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Full Length Research Paper

Improved affinity of a chicken single-chain antibody to avian infectious bronchitis virus by site-directed mutagenesis of complementarity-determining region H3

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Affinity maturation of antibodies using random or site-directed mutagenesis provides the potential to change the properties of antibodies *in vitro*. We used site-directed mutagenesis to improve the binding affinity of an anti-avian infectious bronchitis virus (IBV) single-chain antibody ZL.80 with low affinity. Twelve highly variable residues in the third heavy chain complementarity-determining region (CDR H3) of ZL.80 were mutated to alanine, to determine their contributions to antigen binding. Only mutations at ^{Cys}H105, ^{Asp}H106 and ^{Val}H108 inhibited its binding to IBV in indirect enzyme-linked immunosorbent assays. These three sites were then substituted with tyrosine, arginine and serine, because these were considered to be beneficial to protein-protein interactions. The mutant ^{Val}H108^{Tyr} demonstrated a 12.9-fold increase in affinity compared to parental ZL.80. This study demonstrates that the affinity of single-chain variable fragments can be improved by a single amino acid substitution in the CDR H3 with no change in specificity, indicating that tyrosine could play an important role in antigen binding.

Key words: Single-chain variable fragments (scFvs), affinity maturation, avian infectious bronchitis virus (IBV), third heavy chain complementarity-determining region (CDR H3).

INTRODUCTION

Phage display represents an attractive alternative to conventional hybridoma technology. It has become a powerful tool for selecting recombinant antibodies and antibody fragments with various specificities and affinities from antibody libraries (Bradbury, 2004; Hoogenboom, 2005; Finlay et al., 2011). However, the affinities of recombinant antibodies such as Fabs and single-chain variable fragments (scFvs) obtained by phage display are often lower than those of monoclonal antibodies (mAbs) from hybridomas, or of intact forms of the antibody (Mandrika et al., 2008). This could limit their usefulness for virus diagnosis and treatment, and their affinities need to be improved by affinity maturation. High-affinity antibodies against a conserved epitope could also provide immunity to diverse viral subtypes and protection against future pandemic viruses (Gould et al., 2005; Siegel et al., 2008).

Till date, a variety of mutagenesis strategies for affinity maturation have been proven to enhance the affinity of candidate therapeutic antibodies. Site-directed mutagenesis provides another means of affinity maturation that often focus on the contact residues of the antibody, avoiding the construction of large or multiple small libraries and the drawback of generating a large proportion of nonfunctional species using random mutagenesis approaches. (Putkey et al., 1989; Foster et al., 1992; Zahnd, 2004; Chang et al., 2009). Many studies

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Abbreviation: **IBV**, Infectious bronchitis virus; **CDR**, complementarity-determining region.

have shown that antibody specificity and affinity can be changed by mutagenesis or insertion of a single amino acid in the contact region (Goldstein et al., 1992; Winkler et al., 2000; McCarthy and Hill, 2001; Tachibana et al., 2004; Xu et al., 2009). This suggests that the interactions of a few residues at the centre of the contact interface are responsible for the binding affinity to the antigen (Denzin et al., 1991; Dougan et al., 1998; Chang et al., 2009). The site-directed mutagenesis approach also has limited utility because it relies on the availability of accurate high-resolution antigen-antibody structures, which can be obtained by X-ray or nuclear magnetic resonance, but are often unavailable (Padlan et al., 1989; Valjakka, 2002; Clark et al., 2009). However, the interactions between antibodies and antigens can be predicted using molecular modeling, sequence comparisons and alanine scanning (DeLano, 2002; Kusharyoto et al., 2002; Barderas et al., 2008).

Of an immunoglobulin molecule, the third heavy chain CDR (CDR H3) exhibits the greatest diversity in terms of length and sequence. It is prominent in determining antibody specificity, and is usually responsible for most of the stabilizing contacts (Xu and Davis, 2000). Analysis of the three-dimensional structure of the antigen-antibody complex revealed that each CDR forms a loop and makes an antigen-binding cavity to receive antigens in a specific manner. Most of the six complementarity-determining residues, as well as a few residues outside the hypervariable regions of the antibody, make contact with the antigen. Moreover, most of these contacts are made by the H chain, and in particular by its third CDR. Mutations at these residues are therefore likely to abolish antigen binding. However, mutation may result in an increase in affinity if the native residue exerts a negative effect on the antigen-antibody interaction (Schier et al., 1996; Murakami et al., 2010).

