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ABSTRACT
Amantadine (AMA) is an illegally used antiviral drug in the poultry industry, it is necessary to establish a fast, accurate and time-saving detection method for poultry food. The antibody-based immunoassay can achieve fast and accurate requirements. We developed a recombinant antibody-based specificity immunoassay for AMA. In the recombinant antibody, the heavy chain variable region (VH) is connected covalently with the light chain variable region (VL) by the artificial linker. Here, two recombinant antibodies' single-chain variable fragment (scFv) and scFv-dHLX were constructed and functionally expressed in the periplasm of *Escherichia coli*. The helix-turn-helix peptide was utilized to dimerize VH and VL similar to the IgG counterpart. The ScFv-dHLX protein showed a higher binding ability and affinity resulting in improvement of *in vitro* affinity activity over its corresponding scFv. Our results not only indicated scFv-dHLX as an alternative for scFv in analytical application, but also offered a novel and efficient hetero-dimerization pattern of VH and VL leading to enhanced affinity.

Introduction

Due to the illegal use of these antiviral drugs such as amantadine (AMA), rimantadine, moroxydine, oseltamivir and acyclovir in animal husbandry, the preventive effect of vaccinum has been disturbed, and the inadequate use of these antiviral drugs raises concerns about the increased adverse effects on public health (Cheung et al., 2006; Ilyushina, Govorkova, & Webster, 2005; Pabbaraju et al., 2008).

The development of immunoassay analytical techniques to detect harmful compounds in food has become important (Chen et al., 2014; Guan et al., 2015). Immunoassays based on the specific binding of antibodies and antigens have become well-developed techniques for the determination of trace amounts of low-molecule analytes (Bai et al., 2015; Peng, Song, Liu, Kuang, & Xu, 2016). Monoclonal antibodies against AMA have been achieved, but with the passage of hybridoma cells, the secretory function may be lost. Recombinant

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DNA technology has recently facilitated the cloning of desired antibody genes to obtain the recombinant single-chain variable fragment (scFv). Using overlap extension PCR, it is easy to produce an scFv antibody in bacterial expression vectors (Coia, Hudson, & Irving, 2001; Maragos, Li, & Chen, 2012). However, in some cases, scFvs have demonstrated much lower affinity potency than their parent IgG due to insufficient stabilization (Duan et al., 2012). It may also be attributed to differences in assembly patterns of the heavy chain variable region (VH) and light chain variable region (VL) in the scFv compared to its intact IgG counterpart or Fab. In an intact IgG or Fab, the VH and VL are connected non-covalently via hydrophobic interactions and disulfide bonding between the constant domain of the light chain (CL) and the constant domain one of the heavy chain (CH), so the amino groups at both the terminus of VH and VL are free. In this study, in order to overcome the drawbacks of scFv and simulate the natural structure of Fv fragments in an intact Y-shaped IgG or Fab, we used the pAK500 vector which contains the peptide dHLX formed in a helix-turn-helix motif (Krebber et al., 1997; Plückthun & Pack, 1997), which mediates spontaneous dimerization for the production of dimeric proteins for *in vivo* targeting (Hill, Raleigh, et al., 2000; Willuda et al., 2001) and results in a (scFv)A-hinged HLX-hinged-(scFv)A arrangement. In this case, the affinity, stability and specificity of scFv-dHLX were assessed and compared with scFv *in vitro*. These data provide evidences that the application of scFv-dHLX as a recognization factor for AMA may be enhanced by dimerizing VH and VL with the aid of the helical bundle element dHLX.

Immunoassays for AMA detection have been studied based on monoclonal antibodies (Peng et al., 2017; Xu et al., 2016). However, to our knowledge, an engineered antibody for AMA has not been reported. Previously, we produced a hybridoma cell line (3F2) that secretes an mAb specific for AMA. In the present study, the scFv gene of this mAb was obtained and recombinantly expressed in *Escherichia coli* cells. In the present study, the scFv gene of this mAb was obtained and recombinantly expressed in *E. coli*. Using this engineered antibody, we developed a competitive indirect ELISA for the detection of AMA. This work is helpful for the development and application of engineered antibodies for small molecule detection in food safety.

