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Engineering recombinant chicken antibodies for improved characteristics

By

Joy Sixholo

A dissertation submitted in partial fulfillment of the requirements

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Declaration

I, Joy Sixholo, declare that this dissertation is my own work. It has been submitted to the University of Pretoria for the degree Master of Science. It has not been submitted before for any other degree or examination at any other university.

…….

Joy Sixholo

Date……..…….

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Abbreviations

AP	Alkaline phosphatase
Amp	Ampicillin
BCG	Bacille Calmette Guerin
BSA	Bovine serum albumin
CDR	Complementarity determining region
dNTP	2'-deoxynucleoside-5'-triphosphate
E. coli	*Escherichia coli*
ELISA	Enzyme linked immuno-sorbent assay
Fab	Fragment antigen binding
Fv	Fragment variable
$(Gly_4Ser)_3$	Linker with 12 glycine residues and three serine residues
His-tag	Histidine tag
HIV	Human immunodeficiency virus
IgY	Immunoglobulin class Y
íPr/Cl/C6	4-chloro-6-(isopropylamino)-1,3,5-triazine-2-(6-aminohexane-carboxylic acid)
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
kDa	Kilodalton
mAU	Milli-absorbance unit

M. tuberculosis	Mycobacterium tuberculosis
Ni-NTA	Nickel-nitrilotriacetic
PCR	Polymerase chain reaction
PSB	Protein sample buffer
RT	Room temperature
RU	Response/resonance units
scFv	Single-chain fragment variable
scFv-AP	scFv fused to alkaline phosphatase
sdAb	Single domain antibodies
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sL	Short-linker
SOE	Splicing by overlap extension
SPR	Surface plasmon resonance
TB	Tuberculosis
Tm	Melting temperature
V_H	Variable heavy chain
V_L	Variable light chain
WHO	World Health Organization

Amino acids

A	Alanine
R	Arginine
N	Asparagine
D	Aspartic acid
C	Cystein
E	Glutamic acid
Q	Glutamine
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine

F	Phenylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
Y	Tyrosine
V	Valine

Nucleotide bases

A	Adenine
T	Thymine
G	Guanine
C	Cytosine

Summary

Engineering recombinant chicken antibodies for improved characteristics

by

Joy Sixholo

Promoter : Dr. Jeanni Fehrsen

Department : Department of Veterinary Tropical Diseases, University of Pretoria and New Generation Vaccines Programme, Onderstepoort Veterinary Institute, Onderstepoort, 0110
Republic of South Africa

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Phage libraries are a versatile source of recombinant antibody fragments directed against a wide variety of antigens. Recombinant antibodies have the advantage that they can be engineered to improve their binding or other characteristics. A chicken single chain variable fragment (scFv) phage library was panned against the 16 kDa antigen of *Mycobacterium tuberculosis*. Three phage displayed antibodies were obtained which bound specifically to the antigen. In soluble scFv format, however, they produced low ELISA signals. For this reason they were able to be used as models for antibody engineering. Three mutant sub-libraries were created by random mutagenesis. High stringency panning of the mutant sub-libraries against the target antigen yielded stronger binders which produced ELISA signals of up to eleven times higher than the parent scFvs. An increase in affinity was confirmed by surface plasmon resonance. One mutant scFv with a single amino acid exchange also showed an increase in the yield of scFvs it produced. Upon shortening the linker sequence between the heavy and light chains, size exclusion chromatography showed that multimerisation had occurred. Dimers, trimers and tetramers were formed thus increasing the avidity of the scFvs. Tetramers derived from the unmutated scFv showed the greatest improvement in ELISA binding. To improve expression and purification, an alternate bacterial expression vector with a histidine tag was investigated. For this series of experiments the coding region for a chicken scFv directed against VP7 of bluetongue

virus was transferred from the pHEN1 display vector to the pSANG 14-3F vector, which fuses the scFv gene to a bacterial alkaline phosphatase gene. A bi-functional chicken scFv-alkaline phosphatase fusion protein that exhibits both alkaline phosphatase activity and specific antigen binding was expressed in *Escherichia coli* and purified in a single step via metal affinity chromatography. Furthermore the scFv-AP fusion protein was directly detected in ELISA without the use of secondary detection reagents. This study confirms that the strategies used can efficiently enhance the characteristics of chicken scFvs.

Potential scientific publication

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Chapter 1

Literature review

1.1 Introduction

Phage display is a technology that allows for the display of proteins on the surface of filamentous phage such as M13, fd and f1. Foreign genes are incorporated into the filamentous phage genes that code for coat proteins e.g. gene pIII or gene pVIII (Smith, 1985). The result is a fusion protein that is displayed on the surface of the filamentous phage. Because the phenotype is coupled to the genotype, the displayed peptide can be identified and recovered (Smith, 1985). When this technique is applied to antibodies, the antigen binding site of antibodies can also be displayed by the filamentous phages (McCafferty *et al*., 1990). The displayed phage-bound antibodies have binding specificity and affinity that is similar to that of the parent antibody (Huston *et al*., 1988).

In mammalian antibodies the immunoglobulin (Ig) consists of two large heavy chains and two small light chains connected by disulphide bonds. There are five antibody isotypes known as IgD, IgE, IgG, made up of a single Ig unit (Figure 1.1); IgA (two Ig units) and IgM (five Ig units).

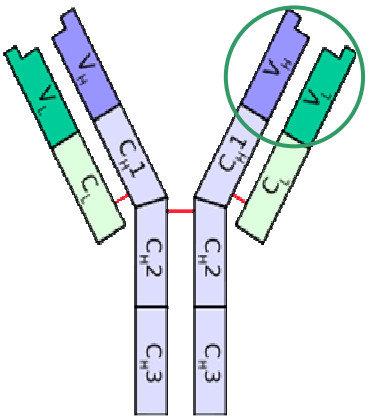


Figure 1.1 A schematic diagram of a mammalian antibody; L is the light chain, H is the heavy chain, C_H2 and C_H3 represent the effector region and V_H and V_L (circled) comprise the antigen binding site. The disulphide bonds are shown in red. The image was obtained from Wikimedia: http://commons.wikimedia.org/wiki/Image:Antibody.JPG

Avian species have a different type of antibody called IgY (Reviewed by Warr *et al*., 1995). IgY differs from IgG in that the heavy chain of IgG contains three constant region domains while IgY has four. Although the general structure of all antibodies is very similar, the region at the tip of the protein is extremely variable. This region is called the hypervariable region and it is here that the antigen binds. The antigen binding site of the antibody is formed from six complementarity determining regions (CDRs). The immune system consists of a diverse population of antibodies and each is distinguished by a unique set of CDRs that confer antigen specificity (Berek *et al*., 1985).

The single chain variable fragment (scFv) format is a small recombinant antibody in which the heavy chain variable region (V_H) and the light chain variable region (V_L) are covalently joined by a flexible linker of variable length (Bird *et al*., 1988; Huston *et al*., 1988; Holliger *et al*., 1996). In this review the word antibody and scFv will be used interchangeably.

The major advantages of using recombinant antibodies are that they can be rapidly and easily generated without relying on animal immunisation (Plückthun, 1991; Winter *et al*., 1994). Purification and identification tag sequences can be introduced with ease (Raats and Hof, 2005). In addition, recombinant antibodies can be produced in large amounts relatively cheaply in *Escherichia coli* (*E. coli*) to generate a continuous, consistent supply (Verma *et al*., 1998). Furthermore, recombinant antibodies can be engineered to improve their characteristics (Winter and Milstein, 1991; Chen *et al*., 1992; Gram 1992 *et al*., 1992; de Kruif *et al*., 1995; Short *et al*., 1995; Low *et al*., 1996). Several specificities can be combined in one antibody molecule to create bi- or tri-specific recombinant antibodies that would have been difficult or impossible to obtain from animals (Holliger *et al*., 1997; Hudson and Kortt, 1999).