The size, shape and chemical features of surface-exposed residues in the antigen-binding region of an antibody are important determinants of binding specificity and affinity (Winkler et al., 2000). However, it is still not known whether the affinity of antibody will be improved by the change of specific amino acids in CDR H3, such as Tyr, Arg, and Ser, which may influence its conformation or the formation of salt links and hydrogen bonds between antigen and antibody (Sharon, 1990). Tyr, Arg and Ser are thought to aid antigen/antibody binding because Tyr, Arg are often “hot spots” of binding energy and Ser could provide conformational flexibility that allows bulky residues to achieve optimal binding contacts (Birtalan et al., 2010). Analysis of structural databases has revealed that co-evolved interfaces are enriched for large Tyr, Trp and Arg residues that are capable of mediating a wide array of intermolecular contacts (Bogan et al., 1998; Daugherty, 2000; Birtalan et al., 2008). Furthermore, Tyr and Ser residues are also highly abundant in natural antibody germ line sequences, and Arg residues become more abundant in the loops of functional antibodies that have undergone affinity maturation (Fellouse, 2004; Fellouse et al., 2006). This suggests that these residues have intrinsic capacities for mediating antigen recognition, and may thus influence antibody binding specificity and enhance affinity (Xiang et al., 1999; Yau et al., 2005; Kossiakoff and Koide, 2008; Birtalan et al., 2010). We previously constructed an scFv expression library using pOPE101-xp vector and mature variable region gene derived from spleen of immunized chicken. Two anti-infectious bronchitis virus (IBV) scFv clones, ZL10 and ZL.80, were screened by indirect ELISA. However, the affinity of scFv ZL.80 was relatively low, only one-tenth of scFv ZL.10. The current study aimed to improve the affinity of scFv ZL.80 by substitution of amino acids in the CDR H3 by site-directed mutagenesis at with two hot spots (Tyr and Arg) and one small residue (Ser).

MATERIALS AND METHODS

Virus strains, vector and scFv

IBV M41 strain was kindly donated by Professor Jianhe Sun of Shanghai Jiaotong University, China. The virus was propagated in 9 to 10 day chick embryos and purified by sucrose density gradient centrifugation. The vector pOPE101-XP was kindly donated by Dr. Ying Zhang of Raybiotech Inc, USA. The anti-IBV scFv clones ZL.10 and ZL.80 were obtained previously (Lin et al., 2011) by screening an immunized chicken library, and identified by enzyme-linked immunosorbent assay (ELISA) and Western blotting.

Sequence comparisons

The nucleotide sequences of ZL-10 and ZL-80 were tested by Sangon Biotech Co., Ltd (Shanghai, China). The deduced amino acid sequences were aligned with three scFvs against the hapten fluorescein (Andris-Widhopf et al., 2000) and three scFvs against allergenic protein [native Amb a 1, recombinant Fel d 1, and whole yellow jacket venom (YJV)] (Finlay, 2005) in previous studies, all from an immunized chicken library.

Site-directed mutagenesis

ZL.80 clone were cultured in Luria-Bertani medium (LB) overnight with 100 μg/ml ampicillin. Plasmids pOPE-101XP-ZL.80 were then extracted and used as templates for amplification of the mutate gene fragment. The DNA encoding mutant scFvs was constructed by overlap polymerase chain reaction (PCR) amplification (SOE-PCR) using a two-step method, as follows: First, two small DNA fragments that overlapped at the site of mutation were generated. Using pOPE-101XP-ZL.80 as templates, fragment (A) was amplified from a unique 5’ restriction site to the site of mutation using P-scFv-NotI and a reverse mutation primer, while fragment (B) was amplified from the site of mutation to a unique 3’restriction site using a forward mutation primer and P-scFv-NcoI. PCR was conducted at 94°C for 4 min, followed by 94°C for 30 s, 62°C for 30 s and 72°C for 1 min, repeated for 30 cycles. Second, the overlapping DNA segments (A + B) were joined to the mutant scFvs by splice overlap extension SOE-PCR. After adding equivalent amounts of segments A and B, without any primer, PCR was conducted at 94°C for 4 min, followed by 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, repeated for 15 cycles. P-scFv-NotI, P-scFv-NcoI were then added and PCR was conducted at 94°C for 4 min, followed by 94°C for 30 s, 62°C for 30 s and 72°C for 1 min,

Table 1. Oligonucleotides used for mutagenesis.

No	Sequence	Description	Mutant
Internal mutation primers			
P-105A	5'-CAT-GGT-AAT-GGT-***GC***T-GAT-GGT-GTT-GGT-3'	Forward primer	^{Cys}H105^{Ala}
P-105Y	5'-CATGGT-AAT-GGT-T***A***T-GAT-GGT-GTT-GGT-3'	Forward primer	^{Cys}H105^{Tyr}
P-105S	5'-CAT-GGT-AAT-GGT-T***C***T-GAT-GGT-GTT-GGT-3'	Forward primer	^{Cys}H105^{Ser}
P-105R	5'-CAT-GGT-AAT-GGT-***C***GT-GAT-GGT-GTT-GGT-3'	Forward primer	^{Cys}H105^{Arg}
P-106A	5'-TAAT-GGT-TGT-G***C***T-GGT-GTT-GGT-CAG-3'	Forward primer	^{Asp}H106^{Ala}
P-106Y	5'-TAAT-GGT-TGT-***T***AT-GGT-GTT-GGT-CAG-3'	Forward primer	^{Asp}H106^{Tyr}
P-106S	5'-TAAT-GGT-TGT-***TC***T-GGT-GTT-GGT-CAG-3'	Forward primer	^{Asp}H106^{Ser}
P-106R	5'-TAAT-GGT-TGT-***CG***T-GGT-GTT-GGT-CAG-3'	Forward primer	^{Asp}H106^{Arg}
P-108A	5'-TTG-TGA-TGGT-G***C***T-GGT-CAG-ATC-GACG-3'	Forward primer	^{Val}H108 ^{Ala}
P-108Y	5'-TTG-TGA-TGGT-***TA***T-GGT-CAG-ATC-GACG-3'	Forward primer	^{Val}H108 ^{Tyr}
P-108S	5'-TTG-TGA-TGGT-***TC***T-GGT-CAG-ATC-GACG-3'	Forward primer	^{Val}H108 ^{Ser}
P-108R	5'-TTG-TGA-TGGT-***CG***T-GGT-CAG-ATC-GACG-3'	Forward primer	^{Val}H108^{Arg}
Amplification primers of scFvs			
P-scFv-Not	5'- GGCCATGGCCGTGACGTTGGACGAGTCCGG-3'	Forward primer to amlifed scFv	
P-scFv-Nco	5'-CTGCGGCCGCTAGGACGGTCAGGGTTGTCCC-3′	Reverse primer to amlifed scFv	