Materials and methods

Materials

The soluble protein expression vector pJB33 used in this study was obtained as a gift from the laboratory of Andreas Plückthun (Biochemical Institute, University of Zurich, Switzerland). *RV308* strains were obtained from ATCC. The oligonucleotide primers and analysis of gene sequence were synthesized and performed at Invitrogen (Shanghai, China). The coating antigen AMD-OVA conjugate was prepared in our laboratory. The hybridoma cell line 3F2, which secretes an mAb against AMA, was previously established in our laboratory.

Construction of the scFv gene

The VH and VL gene fragments were extracted, and the scFv gene was constructed according to our previous report (Tao et al., 2013). Briefly, the total RNA was obtained by

extraction of 1×10^7 hybridoma cells with the RNeasy Mini Kit, and the cDNA was generated using the PrimeScript RT-PCR Kit. The VH and VL gene fragments were obtained by PCR amplification. Then, the two gene fragments with primers VH7F2, VH7R2, VL3R2 and VL3F2 were cycled seven times (98°C for 10 s, 72°C for 3 min) with Pfu DNA polymerase to join the scFv-based DNA fragments. Finally, the scFv gene and the express vector pJB33 were digested with SfiI, and the two digested products were linked to construct the recombinant express vector pJB33-AMA-scFv and pAK500-AMA-scFv (Supplementary Figure 1). All primers used in this study are shown in Supplementary Table 1.

Expression and purification of soluble scFv and scFv-dHLX antibody

E. coli RV308 was transformed with vector pJB33-AMA-scFv and pAK500-AMA-scFv. The single bacterial colony was incubated with 2.5 mL 2× YT medium (16 g tryptone/L, 10 g yeast extract/L, and 5 g NaCl/L) containing chloramphenicol with shaking overnight (37°C, 200 g). Then, a volume of the bacteria solution was diluted 100-fold and cultured in 250 mL of 2× YT medium. When the OD_{600} value reached about 0.6–0.8, 1.0 mM, isopropyl β-D-Thiogalactoside (IPTG) was added to induce scFv expression at 24°C for 12 h. For optimization of anti-AMD-scFv expression, cultivations were performed under different concentrations (0, 0.25, 0.5, 0.75, 1.0 or 1.5 mM) and incubation times (0, 2, 4, 6, 8, 10, 12 h). The induced bacteria were then harvested by centrifugation and dissolved in 5 mL of lysis buffer (50 mM Tris, 300 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, and 200 μg lysozyme/mL, pH 8.0). After the bacterial solution was sonicated for 6 min and centrifuged for 5 min (4°C, 10,000g), the soluble periplasm scFv antibody in the supernatant was purified by a Ni NTA Agarose (Ni-NTA) agarose resin column according to the QIAGEN's protein purification instructions. Briefly, 50% of the Ni-NTA slurry was mixed gently with the supernatant containing the soluble periplasm scFv antibody at a ratio of 1:4 (v/v) by shaking for 2 h at room temperature (RT). Then, the Ni-NTA agarose mixture was carefully loaded into an empty column and washed with 25 mL of washing buffer (50 mM Tris-HCl, 500 mM NaCl, and 25 mM imidazole, pH 8.0). The fusion proteins were eluted with buffer (50 mM Tris-HCl, 150 mM NaCl, and 300 mM imidazole, pH 8.0). Subsequently, the elution was confirmed by SDS-PAGE and Western blotting. After measuring the protein concentration using the Bradford method, the proteins were aliquoted and stored at −80°C.