Using phage display various host systems have been used to produce antibody libraries *in vitro* such as mice, humans, camels, llama, sheep and chickens (Marks *et al*., 1991; Hoogenboom and Winter, 1992; Davies *et al*., 1995; Engberg *et al*., 1996; Arbabi Ghahroudi *et al*., 1997; Charlton *et al*., 2000; Tanha *et al*., 2002; Sapats *et al*., 2003; van Wyngaardt *et al*., 2004). Antibodies against a wide range of antigens of medical and veterinary importance have been selected from these libraries and expressed in various expression systems (Daugherty *et al*., 2000; Kim *et al*., 2004; Kuepper *et al*., 2005; Sapats *et al*., 2005; van den Brink *et al*., 2005; Finlay *et al*., 2006; Johansson *et al*., 2007). ScFvs derived from vast combinatorial libraries are currently being used

extensively in research, imaging, immunotherapy, gene therapy and immunodiagnostics and represent over 30% of all biological proteins that have undergone clinical trials (Reviewed by Presta, 2003).

An ideal scFv for use in practical applications should have characteristics such as high affinity and specificity for the antigen, stability over long periods of storage, and adequate expression yields. However not all antibodies selected from a library will have these characteristics (O' Connell *et al*., 2002). Therefore antibody engineering methods can be applied to the genes coding for the antibodies to allow for the re-selection of fully functional antibodies with improved characteristics. Various methods can be used to improve the characteristics of antibodies namely, random mutagenesis (Gram *et al*., 1992, Short *et al*., 1995; Daugherty *et al*., 2000), directed mutagenesis (Miyazaki *et al*., 1999; Luginbühl *et al*., 2006), parsimonious mutagenesis (Balint and Larrick, 1993), chain shuffling (Finlay *et al*., 2006), the use of *E. coli* mutator strains (Irving *et al*., 1996), DNA shuffling by random fragmentation (Stemmer, 1994) or by a staggered extension process (Sheedy *et al*., 2006).

The avidity of antibodies can also be improved by increasing the number of binding sites available for antigen binding (Crothers and Metzger, 1972, Winter and Milstein, 1991). This is done by a process called multimerisation (Holliger *et al*., 1993; Kortt *et al*., 1997). Multimerisation of the scFv occurs when the linker between the V_H and V_L domains is shortened. This results in an increase in avidity of the scFv (Holliger *et al*., 1993). An alternative method of increasing the avidity of antibodies is by fusion of the recombinant antibody with a bacterial alkaline phosphatase (AP). This fusion results in a dimeric scFv-AP molecule when the enzyme dimerises to its active form (Wels *et al*., 1992; Ducancel *et al*., 1993; Weiss *et al*., 1994). The scFv-AP fusion also allows for direct detection of antigen in ELISA thus eliminating the need for secondary monoclonal reagents.

1.2 Phage display technology

Phage display is a method that was first used in the display of foreign peptide fragments on the surface of M13 filamentous phage particles by insertion of the peptide encoding gene into the gene encoding phage coat proteins (Smith, 1985). M13 is a non-lytic filamentous bacteriophage.

It is cylindrical in shape, 900nm long and 6-9nm in diameter. A narrow (~20nm) protein coat encapsulates a single-stranded DNA genome which is ~6.4 kilobases in length (Cabilly, 1999). The phage genome is composed of eleven genes, five of which are coat proteins. The major coat protein is called pVIII and it is responsible for covering the phage DNA. The proteins pVII and pIX cover the distal ends of the phage particle while pVIII and pIII cover the proximal ends. The minor coat protein pIII is encoded by gene III. It is composed of an amino-terminal and carboxyl domain (Smith and Petrenko, 1997). During bacterial infection the phage protein pIII binds to the F-pilus of F^+ *E. coli* (bacteria that contain the F-plasmid) through its amino-terminal end. The carboxyl terminal half of the protein remains within the virion and is involved in morphogenesis. A foreign sequence can be inserted between these two domains without disrupting the function of pIII, allowing its protein to be displayed on the surface of the phage particle when phage proteins are produced (Smith, 1985).

Phage display represents a cheaper, rapid method for the production of monoclonal antibodies *in vitro*. The fundamental advantage is the linkage of genotype to phenotype which allows for the identification and recovery of the antibodies (Smith, 1985). Also, because the phage-bound antibodies are amplified in *E. coli* during consecutive rounds of panning, the desired antibodies against an antigen of interest can be enriched overnight while hybridoma technology uses eukaryotic cells with rates of reproduction that can take months (Kohler and Milstein, 1975). Hybridoma technology relies heavily on the use of mice as experimental animals but phage display technology allows the use of any animal in addition to the mouse to generate antibodies either spontaneously (Schmitz *et al*., 2000) or by immunisation (Clarkson *et al*., 1991). Billions of antibody clones can be isolated from phage libraries as opposed to thousands in hybridoma technology.

The phage-bound antibodies can be displayed in various formats such as variable fragment (Fv), fragment antigen binding (Fab) and in scFv format. Monovalent display of antibodies is attractive as it allows for the isolation of a single antibody with high affinity. This is because the monovalent display allows for a clear discrimination between antibodies with different affinities to the antigen (O'Connell *et al*., 2002). Monovalent display is achieved by using phage derived plasmids called phagemids (O'Connell *et al*., 2002). Phagemid vectors contain both M13 phage and plasmid origins of replication, as a result they replicate as plasmids in *E. coli.* They also encode phage coat proteins with an N-terminal signal peptide and antibody resistance. The

antibody fragment is cloned upstream of the gene III coat protein sequence. By inserting an amber stop codon between the antibody gene and gene III in the phagemid**,** when phage is grown in a supE suppressor strain of *E. coli* such as TG1 the amber codon is read as glutamine and the antibody fused to pIII is displayed on the surface of the phage (McCafferty *et al*., 1990). When the phage is grown in a non-suppressor strain like HB2151 the amber codon is read as a stop codon, and soluble protein is secreted from the bacteria (Marks *et al*., 1992a). A further advantage of using phagemids is that isopropyl-β-D-thiogalactopyranoside (IPTG) induction of soluble scFv occurs alongside the production of pIII-scFv in supE strains, thus for simple ELISA screening soluble scFv can be produced in TG1 (Hoogenboom, 1996). Unlike in phage vectors where only pIII-scFv fusions are displayed per phage, in phagemid vectors there is a mixture of wild type pIII and pIII-scFv fusions displayed on the phage resulting in low level or monovalent display. These vectors owe their popularity to their high transformation efficiencies as a result of their small size thus enabling the construction of large repertoires (Hoogenboom *et al*., 1991). However, phagemids need the assistance of "helper phage" such as M13KO7 or VCSM13 in order to produce infective phage particles (Reviewed by Carmen and Jermutus, 2002).

Phage display libraries can be produced from different sources including mice, humans, camels, sheep and chickens (Marks *et al*., 1991; Hoogenboom and Winter, 1992; Davies *et al*., 1995; Engberg *et al*., 1996; Arbabi Ghahroudi *et al*., 1997; Sapats *et al*., 2003; van Wyngaardt *et al*., 2004). The chicken is a unique source of antibodies because it is easier to generate a large library from a chicken than other animals. This is due to inherent mechanisms that lead to antibody diversity in chickens (Reynaud *et al.,* 1985, 1987, 1989). Chickens have a single functional heavy and light chain variable region. Diversity is generated by immunoglobulin variable (V), diversity (D) and joining (J) gene rearrangements and gene conversion using non-functional pseudogenes. These pseudogenes are located upstream of the functional genes and they lack elements such as promoter regions. Pseudogenes contain highly conserved regions similar to the functional genes (Reynaud *et al.,* 1985, 1987, 1989; McCormarck *et al*., 1993). As a result chicken V regions have identical amino acid sequences at their termini allowing for the use of only one PCR primer set per heavy and light chain (Yamanaka *et al*., 1996; van Wyngaardt *et al*., 2004).