Mutant forward primers and amplification primers of scFv were listed. While mutant reverse primers were the antisense of mutant forward primers, table not show. Nucleotides mismatches are in bold and italics, restriction enzyme sites are underline. Muntant scFvs are described using the following nomenclature (Dougan et al., 1998): CysH105Ala denotes that residud 105 of the variable region from antibody heavy chain, cysteine in the ZL.80 has been replaced with alaine.

repeated for 25 cycles. Both amplification and mutation primers are listed in Table 1. All PCR reactions were performed with PrimerSTAR HS DNA polymerase (Takara, Japan). The correct nucleotide sequences of mutant scFvs were tested using forward primers 5'-CTATTGCCTACGGCAGC-3' and reverse primers 5'-AAGGCCCAGTCTTTCGAC-3' by Sangon Biotech Co., Ltd (Shanghai, China).

Construction of expression plasmids

The DNA encoding the mutant scFvs was cloned into the pOPE101-XP vector using the NotI and NcoI restriction sites. The correct expression vector was identified by PCR, restriction enzyme digestion and sequencing. The recombinant vector pOPE101-XP-scFv was transformed into *E. coli* JM109 strain (Takara, Japan) for expression. Positive transformants were identified by digestion of plasmid DNA with the appropriate restriction endonucleases.

Expression and extraction of scFv in periplasm

The vector pOPE101-XP, which has a pelB signal peptide in the 5' region of the gene, allows expression of soluble scFv in the periplasm of *E. coli* JM109 (Schmiedl et al., 2000; Hust et al., 2007). Osmotic shock can then be used to extract scFv from the periplasm.

Briefly, the transformed clone was inoculated into 2 ml LB with 100 μg/ml ampicillin and cultured at 37°C and 250 rpm overnight. Then, 20 μl culture medium was removed and added to 2 ml fresh ampicillin LB cultured at 37°C and 250 rpm to an optical density $(OD)_{600}$ of 0.6 to 0.8. The induction was started by adjusting to 50 μM isopropyl-β-D- -thiogalactopyranoside (IPTG, Sigama, USA) and shaking for 6 h at 30°C and 250 rpm. The bacteria were then harvested by centrifugation for 10 min at 4,000 g at room temperature. Pellets were resuspended in 500 μl TES buffer, pH 8 (500 mM sucrose, 30 mM Tris, 1 mM EDTA) at room temperature, with brief vortexing every 5 min. The bacteria were then pelleted for 10 min at 12,000 g at 4°C. The supernatant was abandoned and the pellet was resuspended in 300 μl ice-cold 5 mM $MgSO_4$ incubated for 20 min on ice, with brief vortexing every 5 min. Then the solution was centrifuged for 30 min at 12,000 g and 4°C. The supernatant was collected and stored at -20°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The transformed clone was cultured and induced according to the method mentioned in 'expression and extraction of scFv in periplasm'. The bacteria were harvested after 3 h of induction and boiled in the SDS-PAGE loading buffer with 2-mercaptoethanol for 5 min. The supernatant were then collected as the total bacterial cell lysates by centrifugation for 10 min at 4,000 g at room temperature. To identify the level of scFv expression, the total bacterial cell lysates and periplasmic extracts were analyzed by SDS-PAGE, which was carried out on 12% polyacrylamide gels stained with coomassie brilliant Blue R250.

Indirect ELISA

The specificity of mutant scFvs for binding IBV M41 was tested by ELISA. Firstly, 200 ng antigen (IBV or NDV, BSA) was coated in sodium bicarbonate buffer (pH 9.6) in a 96-well plate (Corning, America) at 4°C overnight. Subsequently, 100 μl of periplasm extracts or purified scFv (500 μM) of each mutants were added and reacted at 37°C for 2 h. The plates were then probed with myc-tag mouse mAb (Raybiotech, America, 1:2,000 dilution) at 37°C for 1 h, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Dingguo, China, 1:5000 dilution) at 37°C for 1 h, both diluted in PBST. The plates were washed three times with

PBST after each step. Finally, 100 µl 3,3′,5,5′-tetramethylbenzidine (TMB) (HRP)-substrate (Tiangen, china) was used for coloration for 30 min and 50 µl H_2SO_4 (2 M) was used to stop the reaction. The absorbance which was proportional to the concentration of free anti-IBV scFv in each well was measured at 450 nm.