Characterization of scFv antibody

Indirect competitive ELISA

A microtiter plate was coated with AMD-OVA overnight at 4°C (100 μL/well) and then blocked with 3% skim milk (100 μL/well) for 1 h at 37°C. After the plate was washed with PBST three times, 70 μL of scFv dilution and 30 μL of AMA standard solution were added into the wells for incubation (37°C, 35 min). The plate was washed and horseradish peroxidase-labeled goat anti-His-tag antibody (1:5000 in PBS) was added and incubated (100 μL/well, 37°C, 35 min). After washing, TMB solution was added to visualize the result (100 μL/well, 37°C, 3 min). Finally, 2 M H_2SO_4 was added to stop the reaction (50 μL/well), and the OD_{450} nm value of each well was read on an ELISA reader. The

analyte concentrations and the $B/B0$ values were used to develop the competitive inhibitory curve.

Relative affinity

The scFv and scFv-dHLX proteins diluted to 3.4×10^{-1} µM in PBS containing 1% (m:v) BSA were added to 96-well ELISA plates pre-coated with the AMD-OVA. Following incubation for 1 h at 37°C, the plate was washed with PBS-T (220 µL/well) five times. The bound protein was eluted with NH_4SCN in PBS at concentrations ranging from 0 to 6 M (100 µL/well). The elution was performed on a shaker at 500 rpm for 1 h at 37°C. The NH_4SCN was washed away with PBS-T (220 µL/well) five times, and the remaining protein was detected by using an anti-His-tag mAb and HRP-conjugated goat anti-mouse IgG. The following steps were the same as those of the ic-ELISA assay described above.

Stability

In order to compare the stabilities of scFv and scFv-dHLX *in vitro*, the antibodies were diluted to 3.4×10^{-1} µM in PBS. Subsequently, the samples were placed at 37°C for different spans of time. The proteins precipitating during the incubation were removed by centrifugation at $10,000 \times g$, and then the supernatants were added to 96-well ELISA plates pre-coated with AMD-OVA. The proteins which did not degrade could be bound by the AMD-OVA and detected by using the anti-His-tag mAb and HRP-conjugated goat anti-mouse IgG as mentioned above.

Results and discussion

Construction of expression plasmids

Total RNA was extracted from the AMA mAb hybridoma cell (Figure 1(b)). The VH and VL genes were amplified separately by PCR approximately 360 and 320 bp, respectively (Figure 1(a)), and the size of the assembled scFv fragments was approximately 750 bp (Figure 1(a)). The anti-AMAhis/c-Myc-ScFv gene was designed in such a way that VL was located in the N terminal to form the resulting expression plasmid of pJB33 and pAK500 (anti-AMA-scFv) (Supplementary Figure 1). The recombinant plasmid was further confirmed using restriction enzyme digestion and sequencing.

Expression and purification of anti-AMA-scFv

Expression of 6XHis-tagged scFv and scFv-dHLX in *E. coli RV308* host strains was identified by SDS-PAGE and Western blotting (Figure 2). A high degree of purity was achieved as indicated by separation on SDS-PAGE and Coomassie blue staining. The molecular weights of two expressed recombinant proteins were expected to be 25 and 54 kDa, respectively. The molecular weight (54 kDa) of dimers was almost twice as much as that of monomers. The present study showed that optimal production of anti-AMA-scFv occurred when 1 mM IPTG was used with a cultivation temperature of 25°C (data not shown). We chose 25°C as the induction temperature, since several *E. coli* chaperones
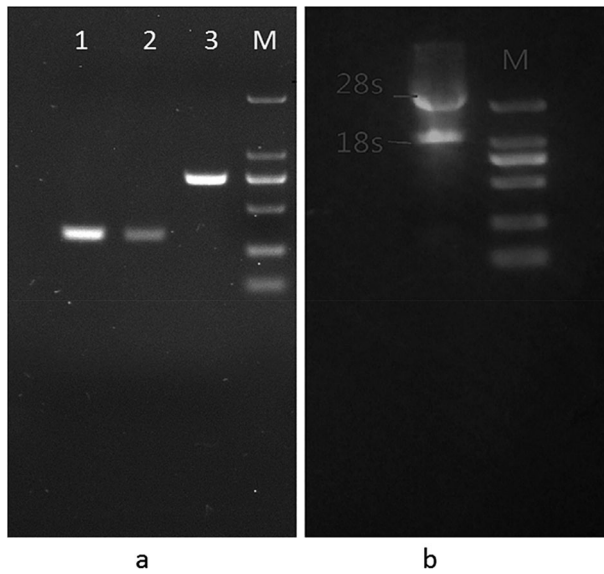
Figure 1. PCR reaction products of the scFv and the variable regions (VL, VH). (a) Extraction of RNA from hybridoma cell and (b) M:DL2000 DNA marker.