In order to create a phage display library the genes encoding the antibody variable genes can be derived from either non-immunised or immunised donors (Clackson *et al*., 1991). An antibody repertoire derived from an immunised donor is biased towards the generation of antigen specific

antibodies (Clarkson *et al*., 1991). Naïve libraries on the other hand, are generated *in vitro* using the genetic material of a donor that has never been exposed to the antigen (Marks *et al*., 1992a; Marks and Marks, 1996, Vaughan *et al*., 1996). Although naïve libraries have more diversity than immune ones, the antibodies selected from these libraries have been said to have lower affinities for their target antigens (Marks *et al*., 1991; Gram *et al*., 1992). However, they have become an important source of antibodies against toxic molecules and human antibodies because of ethical reasons associated with immunising humans (Hoogenboom and Winter, 1992; Marks *et al*., 1992a; Vaughan *et al*., 1996). Synthetic libraries are created by the *in vitro* rearrangement and assembly of the variable antibody domains and introducing random nucleotide sequences encoding CDRs of three to twelve residues in length (Nissim *et al*., 1994). The difference between synthetic and naïve libraries is that naïve libraries are made of natural CDRs while synthetic libraries are made of artificial CDRs (Carmen and Jermutus, 2002). This way the diversity of the naïve repertoire is increased by adding sequences that are not naturally available (Hoogenboom and Winter, 1992; de Kruif *et al*., 1995).

Using a method called panning the antigen specific antibodies are selected and enriched by infection into *E. coli.* There are variations of the panning process depending on the required end result e.g. subtractive panning and sequential antigen panning (Zhang *et al*., 2004a; Zhang *et al*., 2004b). A wide range of antigens can be also used (Winter *et al*., 1994). By changing the concentration of the coating antigen, the washing solution, the number of washes and the washing temperature, the panning can be directed towards the selection of the binders with the highest affinity to the antigen (Irving *et al*., 2001). Low stringency conditions are said to result in the enrichment of scFvs that express well, whereas high stringency conditions favour the selection of high affinity binders (Adams and Schier, 1999). The obtained binders can then be tested in enzyme linked immunosorbent assay (ELISA), western blot, flow cytometry or surface plasmon resonance (SPR) for their binding efficiency. Once the antigen specific antibodies have been isolated, the antibody genes can be cloned into an expression vector and expressed in bacteria. For higher yields the antibodies can be expressed in yeast, plant (Reviewed by Verma *et al*., 1998) or mammalian systems (Dorai *et al*., 1994).

The application of phage display has had a significant impact in medical research and in recent times there has been an increase in its use in veterinary research. The panning of libraries has yielded antibodies against proteins (Yamanaka *et al*., 1996), haptens (Andris-Widhopf, 2000) low

molecular mass toxins (Finlay *et al*., 2006) and viruses (Sapats *et al*., 2003, van Wyngaardt *et al*., 2004, Sapats *et al*., 2006).

1.3 Antibody engineering

ScFvs derived from phage antibody libraries are usually adequate for use in ELISAs, western blots and other immunoassays for research applications, but they sometimes have low affinity, specificity, stability or expression yields (Glockshuber *et al*., 1990; Arbabi Ghahroudi *et al*., 1997). Also, because of their monovalent nature they are usually small in size and are rapidly eliminated from circulation making them unsuitable for use as sensitive diagnostic reagents, cancer targets or as therapeutics (Adams and Schier, 1999; Greunke *et al*., 2006). Another challenge associated with the production of scFvs is the difficulty of obtaining soluble protein. Because of these reasons it thus became essential to find ways to improve these antibodies.

The combination of phage display and antibody engineering thus enables the selection of antibodies against any antigen of choice, manipulation of their physical and chemical properties, and re-selection of high affinity, fully functional antibodies. Changes are targeted at those attributes of the antibody that will improve its performance for a particular application. For instance, the molecular mass and amino acid sequences can be changed in order to influence the binding affinity, specificity, avidity, stability or expression of scFvs. Various procedures for affinity maturation (improvement of binding affinity) of antibodies have been described. These include targeted and non-targeted random mutagenesis by error pone PCR or *E. coli* mutator strains, directed mutagenesis and chain shuffling.

Affinity maturation can be by-passed by forming multivalent molecules thereby increasing the avidity (functional affinity) of the scFv. This can be done by shortening the length of the linker that joins the variable domains of the scFv or fusing the scFv to bacterial alkaline phosphatase. Multimerisation has been shown to have an influence in properties of the scFv like avidity, expression levels, solubility and stability of scFvs (Tang *et al*., 1996; Kortt *et al*., 1997).

1.3.1 Affinity maturation by PCR random mutagenesis

Affinity maturation by random mutagenesis mimics the process of somatic mutation thereby allowing for selection of antibodies with a higher affinity and specificity for the antigen when selective pressure is applied (Gram *et al*., 1992). Here parts of or the whole gene coding for scFvs can be mutated by PCR using a low fidelity, non-proofreading enzyme like *Taq* DNA polymerase (Cadwell and Joyce, 1992). It has been shown that randomly mutating the entire scFv gene sequence of murine antibodies by error-prone PCR can improve the affinity and stability of the scFv (Gram *et al*., 1992; Juárez-González *et al*., 2005). Saviranta *et al*, (1998) showed that PCR random mutagenesis over the entire V_H domain of an anti-hapten Fab can be used to decrease its cross reactivity to testosterone. Other authors however found that when they introduced random mutations to the entire variable heavy region of an anti-p-azophenylarsonate Fab the majority lost their ability to be expressed and had cross reactivity with structurally similar haptens (Casson and Manser, 1995). It is worth noting that the random mutagenesis frequency has to be controlled in order for it to be successful. Although high affinity binders have been selected from a library with an average mutation frequency of 22 mutations per gene, only 0.17% of the antibody clones from that library maintained antigen binding capability (Daugherty *et al*., 2000).

The major disadvantages of this method is the possibility of the emergence of unwanted cross reactivity (Casson and Manser, 1995), nonsense mutations, frameshifts, non-viable sequences and incorrectly folded antibody variants. However a large library in excess of 10^6 clones is usually adequate to overcome this shortcoming (Martinez *et al*., 1996; Christians *et al*., 1999; Zaccolo and Gherardi, 1999). In order to be more effective, random mutagenesis is often followed by the application of selective pressure during the panning process. This can be done by decreasing the coating concentration of the antigen (Juárez-González *et al*., 2005), competitive panning (Saviranta *et al*., 1998), increasing the stringency of washing by either increasing the number of washes, detergent concentration or washing temperatures. In this way only the clones with the highest affinity will be selected.

In vivo, the diversity of the antibody repertoire arises from the combinatorial events of V, D, and J gene segments, somatic hypermutation and selection of high affinity clones. Somatic mutation is driven by antigen stimulation and produces antibodies with increased affinity by optimizing the antibody binding site for its antigen (Yin *et al*., 2003). Most of these hypermutational events are

said to occur in nucleotide sequences called "hot spots" or AGY/RGYW motifs i.e. highly mutable codons which favour affinity maturation (Jolly *et al*., 1996). These are usually located in the six CDRs that shape the antigen binding sites especially the heavy chain CDR1 (Jolly *et al*., 1996; Ho *et al*., 2005). An interesting finding was that although the mechanism of affinity maturation by PCR random mutagenesis is different from the *in vivo* processes, these hot spots were observed when an anti-hapten scFv was affinity matured by error-prone PCR (Gram *et al*., 1992). However mutations in the framework regions flanking the CDRs have also been known to influence binding (Jolly *et al*., 1996), as these sequences may be involved in maintaining the structure, folding, or stability of the scFv which in turn have an influence in antigen binding (David *et al*., 2007).

1.3.2 Affinity maturation using *E. coli* mutator strains

Affinity maturation by random mutagenesis using *E. coli* mutD5 mutator cells is a very rapid and efficient alternative to the method described above. This approach consists of the isolation of phage-antibodies by affinity selection and mutagenesis through infection into the mutator *E. coli*, amplification and reselection of affinity enhanced mutants (Irving *et al*., 1996; Coia *et al*., 2001). The mutD5 mutator strain has a high frequency rate of single base substitutions compared to normal *E. coli* cells (Low *et al*., 1996). This is because the mutD5 strain's error-prone DNA polymerase III has a defective ε-subunit which catalyses the 3' to 5' exonuclease activity (Scheuermann and Echols, 1984).