Purification of scFvs

Nickel affinity chromatography (Yihong, china) was used to purify scFvs, based on the presence of a six-histidine tag at the 3' terminus of scFvs expressed by pOPE101-XP (Hust et al., 2007). Each mutant scFv clone was cultured and induced in LB using the above method, with an amplified volume of no less than 500 ml. The cultured solution was then collected and sonicated for 30 min on ice to break up the *E. coli*, followed by centrifugation for 30 min at 12,000 g at 4°C. The supernatant was then collected and added to a nickel affinity chromatography column for purification of scFvs, according to the manufacturer's instructions. The nickel column was pre-balanced using buffer (20 mM Na_2 HPO_4, 0.5 M NaCl), and non-specific proteins were eluted using one application of 10 mM imidazole-containing buffer (20 mM Na_2HPO_4, 0.5 M NaCl, 10 mM imidazole) and three washes with 50 mM imidazole buffer. The six-histidine-tagged scFvs were eluted using PBS, pH 7.4 with 100 mM EDTA. After dialysis against PBS-buffer, scFv proteins were concentrated to 0.1~0.3 mg/ml by ultrafiltration using Centricon YM-10 concentrators (Millipore, Bedford, MA, USA).

Determination of mutant scFv affinities

The ELISA technique represents a relatively simple and fast method of measuring the strength of antibody/antigen interactions in order to evaluate the affinity constant (K_{aff}). K_{aff} was determined using the method described by Beaty et al. (1987). Briefly, the wells of a 96-well plate (Corning, America) were coated with IBV at two-fold decreasing concentrations from 400 to 50 ng/ml in carbonate–bicarbonate buffer, pH 7.2 at 4°C overnight. The plate was then emptied and washed three times with PBS-Tween (0.1% v/v Tween 20, Dingguo, China). In addition, 100 µl of each two-fold serial dilution of purified scFv (15.6~1000 nM) were dispensed into the IBV-coated wells and the plate was sequentially incubated with myc-tag mouse mAb (1:2,000) and HRP-conjugated goat anti-mouse IgG (1:5,000) for 1 h at 37°C, and with 100 µl TMB in the dark for 30 min. Finally, 50 µl H_2SO_4 (2 M) was used to stop the reaction and the absorbance was measured at 450 nm. K_{aff} was calculated according to Beatty's equation:

$$K_{aff} = (n - 1) / 2 (n[Ab']t - [Ab]t)$$

Where, [Ab′]t refers to scFv concentration at half the maximal OD (OD_{50})for IBV-coated wells at half concentration, and [Ab] refers to scFv concentration at OD_{50} for IBV-coated wells at whole concentration, and n = [Ag]/[Ag′]. Because a scFv has only one binding site for antigen, the affinity can be calculated by the equation of $K_{af\ f}$= 2[Ab′]t −[Ab]t (Zhao et al., 2010). All procedures were performed in triplicate and the mean values calculated.

RESULTS

Expression of soluble scFvs in periplasm

The 726-bp gene of each mutant scFv was amplified by overlap PCR and introduced into the pOPE101-XP vector using the NotI/NcoI restriction sites to yield pOPE101-XP-scFv. The recombinant plasmid was identified by digestion and sequenced, and the sequences are given in (Figure 1). The optimal conditions, such as temperature, host and culture media, for scFv expression using this vector were investigated by Hust et al. (2009). pOPE101-XP-scFv were therefore transformed into JM109 cells and induced for expressing. Reducing the temperature and the concentration of IPTG caused scFv to be expressed in the periplasm. Soluble scFvs with a molecular weight of about 31 kDa on SDS-PAGE could thus be expressed in the periplasm (Figure 2).

Function of residues in CDR H3

Because CDR H3 exhibits the greatest diversity in terms of length and sequence, it is prominent in determining antibody specificity and affinity and is usually responsible for most of the stabilizing contacts. However, only a few residues in CDR H3 contact the antigen and thus contribute to the effect of the antibody. These residues can be identified using alanine scanning to determine the contribution of specific residues to a protein's function by mutating the residues to alanine.

To investigate the contribution of specific residues to antigen binding, we substituted the residues from H99 to H110 in CDR-H3 of ZL.80 with alanine. The effects of the mutations were assessed by analyzing periplasm extracts for each mutagenic clone by indirect ELISA. Alanine substitution of VH Val108 reduced the binding to immobilized IBV (OD_{450} near 0.2), while substitution of VH Cys105 and VH Asp106 reduced it to a low level (OD_{450} below 0.1), similar to the scFv-negative (Figure 3). Substitutions in other positions had no apparent effect compared to unmutated ZL.80 (OD_{450} among 0.4~0.5). These three residues in ZL.80 may therefore play important roles in antigen recognition and were assumed to be involved in contacting the antigen and affecting its affinity.

Effects of mutagenesis on specificity and affinity

Comparison of the ZL.80 CDR amino acid sequences with those of the high-affinity scFv ZL.10 and with five functional chicken scFvs reported in other studies indicate that ZL.80 has fewer tyrosines in CDR H3 and arginines in CDR H2 (Figure 4), In addition，many reports have discussed the effects of hot-spot residues on the binding ability of antibodies. It's revealed that Tyr, Arg and Ser residues have intrinsic capacities for mediating antigen recognition, and thus may affect antibody binding specificity and enhance the affinity. By alanine scanning, CysH105, AspH106 and ValH108 were identified as the functional residues which influenced antigen recognition. These residues were then replaced by Tyr, Arg and Ser to determine their function in improving the affinity of ZL80.

