.org/10.1016/j.ab.2019.02.022.

Jiang, D., B. Cao, M. Wang, H. Yang, K. Zhao, J. Li, M. Li, L. Sun, and A. Deng. 2017. Development of a highly sensitive and specific monoclonal antibody based enzyme-linked immunosorbent assay for the detection of a new β-agonist, phenylethanolamine A, in food samples. *Journal of the Science of Food and Agriculture* 97 (3): 1001–1009. https://doi.org/10.1002/jsfa.7826.

Jiang, W., L. Zeng, L. Liu, S. Song, and H. Kuang. 2018. Immunochromatographic strip for rapid detection of phenylethanolamine A. *Food and Agricultural Immunology* 29 (1): 182–192. https://doi.org/10.1080/09540105.2017.1364709.

Lee, W., A.A. Syed, C.Y. Leow, S.C. Tan, and C.H. Leow. 2018. Isolation and characterization of a novel anti-salbutamol chicken scFv for human doping urinalysis. *Analytical Biochemistry* 555: 81–93. https://doi.org/10.1016/j.ab.2018.05.009.

Li, M., H. Yang, S. Li, K. Zhao, J. Li, D. Jiang, L. Sun, and A. Deng. 2014. Ultrasensitive and quantitative detection of a new beta-agonist phenylethanolamine A by a novel immunochromatographic assay based on surface-enhanced Raman scattering (SERS). *Journal of Agricultural and Food Chemistry* 62 (45): 10896–10902. https://doi.org/10.1021/jf503599x.

Li, X., W. Wang, L. Wang, Q. Wang, X. Pei, and H. Jiang. 2015a. Rapid determination of phenylethanolamine A in biological samples by enzyme-linked immunosorbent assay and lateral-flow immunoassay. *Analytical and Bioanalytical Chemistry* 407 (25): 7615–7624. https://doi.org/10.1007/s00216-015-8917-6.

Li, Y., S. Lu, Z. Liu, L. Sun, J. Guo, P. Hu, J. Zhang, Y. Zhang, Y. Wang, H. Ren, X. Meng, and Y. Zhou. 2015b. A monoclonal antibody based enzyme-linked immunosorbent assay for detection of phenylethanolamine A in tissue of swine. *Food Chemistry* 167: 40–44. https://doi.org/10.1016/j.foodchem.2014.06.085.

Li, L., S. Wu, Y. Si, H. Li, X. Yin, and D. Peng. 2022. Single-chain fragment variable produced by phage display technology: Construction, selection, mutation, expression, and recent applications in food safety. *Comprehensive Reviews in Food Science and Food Safety*. https://doi.org/10.1111/1541-4337.13018.

Liu, Y., S. Liu, C. Xu, M. Lin, Y. Li, C. Shen, Y. Liang, X. Sun, D. Wang, P. Lü, and X. Liu. 2021. Epitopes prediction for microcystin-LR by molecular docking. *Ecotoxicology and Environmental Safety* 227: 112925. https://doi.org/10.1016/j.ecoenv.2021.112925.

Liu, M., Y. Bai, L. Dou, Y. Kong, Z. Wang, K. Wen, and J. Shen. 2022. A highly salt-tolerant monoclonal antibody-based enzyme-linked immunosorbent assay for the rapid detection of phenylethanolamine A in urine. *Food and Agricultural Immunology* 33 (1): 575–587. https://doi.org/10.1080/09540105.2022.2084043.

Lu, Q., Y.Y. Hou, X.X. Liu, H. Wang, J.J. Hou, J.L. Wei, S.S. Zhou, and X.Y. Liu. 2020. Construction, expression and functional analysis of anti-clenbuterol codon-optimized scFv recombinant antibody. *Food and Chemistry Toxicology* 135: 110973. https://doi.org/10.1016/j.fct.2019.110973.

Ouyang, S., S. Yu, and Y. Le. 2022. Current advances in immunoassays for the detection of β2-agonists. *Foods* 11 (6): 803. https://doi.org/10.3390/foods11060803.

Peltomaa, R., R. Barderas, E. Benito-Peña, and M.C. Moreno-Bondi. 2022. Recombinant antibodies and their use for food immunoanalysis. *Analytical and Bioanalytical Chemistry* 414 (1): 193–217. https://doi.org/10.1007/s00216-021-03619-7.

Peng, D., L. Zhao, L. Zhang, Y. Pan, Y. Tao, Y. Wang, F. Sheng, and Z. Yuan. 2019. A novel indirect competitive enzyme-linked immunosorbent assay format for the simultaneous determination of rRactopamine and phenylethanolamine A residues in swine urine. *Food Analytical Methods* 12 (5): 1077–1085. https://doi.org/10.1007/s12161-019-01445-3.

Pierce, B.G., K. Wiehe, H. Hwang, B.H. Kim, T. Vreven, and Z. Weng. 2014. ZDOCK server: Interactive docking prediction of protein-protein complexes and symmetric multimers. *Bioinformatics* 30 (12): 1771–1773. https://doi.org/10.1093/bioinformatics/btu097.