A one hundred-fold increase in affinity was obtained for an anti-2-phenyl-5-oxazolone human scFv after multiple rounds of growth in mutator cells and stringent selection (Low *et al*., 1996). The apparent affinity of an anti-glycoporin scFv with mutations in the light chain framework region close to the light chain CDR3 increased ten thousand times after mutator strain mutagenesis (Irving *et al*., 1996). A ten-fold increase in the expression levels of a Hepatitis B specific scFv was observed after mutator strain mutagenesis although there was no apparent increase in affinity (Coia *et al*., 1997). An advantage of the mutator cell system is that there is no re-cloning necessary after selection of the phage bound antibodies (Irving *et al*., 1996). The major disadvantages are that repetitive rounds of mutation are required before high affinity mutants can be identified (Sheedy *et al*., 2007) and constant monitoring of the ratios of scFv expressers to non-expressers (Irving *et al*., 1996). Also mutations of the vector sequences other than the

antibody gene may occur resulting in loss of either antibiotic resistance or the ability of the vector to express protein (Irving *et al*., 1996).

1.3.3 Affinity maturation by site-directed mutagenesis

A more difficult and involved approach to affinity maturation is that of directed mutagenesis where selected parts or individual residues of the gene encoding the antibody are selected for mutation. When mutating antibodies *in vitro* via directed mutagenesis, decisions about where and how to introduce mutations into the antibody variable domains are of importance. Antibodies with improved affinity and specificity can be generated by gaining information on the molecular structure of the antibody-antigen interactions and increasing the number of favourable interactions (Doná *et al*., 2007, Birtalan *et al*., 2008). Information from the natural somatic hypermutation mechanisms can also be used as a guide for targeting positions that have a high mutational frequency and more likely to generate improved affinity (Hoogenboom and Chames, 2000).

A Fab against human interleukin-1 with two amino acid substitutions in the heavy chain CDR3 was found to have a ten-fold increase in affinity after site directed mutagenesis (Jackson *et al*., 1995). A forty-fold increase in affinity was obtained after the directed mutagenesis of the light and heavy chain CDR3 regions of an anti-testosterone Fab. Among the observed mutations only one amino acid exchange from glutamine^{95} to alanine in the heavy chain CDR3 had direct contact with the antigen while eight other mutations in the light chain CDR1 had no direct contact with the testosterone. Crystal structure analysis showed that the light chain CDR1 mutations caused a rearrangement in the antibody conformation thus reshaping the binding site, proving that CDR regions that are not in direct contact with the antigen can have a positive effect on binding (Valjakka *et al*., 2002). By combining selected mutations that were known to increase affinity in four CDRs of an anti-HIV gp120 Fab the affinity was increased four hundred and twenty-fold (Yang *et al*., 1995). A similar strategy of combining different mutations from individual CDR libraries was applied by Hemminki *et al*, (1998) and an antibody clone with a twelve-fold increase in affinity and reduced cross reactivity was obtained. A study showed that out of forty six mutant antibodies containing point mutations in the heavy chain CDR2 all had diminished affinity and specificity for the antigen suggesting that the residues at these positions could possibly be essential for antigen binding (Chen *et al*., 1992). Another study used site directed

mutagenesis to investigate the molecular basis of the specificity of an antibody specific for 4-chloro-6-(isopropylamino)-1,3,5-triazine-2-(6-aminohexane-carboxylic acid) (iPr/Cl/C6) and to improve its affinity. The study revealed interesting findings that certain amino acid residues at specific positions can improve, reduce or totally diminish the affinity of the anti-hapten antibody (Kusharyoto *et al*., 2002).

The combination of site specific mutagenesis and random mutagenesis was used to change the specificity of a monoclonal antibody against 11-deoxycortisol to a new specificity for cortisol. This was done by introducing mutations at fourteen amino acid positions in the three heavy chain CDRs that form the steroid binding pocket followed by random mutagenesis in the entire heavy domain (Miyazaki *et al*., 1999). Antibodies with fifteen to fifty five-fold improved affinities were obtained from a mutant scFv library by randomising three CDR codons in the AGY/RGYW motifs, while only one antibody clone with four-fold improvement in affinity was obtained by randomly mutating CDR non-hotspots (Chowdhury and Pastan, 1999). An anti-parathyroid hormone single domain antibody (sdAb) with thirty-fold improvement in affinity was selected from a library constructed by randomising nine heavy chain CDR2 and CDR3 hotspot codons (Yau *et al*., 2005).

1.3.4 Affinity maturation by chain shuffling

Chain shuffling offers an efficient alternative strategy to random mutation. This method is achieved by mixing fragments of the heavy and the light chain variable regions of an antibody (Marks *et al*., 1992b). Just one V domain is altered at a time. In light chain shuffling the V_H of the original scFv against an antigen of choice is recombined with a diverse set of naïve V_L regions. The resulting library contains antigen specific V_H and random V_L chains. The opposite is true for heavy chain shuffling. Panning is then carried out to select antibodies with improved characteristics. Chain shuffling relies largely on the promiscuous pairing (same V gene is found with a number of different partners) of V domains to create new functional Fv domains (Kang *et al*., 1991).

Chain shuffling allows for the generation of panels of antibodies with related specificity but unique CDR residues (Kang *et al*., 1991). Using this methodology an increase in the affinities of anti-2-phenyloxazol-5-one (Marks *et al*, 1992b) and anti-hepatitis B virus (Park *et al*., 2000)

antibodies was obtained after their natural light and heavy chains were shuffled with light and heavy chains from non-immunised donors. In addition to the attainment of affinity matured variants, the humanization of a mouse monoclonal antibody against N. glycol GM3 and the improvement of its expression in bacterial cells was attained using this method (Rojas *et al*., 2004). The disadvantage of chain shuffling is that pre-isolation of an antigen specific antibody is mandatory thus naïve libraries cannot be used alone (Park *et al*., 2000).

1.3.5 Improving the avidity of scFvs by multimer formation

A typical scFv is made up of the V_H and V_L domains of an antibody joined by a peptide linker e.g. a fifteen amino acid $(Gly_4Ser)_3$ linker. This type of linker is the most commonly used and its glycine residues give flexibility to the linker while the serine residues allow for solubility (Bird *et al.*, 1988; Huston *et al*., 1988). In this form, the scFv is usually found to be monomeric (Hudson and Kortt, 1999). This mono-valency is due to linker flexibility. Multimerisation of the scFv occurs when the linker length is shortened. As the linker is shortened to less than twelve residues the natural Fv orientation cannot be maintained and the V_H and V_L domains cannot associate with each other. This causes association or pairing of complementary V_H and V_L from two adjacent scFvs thus forming a bivalent molecule called a diabody (Holliger *et al*., 1993). Holliger *et al*, (1993) constructed a bivalent scFv by shortening the linker peptide to five amino acid residues.

The x-ray crystal structure of a diabody with a five residue linker showed that when the linker is further reduced to one or two residues the diabody association is strained and can no longer be maintained (Perisic *et al*., 1994). This results in further interactions between three or four scFvs, forming trimers and tetramers which are called triabodies and tetrabodies (Kortt *et al*., 1997). When the linker of an anti-neuraminidase antibody NC10 was shortened to three or four glycine residues there was a strict transition to diabodies, whereas a linker length of one or two residues formed exclusively trimers (Atwell *et al*., 1999). An anti-human B cell antigen CD19 scFv with a single amino acid linker was found to exclusively form tetramers (Le Gall *et al*., 1999). An scFv without a linker was constructed by directly joining V_H to V_L to form stable trimers which still had active antigen binding sites (Iliades *et al*., 1997; Le Gall *et al*., 1999).

Different patterns of oligomerisation were observed when the scFv domain orientation was changed from V_H-V_L to V_L-V_H (Dolezal *et al*., 2000). An scFv without a linker was found to be

more stable when the domain orientation was inverted to V_L-V_H (Arndt *et al*., 2004). It has also been shown that inversion of the Fv domain of the scFv from V_H-V_L to V_L-V_H can destroy antigen binding to the antigen and also minimize the amount of protein expressed (Albrecht *et al.*, 2006).