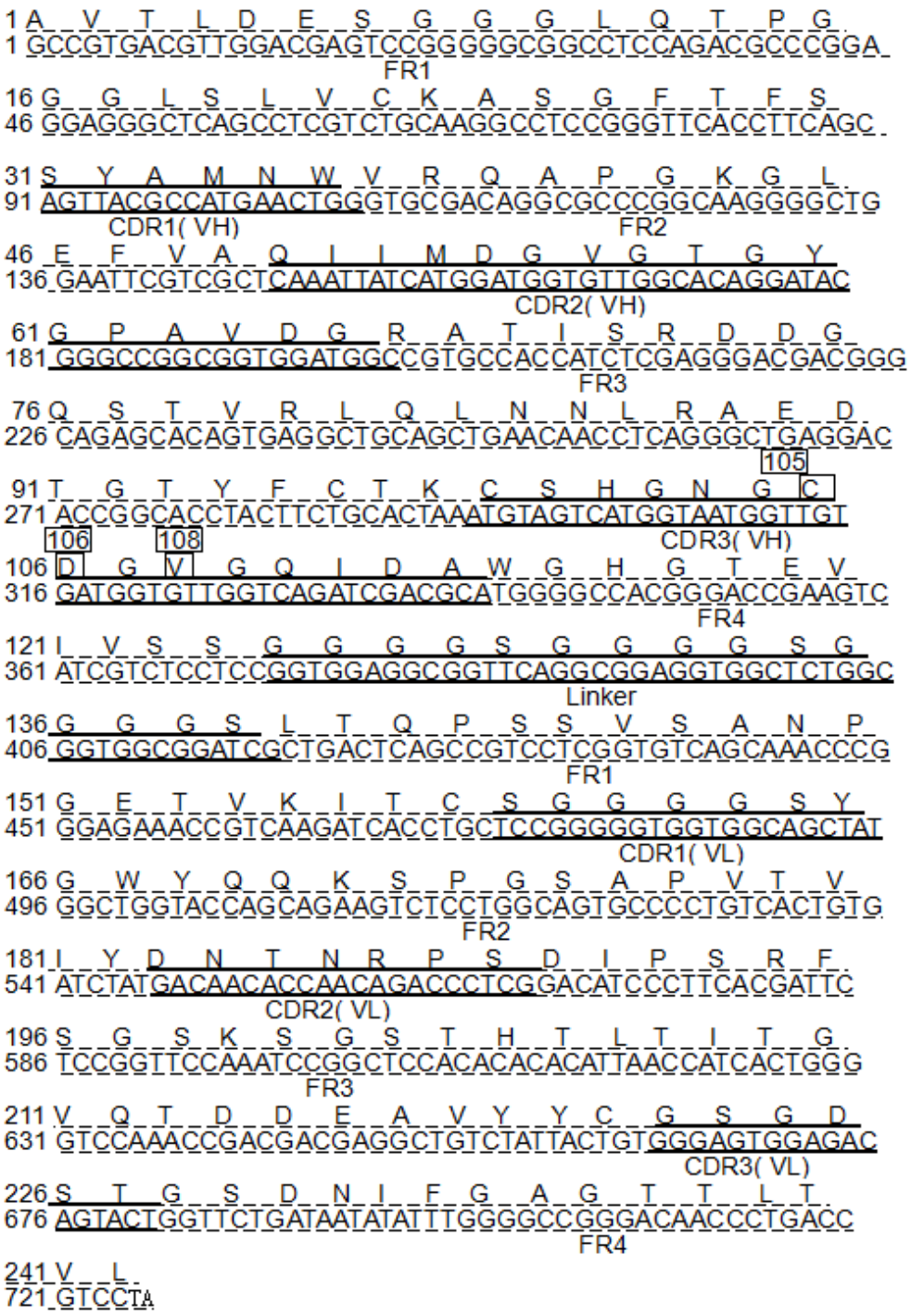

Figure 1. Necleotide and deduced amino acid sequence of anti-IBV scFv (ZL.80). The complementarity determining region of the heavy and light chain variable region are underlined. The three positions identified and determined to introduce Tyr, Arg, and Ser mutations are boxed and numbering according to their position in ZL.80.

The influence of each mutation on the binding of IBV M41 was measured by indirect ELISA. OD values were reduced in four of the scFv mutants ($^{Cys}H105^{Tyr}$, $^{Cys}H105^{Ser}$, $^{Val}H108^{Ser}$ and $^{Val}H108^{Arg}$), and were undetectable in another four mutants ($^{Cys}H105^{Arg}$, $^{Asp}H106^{Tyr}$, $^{Asp}H106^{Ser}$ and $^{Asp}H106^{Arg}$). However, the OD value was improved in the $^{Val}H108^{Tyr}$ mutant. $^{Val}H108^{Tyr}$ mutant reacted exclusively with IBV as well as ZL.80, whereas neither of them reacted to Newcastle disease virus (NDV) or bovine serum albumin (BSA) (Figure 5).