Rahbarnia, L., S. Farajnia, H. Babaei, J. Majidi, K. Veisi, A. Tanomand, and B. Akbari. 2016. Invert biopanning: A novel method for efficient and rapid isolation of scFvs by phage display technology. *Biologicals : Journal of the International Association of Biological Standardization* 44 (6): 567–573. https://doi.org/10.1016/j.biologicals.2016.07.002.

Reader, R.H., R.G. Workman, B.C. Maddison, and K.C. Gough. 2019. Advances in the production and batch reformatting of phage antibody libraries. *Molecular Biotechnology* 61 (11): 801–815. https://doi.org/10.1007/s12033-019-00207-0.

Roth, K.D.R., E.V. Wenzel, M. Ruschig, S. Steinke, N. Langreder, P.A. Heine, K.T. Schneider, R. Ballmann, V. Fuhner, P. Kuhn, et al. 2021. Developing recombinant antibodies by phage display against infectious diseases and toxins for diagnostics and therapy. *Frontiers in Cellular and Infection Microbiology* 11: 697876. https://doi.org/10.3389/fcimb.2021.697876.

Safarpour, H., M. Shahmirzaie, E. Rezaee, M. Barati, M.R. Safarnejad, and H.S. F. 2018. Isolation and characterization of novel phage displayed scFv fragment for human tumor necrosis factor alpha and molecular docking analysis of their interactions. *Iranian Journal of Pharmaceutical Research* 17 (2): 743–752. https://doi.org/10.22037/IJPR.2018.2229.

Shi, F.S., L. Zhang, W.Q. Xia, J. Liu, H.C. Zhang, and J.P. Wang. 2017. Production and evolution of a ScFv antibody for immunoassay of residual phenothiazine drugs in meat based on computational simulation. *Analytical Methods* 9 (30): 4455–4463. https://doi.org/10.1039/C7AY01103B.

Sompunga, P., N. Pruksametanan, K. Rangnoi, K. Choowongkomon, and M. Yamabhai. 2019. Generation of human and rabbit recombinant antibodies for the detection of Zearalenone by phage display antibody technology. *Talanta* 201: 397–405. https://doi.org/10.1016/j.talanta.2019.04.034.

Tang, Q., F. Cai, A. Deng, and J. Li. 2015. Ultrasensitive competitive electrochemiluminescence immunoassay for the β-adrenergic agonist phenylethanolamine A using quantum dots and enzymatic amplification. *Microchimica Acta* 182 (1): 139–147. https://doi.org/10.1007/s00604-014-1292-8.

Tao, X., Y. Huang, C. Wang, F. Chen, L. Yang, L. Ling, Z. Che, and X. Chen. 2020. Recent developments in molecular docking technology applied in food science: A review. *International Journal of Food Science & Technology* 55 (1): 33–45. https://doi.org/10.1111/ijfs.14325.

Toride, King.M., and C.L. Brooks. 2018. Epitope mapping of antibody-antigen interactions with X-ray crystallography. *Methods in Molecular Biology* 1785: 13–27. https://doi.org/10.1007/978-1-4939-7841-0_2.

Wang, J.P., J. Dong, C.F. Duan, H.C. Zhang, X. He, G.N. Wang, G.X. Zhao, and J. Liu. 2016. Production and directional evolution of antisarafloxacin ScFv antibody for immunoassay of fluoroquinolones in milk. *Journal of Agricultural and Food Chemistry* 64 (42): 7957–7965. https://doi.org/10.1021/acs.jafc.6b03356.

Wang, Z., Q. Zhou, Y. Guo, H. Hu, Z. Zheng, S. Li, Y. Wang, and Y. Ma. 2021. Rapid detection of Ractopamine and Salbutamol in swine urine by immunochromatography based on *Selenium Nanoparticles*. *International Journal of Nanomedicine* 16: 2059–2070. https://doi.org/10.2147/IJN.S292648.

Xie, S., J. Wang, X. Yu, T. Peng, K. Yao, S. Wang, D. Liang, Y. Ke, Z. Wang, and H. Jiang. 2020. Site-directed mutations of anti-amantadine scFv antibody by molecular dynamics simulation: Prediction and validation. *Journal of Molecular Modeling* 26 (3): 49. https://doi.org/10.1007/s00894-020-4286-y.

Xu, C., W. Miao, Y. He, Y. Zu, X. Liu, J. Li, and X. Liu. 2019. Construction of an immunized rabbit phage display antibody library for screening microcystin-LR high sensitive single-chain antibody. *International Journal of Biological Macromolecules* 123: 369–378. https://doi.org/10.1016/j.ijbiomac.2018.11.122.

Yan, P., J. Zhang, Q. Tang, A. Deng, and J. Li. 2014. A quantum dot based electrochemiluminescent immunosensor for the detection of pg level phenylethanolamine A using gold nanoparticles as substrates and electron transfer accelerators. *The Analyst* 139 (17): 4365–4372. https://doi.org/10.1039/c4an00378k.

Li, Y., Y. Hu, Z. Tu, Z. Ning, Q. He, and J. Fu. 2020. Research on the mechanism of action of a citrinin and anti-citrinin antibody based on Mimotope X27. *Toxins (Basel)* 12 (10). https://doi.org/10.3390/toxins12100655.

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