It is also possible to reconstitute scFvs into full IgG constructs in order to increase avidity. Vectors were generated for the generation of bivalent IgY constructs. Using these vectors chicken scFvs can be reassembled into a homodimeric or heterotetrameric IgY antibody (Greunke *et al*., 2006).

1.3.6 scFv-alkaline phosphatase fusion proteins

An alternative method of creating multivalent scFvs can be by genetically fusing the scFv with bacterial alkaline phosphatase to obtain scFv-alkaline phosphatase fusion proteins. Genetically fused scFv-enzyme conjugates have several advantages over their chemically coupled counterparts. They can be produced rapidly and in a controlled way. Also, because they are mono-substituted they show better assay performance compared to that of multiply substituted conjugates generated by chemical coupling (Kershbaumer *et al*., 1996).

A number of expression vectors have been developed for cloning and production of scFv-AP fusion proteins i.e. pDAP2 (Kerschbaumer *et al*., 1996), pScFV(NP)AP (Suzuki *et al*., 1997), pEL-DBAP (Furuta *et al*., 1998), pSKAP/S (Griep *et al*., 1999), pLIP6v (Muller *et al*., 1999), and pSANG 14-3F (Martin *et al*., 2006). All the vectors link the scFv to the N-terminus of AP. All authors reported production of high amounts of soluble protein, and all but Muller used a hexa-histidine-tag (6x His-tag) for protein purification.

The binding of an scFv to its antigen is usually visualized by detection of anti-mouse HRP to a secondary antibody e.g. 9E10 which recognises the c-myc peptide tag fused to the scFv. The scFv-AP fusion allows direct detection of scFv binding with the antigen thus eliminating the need for secondary reagents in ELISA. This has an obvious cost and time advantage as fewer reagents are used.

The vector pSANG 14-3F was used in this study (Figure 1.2). It was constructed on a pET26 backbone and protein expression is driven by a T7 lac promoter (Martin *et al*., 2006). The vector allows for the fusion of the scFv to AP. The resultant scFv-AP construct is a dimeric molecule with increased avidity. The vector contains an N-terminal *pelB* leader sequence which directs scFvs to the periplasmic space. In addition to that it contains a 6x His-tag for purification using metal affinity chromatography and a tri-FLAG tag as an additional tag.

Figure 1.2 A schematic diagram representing the pSANG 14-3F vector. Protein expression is driven by a T7 promoter. The scFv is inserted into the *NcoI* and *NotI* restriction sites. The vector adds a *pelB* leader sequence and alkaline phosphatase to the scFv. The 6x-His and tri-FLAG tags allow for purification of the fusion protein. The image was obtained from Martin *et al*., 2006.

1.4 ScFvs against targets of medical and veterinary importance

Recombinant antibody fragments directed against proteins of medical and veterinary importance have been selected from large combinatorial libraries (Nissim *et al*., 1994; Vaughan *et al*., 1996; van Wyngaardt *et al*., 2004). These may be useful for a number of applications including laboratory diagnosis, antigenic characterisation and immunotherapy. The recombinant antibodies that were used in this study were obtained from the *Nkuku*® library (van Wyngaardt *et al*., 2004). The scFvs B4, B8, D4 and their mutants are directed against the 16 kDa antigen of *Mycobacterium tuberculosis* (*M. tuberculosis*) and scFv F10 (Genbank AY631243) is directed against VP7 of bluetongue virus (Fehrsen *et al*., 2005). The anti-16 kDa and anti-VP7 scFvs are potentially useful reagents in the detection of antigens for research and diagnostic purposes. They can be used in a variety of assays including ELISA, SPR, flow cytometry and immunochromatographic tests. This section will give a brief outline of the importance of these antigens.

1.4.1 Tuberculosis

The effective and timely diagnosis of tuberculosis (TB) is one of the major challenges of public health especially in developing countries. The World Health Organization (WHO) reported that annually there are eight to ten million cases of new active TB and three million fatalities worldwide (WHO, 2006). The incidence of disease has increased over the past 20 years mainly due to its association with human immunodeficiency virus (HIV) and the occurrence of multi-drug resistant strains (WHO, 2008). This points to the need for new, more reliable and rapid tests for TB in order to fight this disease. In fact, improved new diagnostics for TB are listed as one of the six elements of the Global Plan to Stop TB (WHO, 2006).

TB is primarily an illness of the respiratory tract, but it can also occur in the kidney, spine and brain. It is caused by a bacterium called *Mycobacterium tuberculosis*. Mycobacterium was first viewed under the microscope by Robert Koch in 1882. *M. tuberculosis* is a large, non-motile, acid-fast gram positive rod-shaped bacillus that lacks an outer cell membrane. It is an obligate aerobe and a facultative intracellular parasite. Tuberculosis occurs in humans and animals and the progression of the disease has been well documented (Reviewed by Cunningham and Spreadbury, 1998).

The tests currently used for diagnosis of TB are chest radiography, sputum smear microscopy, mycobacterial culture, PCR, serological detection, phage based detection, mantoux test and cytokine detection (Albay *et al*., 2003). Direct smears, although easy to perform, have been found to be relatively insensitive, while culture techniques currently in use are both time consuming and expensive (Raja *et al*., 2008). Because of the extensive variation in antibody response in individuals, serodiagnostic tests like ELISA have been found to be unreliable (Uma Devi *et al*., 2003). Despite the continuous development of new diagnostic tests, most of these have met with problems either of specificity or cost.

Certain mycobacterial antigens have been identified as crucial in the serodiagnosis of tuberculosis. Many studies have reported on the use of three immunodominant mycobacterial antigens namely the 38, 30 and 16 kDa antigens (Verbon *et al*., 1993; Lim *et al*., 1999; Hendrickson *et al*., 2000; Raja *et al*., 2002; 2006). The 38 kDa antigen is a phosphate binding protein that acts as a receptor in active transport (Chang *et al*., 1996). This antigen contains species specific epitopes, is secreted in the early phase of infection and makes up the main

secretory constituent of the mycobacterial culture fluid which makes it an ideal candidate for serodiagnosis (Raja *et al*., 2008). The 30 kDa antigen is suitable to be used in early diagnosis as it is found in culture within the first three days (Uma Devi *et al*., 2003).

The 16 kDa antigen is a member of the α-crystallin super-family of small heat shock proteins whose primary function is to act as a molecular chaperone. This is based on its activity in preventing thermal denaturation of alcohol dehydrogenase (Yuan *et al*., 1998) and aggregation of citrate synthase *in vitro* (Chang *et al*., 1996). Various names have been used for this protein such as Hsp 16, Hsp.16.3, Acr and MPT63 (Chang *et al*., 1996; Yuan *et al*., 1998; Goulding *et al*., 2002). It has been found to occur as oligomers consisting of trimers making up a nine-subunit complex (Chang *et al*., 1996). The 16 kDa antigen is expressed during the transition from log to stationery phase of mycobacterial growth (Yuan *et al*., 1998) and accumulates as the dominant protein in the latent phase of infection. High levels of expression of the 16 kDa protein have been observed in microaerobic and anaerobic cultures suggesting that the protein may be used as a marker for the dormant state of *M. tuberculosis*. The results of a serological assay performed in 1988 showed that the greatest potential for this antigen was in the detection of infection in a population for which tuberculin testing is unreliable as the highest titres of antibody binding to this antigen were raised in patients who had been vaccinated with *Mycobacterium bovis* Bacille Calmette Guerin (BCG; Jackett *et al*., 1988). A new serological test for pulmonary TB with 91% sensitivity for known smear and culture positive samples was reported. This ELISA test uses four antigens in combination i.e. the 38, 30, 27 and 16 kDa. The results show that serodiagnostic assays for TB must be based on a rational combination of antigens and that the 16 kDa antigen is valuable when used in conjunction with the other antigens in the serodiagnosis of TB (Raja *et al*., 2008).