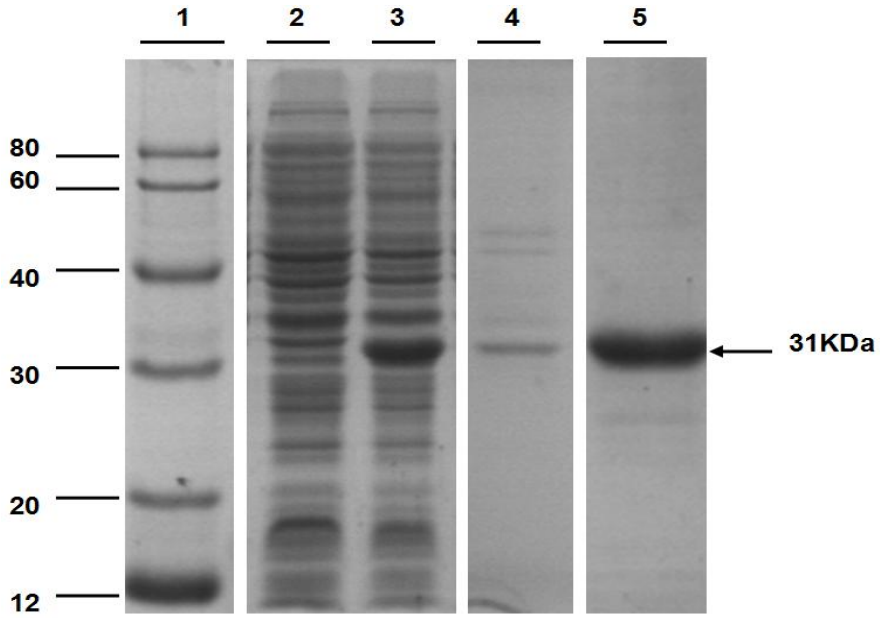

Figure 2. SDS-PAGE analysis of E.coli co-transfected with pOPE101-xp-ZL.80. Lane 1, molecular mass markers; lanes 2 and 3, analysis of total bacterial cell lysates after 3 h of shaking with and without induction with IPTG; lane 4, analysis of periplasmic extraction after 3h of shaking with induction with IPTG; lane 5, purified ZL.80.

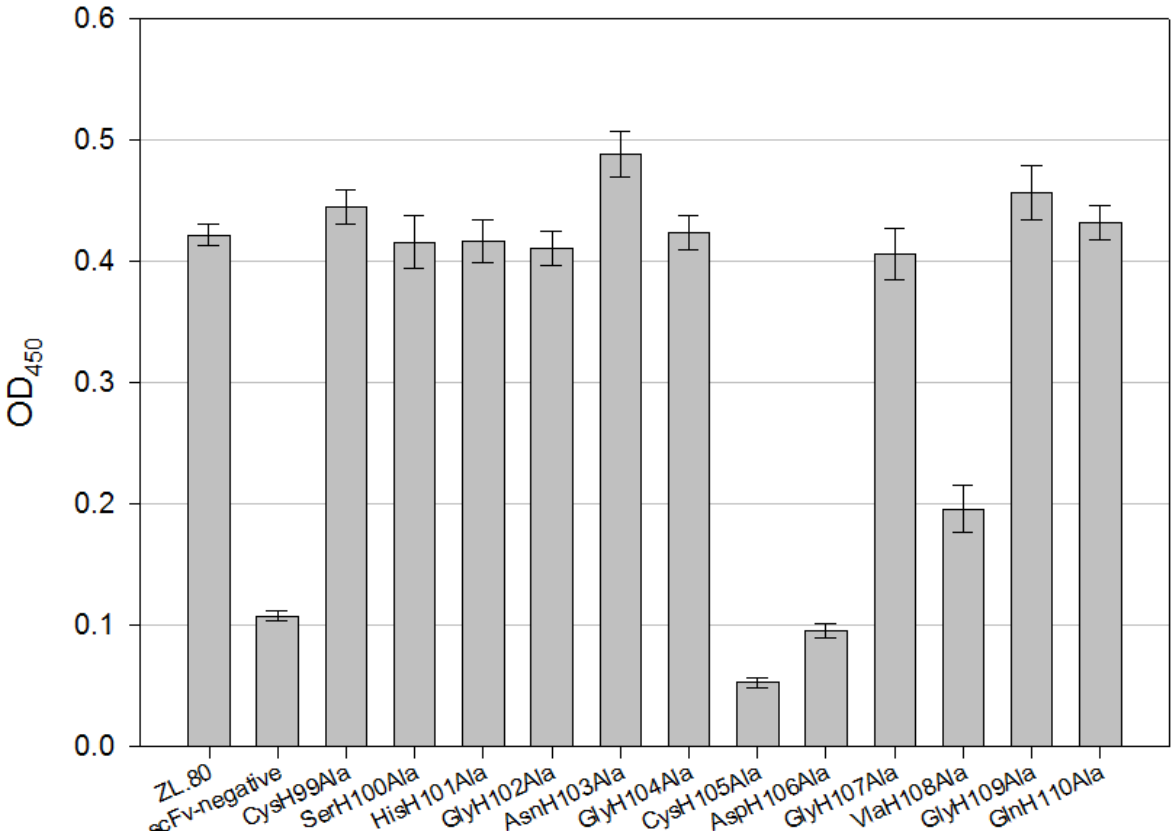

Figure 3. ELISA analysis of alaine mutant scFvs. Periplasm extraction of alaine mutants substituted from H99 to H110 in CDR-H3 of ZL.80 were tested their reaction to IBV in 96-well plate using indirect ELISA method. ZL.80 is a positive control and scFv-negative is a negative control. The data represent the average of three experiments, with the standard deviation indicated by error bars.