are actively expressed at low temperatures, the stability and folding of recombinant scFv were enhanced at 25°C (Jung, Honegger, & Plückthun, 1999). We chose to express and purify the soluble, *in vivo* proper folding and functionally active scFv in an enhanced expression and high-copy vector pJB33 and pAk500, instead of isolating it from inclusion bodies under denaturing conditions and performing *in vitro* refolding procedures.
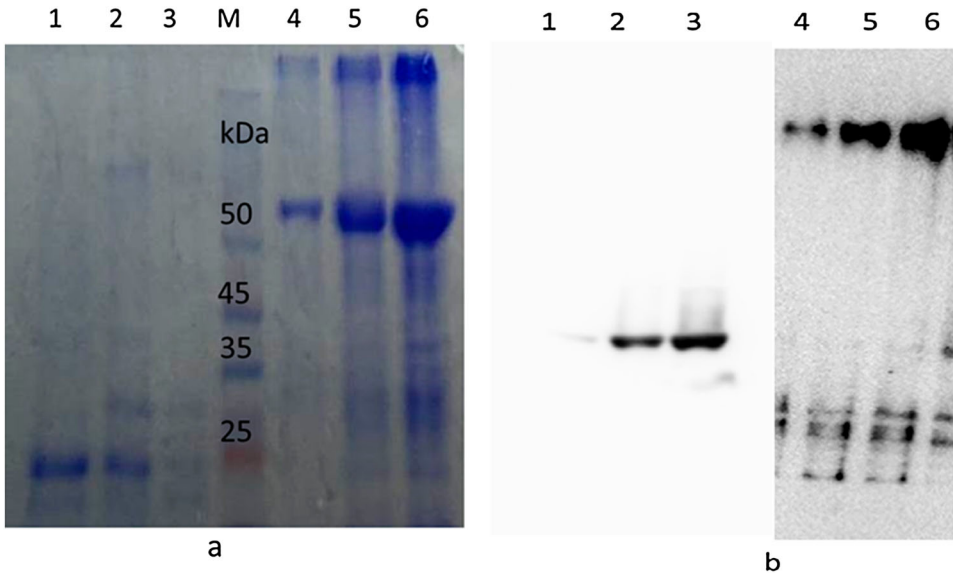

Figure 2. Elution proteins (a) were separated on a 12% SDS-PAGE gel and visualized by Western blot. (a) Lane 1, lane 2, lane 3 were anti-AMA-ScFv elutions protein and lane 4, lane 5, lane 6 were AMA-scFv-dHLX elutions protein and (b) Western blot results corresponding to figure (a).

Producing scFvs at high levels in *E. coli* often results in the formation of inclusion bodies, it is common to encounter problems with solubility and proper folding (Worn & Plückthun, 2001), however in this study, the T7 promoter expression system is so efficient that mRNA synthesis is fast and directly coupled to translation, and the pelB signal peptide directs scFv secretion into the periplasmic space. which provide an oxidative environment suitable for functional proteins production and disulfide bond formation (Dreier & Plückthun, 2011; Jost & Plückthun, 2014; Kang et al., 2016; Plückthun 2015; Tamaskovic, Simon, Stefan, Schwill, & Plückthun, 2012). The oxidized environment of the periplasmic space, rich in disulfide bond formation proteins (PDI, DsbA and DsbC) and chaperones SKp (Lindner et al., 2014; Liu et al., 2008), is most suitable for natural folding of the scFv fragment with high yield. Additionally, extraction and purification of periplasmically expressed proteins can easily be performed by a simple osmotic shock procedure without denaturation/renaturation steps.