1.4.2 Bluetongue

Bluetongue is an arthropod borne disease of domestic and wild ruminants. It occurs mainly in sheep and occasionally in cattle and some species of deer (Reviewed by Roy, 1992). It is caused by a virus of the genus *Orbivirus* in the family *Reoviridae*. It is transmitted by the bite of the vector midge of the genus *Culicoides*. The virus can also be transmitted sexually in infected semen. Currently twenty four serotypes have been reported from all over the world (Reviewed by Schwartz-Cornil *et a*l., 2008). The disease is characterised by inflammation, haemorrhage, coronitis, laminitis, oedema of the head, neck and torticollis and cyanosis of the oronasal mucous

membranes (Reviewed by Verwoerd and Erusmus, 2004). Actually it is from the cyanosis of the tongue that the disease derives its name.

Bluetongue was first described in South Africa in the 19th century. The disease was first thought to be confined to the African continent until its emergence in Cyprus in 1924 (Polydorou, 1978). Outbreaks of the disease have subsequently been reported in the USA, Europe, Middle East, Australia and Asia (Reviewed by Verwoerd and Erusmus, 2004). Due to its economic impact bluetongue is an Office International des Eppizooties (OIE) listed disease. The economic losses associated with bluetongue disease are directly due to decreased productivity and death and indirectly due to the restriction of movement of sheep and cattle semen to bluetongue free countries and the costs of controlling the disease (Reviewed by Schwartz-Cornil *et al*., 2008).

The bluetongue virion has a double-layered capsid which encapsulates ten double stranded RNA segments. The inner core consists of two major proteins VP3, VP7 and three minor proteins VP1, VP4 and VP6. VP7 is a 38 kDa hydrophobic protein that plays an important role in maintaining the structural integrity of the viral core (Roy, 1992). It is the immunodominant, group reactive antigen and is highly conserved among all BTV serotypes hence it is used for BTV antibody detection (Huismans and Erasmus, 1981; Gumm and Newman, 1982). The protein can be expressed at high levels in recombinant Baculovirus and purified to 95% homogeneity (Oldfield *et al*., 1990). Assays used for the diagnosis of exposure to bluetongue virus are primarily based on detecting antibodies to a group-specific antigen. The agar gel immunodiffusion (AGID) test has been widely used because it is simple and rapid (Pearson *et al*., 1985). However this test is insensitive and gives cross-reactions with other orbiviruses (Chandel *et al*., 2003). This has led to the development of hybridoma derived monoclonal antibody based ELISAs (Oldfield *et al*., 1990; Afshar *et al*., 1991; 1992; 1993a; 1993b). More recently recombinant antibody technology has been used to make antibodies against BTV and its antigens. As mentioned earlier, recombinant antibodies have many advantages over hybridoma derived monoclonals. The potential of human (van Wyngaardt and Du Plessis, 1998), chicken (Fehrsen *et al*., 2005; van Wyngaardt *et al*., 2004), and mouse (Nagesha *et al*., 2001; Owens *et al*., 2004) derived scFvs as useful reagents in the serodiagnosis of BTV has been shown. The chicken anti-VP7 scFv F10 has been characterised and shown to be blocked by antisera to all twenty four bluetongue virus serotypes in an inhibition ELISA (Fehrsen *et al*., 2005). This scFv was used in this study to

investigate whether expression and purification of chicken scFvs can be improved by using an alternative expression vector.

1.5 Problem and hypothesis

Although large combinatorial phage display libraries are a good source of scFvs against antigens of medical and veterinary importance, sometimes scFvs with poor characteristics are obtained. These binders may be specific for the antigen, but their binding affinity, stability or expression levels may make them inadequate for use in any application. Antibody engineering methods like random mutagenesis, shortening the linker and constructing scFv-AP immuno-conjugates have been shown to be effective in the improvement of mouse and human scFvs. This study aims to explore whether these methods will also improve the characteristics of chicken scFvs. We thus hypothesize that engineering these chicken scFvs with poor characteristics by error-prone PCR and shortening or removing the linker will improve their specificity, affinity, avidity, stability or expression levels in *E. coli*. Furthermore we hypothesize that by genetically fusing the scFvs to the enzyme alkaline phosphatase and adding a histidine tag we can improve the avidity, expression and purification of the scFvs and be able to detect antigen by direct ELISA. The benefits arising from this study would be the establishment and application of antibody engineering methods to improve scFv characteristics. The information obtained will enable the compilation of a database of the amino acid residues identified to be essential in the improvement of antibody characteristics in order to design chicken scFvs with desirable properties in the future.

1.6 Aims and objectives

To investigate whether the characteristics of chicken scFvs can be improved by introducing random mutations to the genes encoding the scFvs by error-prone PCR.

To investigate whether the avidity of chicken scFvs can be improved by either shortening or removing the linker between the variable domains of the scFvs.

To investigate an alternative vector for improved expression and purification of scFvs and direct antigen detection.

Chapter 2

Affinity maturation of anti-16 kDa scFvs by PCR random mutagenesis

2.1 Introduction

A semi synthetic chicken scFv library (*Nkuku*®) with 2 x 10^9 clones (van Wyngaardt *et al*., 2004) was created at the Onderstepoort Veterinary Institute (OVI). To date panning this library has yielded scFvs against a wide variety of antigens of veterinary and medical importance. Most pannings yielded good quality antibodies, however sometimes poor binders were obtained. Unlike serum antibodies or traditional monoclonals, recombinant antibodies can be engineered using standard molecular biology methods in order to improve their characteristics. Error-prone PCR followed by a panning with high selection pressure is an efficient method for increasing both specificity and affinity of antibodies (Miyazaki *et al*., 1999). It uses a low-fidelity *Taq* DNA polymerase to introduce a low level of point mutations randomly over a gene sequence (Stemmer, 1994).

When the *Nkuku*® library was panned against the 16 kDa antigen of *M. tuberculosis* three binders (B4, B8 and D4) with high ELISA signals in phage format and weak in scFv format were obtained (Figure 2.1). Although a high ELISA signal may be obtained for phage bound antibodies the signal may diminish when the scFv is expressed as soluble protein. This is because of multiple binding of secondary antibody on the phage particle thus increasing the ELISA signal. The greater binding avidity due to multivalent display on phage may also play a role in the signal obtained (O'Connell *et al*., 2000). Thus soluble scFv gives a more accurate and sensitive assay. The weak binding anti-16 kDa scFvs were chosen to be used as models for antibody engineering by PCR random mutagenesis.

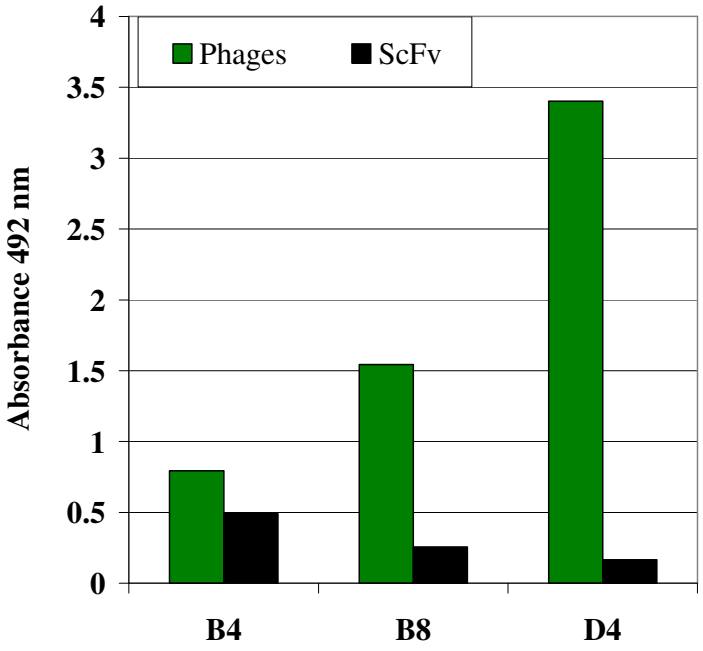


Figure 2.1 ELISA of the scFvs B4, B8 and D4 from the *Nkuku*® library in phage displayed and soluble scFv formats reacting to the 16 kDa antigen.