	CDR1	CDR2	CDR3
GL	SYDML	GIDNTG-GYTHYGAAVKG	RTAG----------SIDA
ZL.80	SYAMN	QIIMDG-VGTGYGPAVDG	CSHG-----NGCDGVGQIDA
ZL.10	SHGMG	AISSTG-TYTNYGAAVKG	SAGG-------RWYPYNIDA
84/85-S-1	SHGMQ	SISYSG-RYTGYGAAVKG	SACA------HSYESGCIDA
84/85-S-9	SYGMH	YISYTG-SYTYYGSAVKG	RGSA-----CYNGYTGCIDA
84/85-F-S	NNGMM	GISRTG-RYTSYGAAVKG	AADV---DGSYSYRAGTVDA
Amb.a.1-6	SYAMN	GIDSVGSNRTYYGAAVKG	SDYG--YSSSGRWYGGRIDA
Fel.d.1-10	DYAMG	GISGNTGRYTNYGSAVKG	SATG---YGYVVHIPGYMDA
YJV-8	SYQMN	AINKFG-NSTGYGAAVKG	DVYAYCGSGSLCTGLGHIDA

Figure 4. Alignment of complementary determinant region of heavy chains. The amino acid sequences of scFv ZL.80 and ZL.10 were aligned with six scFvs, 84/85-S-1, 84/85-S-9, 84/85-F-S against the hapten fluorescein, and Amb.a.1-6, Fel.d.1-10, YJV-8 against native Amb a 1, recombinant Fel d 1, and whole yellow jacket venom (YJV) respectively. All these scFvs were obtained from immunized liabrary of chicken. GL is the germline gene of chicken and one the top. The complementary determinant regions are indicated. The increased Tyr (Y) and Arg (R) residues in CDR H2 or CDR H3 of each scFvs compared with GL were shadowed.

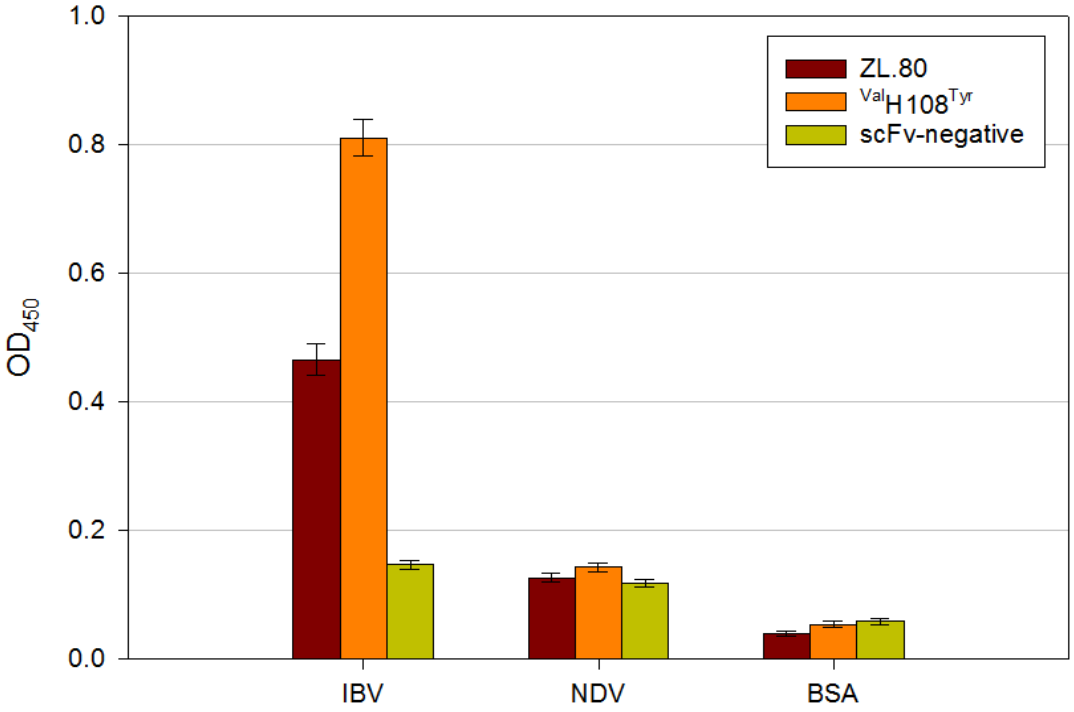

Figure 5. Specificity analysis of $^{Val}H108^{Tyr}$ mutant scFv. Purified $^{Val}H108^{Tyr}$ mutant scFv was tested the binding ability to IBV, NDV and BSA respectively by indirect ELISA method. Briefly, 96-well plate were coated with IBV (200 ng), NDV (200 ng) and BSA (200 ng) respectively overnight, 100µl mutant scFvs (500 µM) were then added and incubated at 37 °C for 2h. After scrubbing with PBST, myc-tag mouse mAb and HRP-conjugated goat anti-mouse IgG were added for detection, and colored reaction was performed using TMB. ZL.80 is a positive control and scFv-negative is a negative control. The data represent the average of three experiments, with the standard deviation indicated by error bars.