The periplasmic fractions were extracted from a large volume of induced *RV308* cells and loaded onto a Ni-NTA column for the purification of the target proteins. SDS-PAGE analysis demonstrated that the scFv and scFv-dHLX fusion proteins were highly purified (Figure 2(a)); the envisioned molecular weight of anti-AMA-scFv was approximately 27 kDa (Figure 2(a)). The result of Western blotting analysis using the anti-c-Myc (C-terminal) antibody is shown in Figure 2(b), which indicates that the expressed anti-AMA-scFv was the c-Myc-tagged fusion protein.

Relative affinity

The affinities of scFv and scFv-dHLX for AMA were evaluated and compared using the NH_4SCN elution test. When the OD_{450} decreased to 50% of the initial amount of scFv-dHLX without eluting by NH_4SCN, the corresponding concentration of NH_4SCN was 5.28 M, while that of scFv was 2.83 M (Figure 3). Thus, this result showed that the affinity of scFv-dHLX for AMA was higher than that of scFv. The obvious explanation was the greater similarity in structure of scFv-dHLX with that of the natural IgG or Fab. The scFv-dHLX contained the identical assembly pattern of VH and VL as that of the IgG or Fab aided by efficient interaction between the helical bundle element dHLX and the disulfide bond. Meanwhile, VH and VL in an scFv was connected covalently by an artificial polypeptide linker, resulting in a non-free amino at the terminus of VH or VL. Furthermore, the design of scFv's linker was not easy because an inappropriate linker could interfere with the antigen-binding domain, in this study the actual assembly pattern of VH and VL in three-dimensional space to some extent forms the correct conformation. Surprisingly, the binding behavior indicated an increase in affinity, which was attributed to avidity.

Stability

To evaluate the stability of scFv and scFv-dHLX, the samples were diluted to the same molar quantities in PBS placed at 37°C for different spans of time. The half time for scFv-dHLX in PBS was 73.9 h, which was 4.42 times higher than that for scFv (16.7 h) (Figure 4). These results clearly demonstrated the greater stability of scFv-dHLX compared with scFv, the generation of dimeric miniantibodies is simply achieved by adding
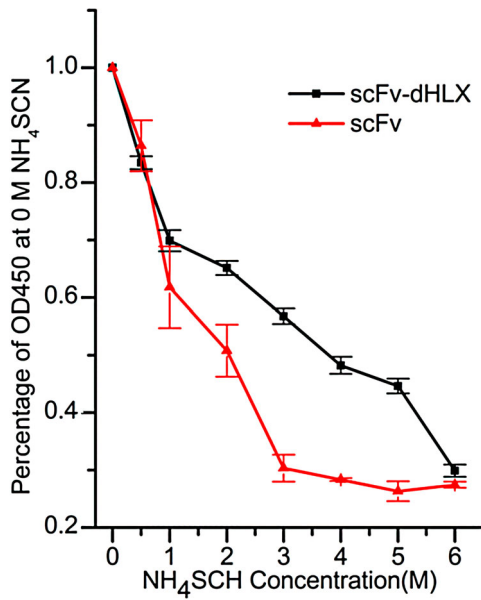
Figure 3. Relative affinities of scFv and scFv-dHLX.