2.2 Materials and methods

2.2.1 Antigen and chicken derived scFvs

The purified 16 kDa antigen of *M. tuberculosis* was obtained from Vision Biotech (Pty) Ltd, Cape Town, South Africa. Instructions were given not to vortex or re-freeze the antigen. Once thawed the antigen was kept at 4°C. The three scFvs used in this study were selected from the OVI's *Nkuku*® library (van Wyngaardt *et al*., 2004). The 9E10 monoclonal antibody was provided by Mr. Wouter van Wyngaardt.

2.2.2 Mutant library construction

The first strategy was to attempt to improve the characteristics of the scFvs B4, B8 and D4 by introducing random mutations over the entire gene coding for these scFvs by error-prone PCR using the Diversify ™ PCR Random Mutagenesis kit as described by the manufacturer (BD Biosciences, California, USA). DNA (1 ng) of each scFv and 0.4 pmol/µl LCNOT1 and Sfi1L

(van Wyngaardt *et al*., 2004) primers were added to the PCR reaction. Conditions were used to obtain seven mutations per 1000 base pairs. The PCR products were digested with 40 U of both the enzymes *SfiI* (Roche Diagnostics, Mannheim, Germany) at 50ºC overnight followed by *NotI* (Roche) at 37ºC overnight. The digested products were purified with the QIAquick PCR kit (QIAGEN, Hilden, Germany). Primers Sfi1L and LCNOT1 (0.4 pmol/µl) were used to allow cloning into the *SfiI* and *NotI* sites of the phage display vector pHEN1 (1 µg) which was cut with 40 U of the same restriction enzymes and purified with the QIAquick PCR kit (van Wyngaardt *et al*., 2004). To remove the stuffer fragment the vector was further purified with a crystal violet gel (Rand, 1996). The genes were ligated into the vector with 1 U *T4 DNA Ligase* (Roche). The ligations were electroporated into *E. coli* TG1 and plated as described before (van Wyngaardt *et al*., 2004). Serial dilutions of 10^{-1}-10^{-4} were plated onto TYE agar (see Appendix) with 100 µg/ml Ampicillin (Amp) to determine the size of the library. The plates were incubated overnight at 30°C, thereafter the colonies were scraped off the plates with 2XTY (see Appendix). The bacterial stocks were stored in 15% glycerol at -70°C.

2.2.3 Sequencing

To determine for mutations phagemid DNA was isolated from single *E. coli* TG1 transformant colonies grown up in 5 ml 2XTY supplemented with 100 µg/ml Amp and 2% glucose (2XTY-AG) at 30°C, 240 rpm by using QIAprep Spin Miniprep plasmid purification kit (QIAGEN). Sequencing primers OP52 and M13rev were used (van Wyngaardt *et al*., 2004). Automated sequencing was done by the Molecular Biology Division, OVI. Sequences were analysed using the Staden and BioEdit software packages (Staden, 1994a; 1994b; 1994c; Hall, 1999)

2.2.4 Screening mutant libraries

The mutant libraries were panned using stringent conditions in order to select for scFvs which bound to the antigen with higher affinity than the parent scFvs. Panning was done as described previously with some modifications (van Wyngaardt *et al*., 2004). For the first two rounds of panning 20 µg/ml of 16 kDa *M. tuberculosis* antigen in PBS was used to coat the wells of a microtitre plate (Nunc, Maxisorp) and for the third round 2 µg/ml was used. For the first round the wells were washed 19 times with PBS containing 0.1% Tween-20 (Tween-PBS) (Merck,

Schardt, Germany) followed by one long wash of 20 minutes on a rocker thereafter 20 washes with PBS. For the second and third rounds the wells were washed 20 times with 0.5% Tween-PBS followed by 20 washes with PBS. The phages were eluted with 0.1 N HCl for all rounds. In order to determine input phage titres dilutions of 10^{-9}-10^{-11} phages in PBS were made. From each phage dilution 10 µl was added to 40 µl *E. coli* TG1 cells (OD_{600} 0.4-0.6) and 50 µl 2XTY. This was incubated at 37°C for 30 minutes, plated on TYE plates containing 100 µg/ml Amp and incubated at 30°C overnight. The eluted phages (output) were titred by plating ten-fold dilutions (10^{-1}-10^{-4}) and the rest plated on a 16 cm petri dish.

2.2.5 Production of scFvs

To produce scFvs, individual TG1 transformant colonies were inoculated into 5 ml 2XTY-AG and incubated overnight at 30ºC. A $^1/_{100}$ dilution was made in the same medium and grown until the OD_{600} was 0.9. The culture was centrifuged at 2000 x g for 10 minutes and the pellet resuspended in $^1/_5$ volume 2XTY with 100 µg/ml Amp and 1 mM IPTG to induce scFv production. The final induction volumes were 10ml or 2 ml and left shaking at 30ºC overnight. To remove bacteria the culture was spun at 2000 x g for 15 minutes. The supernatant containing the secreted antibodies was used directly in ELISAs. In some instances the scFvs were extracted from the periplasmic space by resuspending the cell pellet in $^1/_{10}$ volume of ice cold 1M NaCl and 1 mM EDTA in PBS and incubating it on ice for 30 minutes. The suspension was centrifuged at 4°C, 7500 x g for 10 minutes. The supernatant was transferred to a new tube and centrifuged again at 4°C, 16 000 x g for 10 minutes to remove cell debris and diluted $^1/_5$ in 2XTY before use. Alternatively, scFvs were affinity purified from the supernatant fluid using an anti-c-myc tag monoclonal antibody 9E10 by J. Frischmuth from the National Bioproducts Institute, Pinetown. The concentration of the purified scFvs was determined spectrophotometrically with OD_{280} of 1.0 corresponding to 0.7 mg/ml scFv and 1 mg/ml equal to 40 nM scFv. The secreted scFv and periplasmic fractions were stored at 4°C or in 2% sucrose solution at -20°C.

2.2.6 ELISA

The binding of the mutant scFvs to the antigen was characterised by ELISA and SPR. An immunoplate (Nunc, Maxisorp) was coated with 50 µl per well of 10 µg/ml of 16 kDa *M.*

tuberculosis antigen diluted in PBS at 4°C overnight. The plate was blocked with 300 µl per well of 2% bovine serum albumin (BSA) in PBS at 37°C for one hour followed by three washes with 0.05% Tween-PBS. The unpurified scFvs from the supernatant were diluted 1:1 with 4% BSA-PBS and 50 µl per well was incubated at 37°C for one hour followed by three washes. An anti-c-myc tag monoclonal antibody 9E10 in Dulbecco's Modified Eagles Medium (GIBCO, Grand Island, USA) diluted 1:1 in 4% BSA-PBS was added, incubated at 37°C for one hour and washed three times with 0.05% Tween-PBS. For detection a $^{1}/_{1000}$ dilution of polyclonal rabbit anti-mouse immunoglobulins-HRP (DakoCytomation, Ely, UK) in 2% BSA-PBS was added and incubated at 37°C for one hour and washed as in the previous step. After the final wash 50 µl of substrate made up of 1 mg/ml o-phenylene diamine and 0.5 µl/ml of 30% ($^{v}/_{v}$) H_2O_2 in 0.1 M citrate buffer (pH 4.5) was added and left to stand at room temperature (RT) for 40 minutes. The ELISA was stopped with 50 µl 2 N H_2SO_4 and the absorbance measured at 492 nm. All ELISAs in the study were done in duplicate.

In addition, an ELISA with a short incubation time of 10 minutes was used to determine how quickly the scFvs bind to the antigen. The scFvs were diluted 1:1 with 4% BSA-PBS, and incubated at 37°C for 10 minutes for the short ELISA and washed as above.

An ELISA with harsh washing conditions was also used to determine the strength with which the scFvs bind to the antigen. Here the scFvs diluted 1:1 with 4% BSA-PBS were incubated at 40°C for one hour and washed 10 times with 0.05% Tween-PBS at 40ºC, followed by 30 minutes incubation with 0.05% Tween-PBS at 40 ºC.

The scFvs were also stored under different conditions to determine their stability. They were stored at 4°C and -20°C for two weeks in culture medium containing 2% sucrose. The ELISA consisted of one hour incubations at 37°C followed by 3 washes with 0.05% Tween-PBS.