The affinity of the mutant scFvs that still bound to IBV in ELISAs were tested after purification, according to the method of Beatty et al. (1987). The affinity of the $^{Val}H108^{Tyr}$ mutant was increased approximately 12.9-fold compared with parental ZL.80, while those of the $^{Cys}H105^{Tyr}$, $^{Cys}H105^{Ser}$, $^{Val}H108^{Ser}$ and $^{Val}H108^{Arg}$ mutants

Table 2. The affinity constants of wild-type and mutant antibodies for IBV.

Mutant	K_{aff}, $M^{-1} \times 10^{-5}$ (relative affinity)
ZL.80	1.64 ± 0.39 (1.0)
ZL.10	17.55 ±1.35 (10.7)
^{Cys}H105^{Ala}	—
^{Cys}H105^{Tyr}	1.43 ± 0.26 (0.87)
^{Cys}H105^{Ser}	0.41 ±0.17 (0.25)
^{Cys}H105^{Arg}	—
^{Asp}H106^{Ala}	—
^{Asp}H106^{Tyr}	—
^{Asp}H106^{Ser}	—
^{Asp}H106^{Arg}	—
^{Val}H108^{Ala}	0.61 ± 0.13 (0.37)
^{Val}H108^{Tyr}	21.16 ± 1.87 (12.9)
^{Val}H108^{Ser}	1.38 ± 0.32 (0.84)
^{Val}H108^{Arg}	1.13 ± 0.28 (0.69)

Mutant scFvs in H105, H106 and H108 were purified, quantitated to 0.1~0.3mg/ml and equalized before determination of the Kaff using Beatty's method. ZL.80 and ZL.10 were positive control. ScFvs which failed in Kaff determination for their poor reaction in ELISA were marked with "—". The Kaff values were average value of three determinations and their relative affinity were calculated by comparing with ZL.80.

were decreased by about 1.1-fold, 4-fold, 1.2-fold and 1.5-fold, respectively (Table 2). These results regarding binding affinity are consistent with the results of the ELISAs.

DISCUSSION

Antibodies obtained by phage display technology often have low affinities and thus require *in vitro* affinity maturation to improve their utilities for diagnosis, immunoprophylaxis and therapy (Lefrancois, 1984; Gould et al., 2005). The results showed that the affinity of single-chain variable fragments can be improved by a single amino acid substitution in the CDR H3 with no change in specificity, indicating that tyrosine could play an important role in antigen binding. These results may be helpful in identifying the relationship between antibody affinity and amino acid properties of antigen binding site, thus providing theory complements for the development of high-performance reagents of antibody.

All alanine mutants of ZL.80, from H99 to H105 in CDR H3, performed decreased affinity, which was consistent with the previous studies that mutant ^{Tyr}H32^{Ala} and ^{Phe}L94^{Ala} reduced the affinity of the epitope peptide by 250-fold and of the non homologous peptides by 10-fold, respectively (Winkler et al., 2000). A single-tyrosine replacement at ^{Val}H108 in CDR H3 improved the affinity of ZL.80 approximately 12.9-fold, while replacement of this site with other residues had no effect, confirming that tyrosine aids antigen binding in accord with the conclusion that tyrosine residues, combined with flexible Ser/Gly residues, are capable of mediating antigen recognition with both high affinity and specificity (Birtalan et al., 2008). In contrast, substitution of ^{Cys}H105 or ^{Asp}H106 with these residues all decreased the affinity of ZL.80. These results may reflect the importance of the Cys residue at H105 and the Asp residue at H106 for antigen binding. It is generally believed that Cys in CDR H3 can form loops by disulfide bonds and thus change the structure of the CDR H3 (Koti et al., 2010), while Asp and other charged residues contribute specificity to molecular recognition by establishing precise electrostatic and hydrogen-bonding interactions (Sheinerman et al., 2000; Sasamori et al., 2011). These findings suggest that this protocol may represent an effective strategy for improving the affinity of scFvs.

Of Tyr, Arg and Ser mutants, only mutant ^{Val}H108^{Tyr} had improved affinity by 12.9 fold. This result indicted that only few mutant possessed improved affinity after site-indirect mutagenesis, which was consistent with previous studies (Tachibana et al., 2004). Despite the effectiveness of site-directed mutagenesis, many obstacles in improving antibody affinity still exist. These include the difficulty of identifying the contact residues suitable for mutagenesis. Accurate high-resolution antigen-antibody structures determined by X-ray analysis or nuclear magnetic resonance are often not available (Padlan et al., 1989; Valjakka, 2002; Clark et al., 2009), and the prediction of contact residues by three- dimensional modeling or alanine mutagenesis may not be correct. In addition, Tyr may only be effective when combined with other residues,

making the results unpredictable.

In summary, our study demonstrates the feasibility of single-tyrosine replacement of functional residues in CDR H3 for improving the affinity of ZL.80. This demonstrates that in vitro site-directed mutagenesis can be used to improve antibody affinity without affecting specificity. The results also suggest that tyrosine in CDR H3 may make a significant contribution to antibody affinity. Further studies are required to determine if substitution of tyrosine, arginine or serine in other CDR regions could also improve the affinity of ZL.80 for IBV.

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