Notes: The scFv and scFv-dHLX proteins were incubated at the same molar quantities with pre-coated AMD-OVA. The bound proteins were then eluted with various concentrations of NH4SCN ranging from 0 to 6 M. Data are means ± SD of three independent experiments.
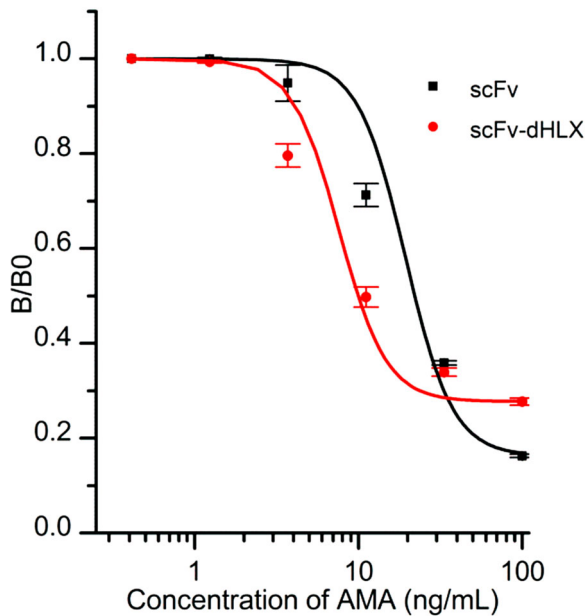
Figure 4. Molecular stabilities of scFv and scFv-dHLX.

Notes: Stabilities of the purified proteins were determined by ELISA using plates pre-coated with AMD-OVA and detected with an anti-myc mAb and HRP-conjugated goat anti-mouse IgG. The proteins in PBS were incubated at 37°C for 0–40 h and then added to the ELISA plates. Absorbance values of samples at 450 nm were determined and presented as percentages relative to those of samples incubated for 0 h. Data are means ± SD of three independent experiments.

Figure 5. Comparison of scFv to scFv-dHLX.

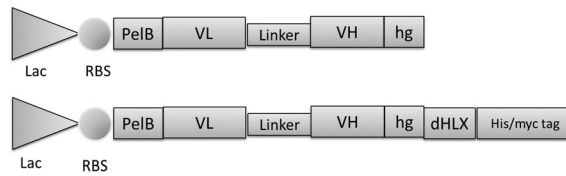Notes: Binding of scFv-dHLX (red sphere) or scFv (black square) to AMD-OVA in the presence of AMA as measured by indirect ELISA. Each point represents the average of three replicate assays standard deviation.

an oligomerizing sequence to the C-terminus of the scFv fragment. The self-associating modules consist of a helix-turn-helix motif fused to the scFv with two of them "clasping" each other (Hill, Bracken, et al., 2000). Homodimerization of scFv resulting in coiled coil helices showed very good behavior with regard to stability, heterospecificity and resistance to proteases (Arndt, Muller, & Plückthun, 2001). The presence of the dimerization motif dHLX was shown to cause the formation of covalent cross-links of the self-associated peptides, thus resulting in an increase in their stability (Kubetzko, Balic, Waibel, Zangemeister-Wittke, & Plückthun, 2006; Schaefer, Lindner, & Plückthun, 2010).

Optimization and specificity of ic-ELISA

To evaluate the binding activities of purified soluble scFv with AMA, different concentrations of AMA were detected by ELISA. As shown in Figure 5, the recognition and binding of purified soluble scFv to AMA were dose dependent, while no nonspecific binding of target scFv to negative antigen control (BSA) was detected. Furthermore, the soluble scFv and scFv-dHLX presented a nearly identical ELISA signal curve with the mAb-AMA, indicating that they perform identical binding activity to AMA. This result revealed that the soluble expressed scFv and scFv-dHLX proteins retained full antigen-binding activities with specific antigen AMA, suggesting correct folding of the scFv and scFv-dHLX.

Conclusion

In conclusion, we cloned the VH/VL gene from the hybridoma cell line 3F2 and constructed two expressed vectors pJB33-AMA-scFv and pAK500-AMA-scFv-dHLX in *E. coli RV308*. The results demonstrated that the scFv-dHLX protein showed a higher binding ability and stability corresponding to scFv. This study provides a theoretical basis for scFv molecular designing aimed at enhancing its specificity and affinity, which is helpful for developing immunoassay of AMA residue in the food industry.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

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