2.2.7 Surface plasmon resonance

SPR was used to compare the binding kinetics of the mutant scFvs on a Biacore X (Biacore, Uppsala, Sweden). Experiments were performed at 25°C using HBS-EP running buffer (see Appendix). Bound scFvs were removed with 10 µl of 0.1 M glycine, pH 2. The 16 kDa protein of

M. tuberculosis was covalently bound to the dextran surface of a CM5 chip via its primary amine groups (BIApplications handbook, Biacore). A volume of 35 µl of 16 kDa (50 µg/ml in 10 mM acetate buffer, pH 4) was injected and un-reacted ester groups were blocked with 1 M ethanolamine-HCL, pH 8.5. These conditions resulted in 3, 300 RU being immobilized. The control flow cell was left empty. Dilutions of the scFvs B4 (860 nM), B4m1 (860 nM), B4m2 (900 nM), B4m3 (1000 nm), B8m1 (900 nM) and B8m3 (860 nM) in HBS-EP were passed over the chip at a flow rate of 30 µl/min for 70 seconds and allowed to dissociate for the same time. All kinetic analyses were done with the BIAevaluation software according to the 1:1 Langmuir model using values obtained after subtracting the reference signal. SPR analysis was performed by J. Fehrsen from Immunology section, OVI.

2.2.8 SDS-PAGE and immunoblot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot was used to compare the amount of scFvs secreted into the culture supernatant (Laemmli, 1970; Schagger *et al*., 1987). Equal volumes of each sample diluted 1:1 in 2x protein sample buffer was loaded into a 12.5% PAGE gel and electroporated using 1x SDS buffer (see Appendix) at 100 V for one hour. Samples were compared with 5 µl of Precision Plus Protein Kaleidoscope standards (Bio-Rad, California, USA) to estimate the size of the protein. After electrophoresis the proteins were transferred to a PVDF membrane (Invitrogen, California, USA) overnight (Burnett, 1981). The membrane was blocked with 2% BSA-PBS at RT for one hour followed by three washes with 0.05% Tween-PBS. The 9E10 monoclonal antibody was diluted 1:1 with 4% BSA-PBS and incubated at RT for one hour thereafter the membrane was washed. A $^{1}/_{1000}$ dilution of polyclonal rabbit anti-mouse immunoglobulins-HRP (DakoCytomation) in 2% BSA-PBS was added and incubated at 37°C for one hour. The membrane was washed as above. For detection the membrane was incubated at RT for five minutes in equal volumes of SuperSignal West Pico substrate (Pierce, Illinois, USA). The image was captured by a Lumi Imager F1 (Roche).

2.3 Results

2.3.1 Mutant library construction

The first strategy used to determine if it was possible to improve antibody characteristics was random mutagenesis. A low fidelity *Taq* DNA polymerase was used to introduce random mutations over the entire gene sequences coding for the B4, B8 and D4 scFvs. Conditions that allowed seven mutations per 1000 bases were applied to maintain the integrity of the scFvs binding to antigen. Five colonies were picked from each library and their DNA was isolated for sequencing to confirm if mutations were introduced. Upon finding mutations in all mutant libraries including deletions in the mD4 library we carried on and panned the library against the 16 kDa antigen. Three mutant libraries each greater than 10^6 clones were obtained (Table 1). The B4, B8, and D4 mutant libraries were named mB4, mB8 and mD4 respectively. The obtained library sizes were found to be adequate as most secondary libraries generated from random mutagenesis were between 10^5 and 10^7 clones and antibodies with improved characteristics were isolated from these libraries (Gram *et al*., 1992; Martinez *et al*., 1996; Christians *et al*., 1999; Zaccolo and Gherardi, 1999; Daugherty *et al*., 2000).

Table 1: Size of anti-16 kDa mutated libraries

Mutant library	**mB4**	**mB8**	**mD4**
Library size	3.3×10^7	7.1×10^6	1.2×10^7

2.3.2 Selection of scFvs from mutant libraries by panning

To select for higher affinity binders, the mutant libraries were re-panned against the 16 kDa *M. tuberculosis* antigen. The mB4 library was subjected to three rounds of panning. To ensure that only the strongest binders were selected, the antigen coating concentration was decreased ten-fold from the second to the third round. The stringency of the washing conditions was also increased. Due to these factors there was no enrichment in the number of phages after each round as all the weak binders were washed away. Out of 92 phage clones tested in the monoclonal phage ELISA,

only five showed ELISA signals with an absorbance at 492 nm of higher than 1.0 (Figure 2.2) and with background of less than 0.1 on the milk powder control (Figure 2.3). Upon sequencing these were found to be identical in sequence and were named B4m1. Because only a single scFv binder from the third round of panning was obtained, scFv clones from round one were tested by monoclonal phage ELISA. A total of 18 scFv binders were obtained using the selection criteria above (Figure 2.4). These were sequenced and two additional binders, B4m2 and B4m3 were obtained. When expressed as scFvs B4m1, B4m2, and B4m3 were found to have ELISA signals 10.8, 6.9 and 9.6 times that of the parent antibody B4 (Figure 2.5).

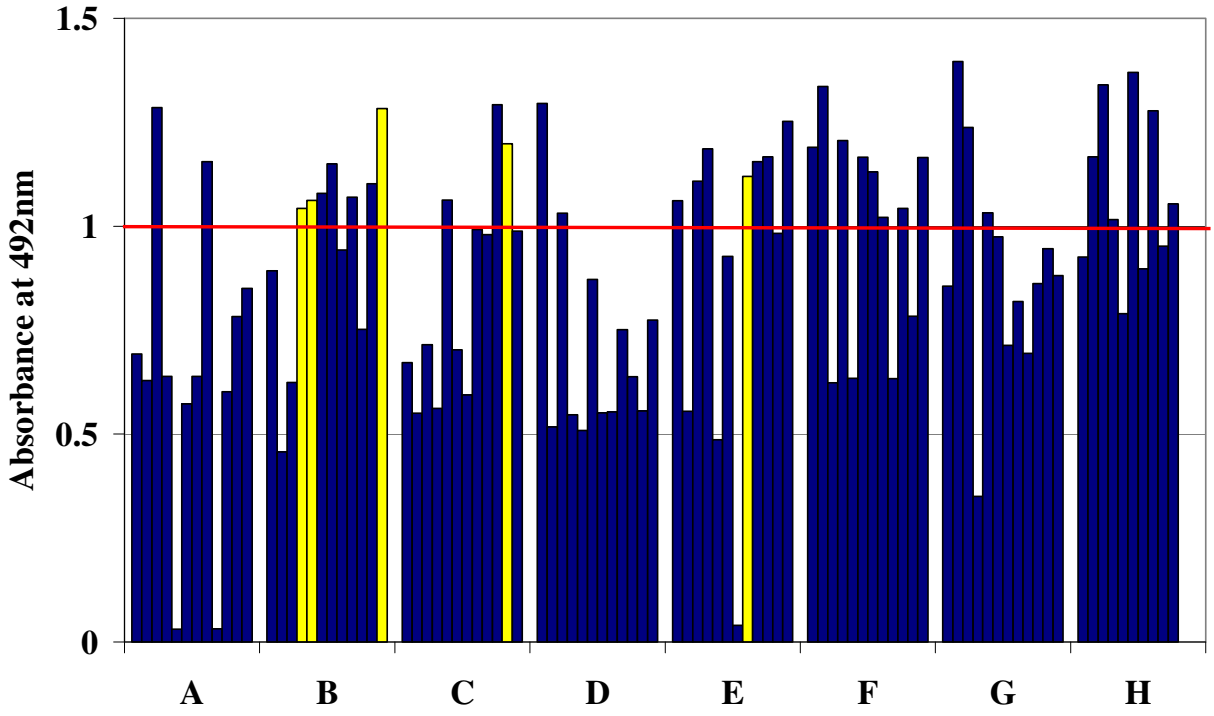


Figure 2.2 A monoclonal phage ELISA of randomly picked mB4 phage clones from the third round of panning reacting with the 16 kDa antigen. The yellow bars represent the clones that were sequenced.

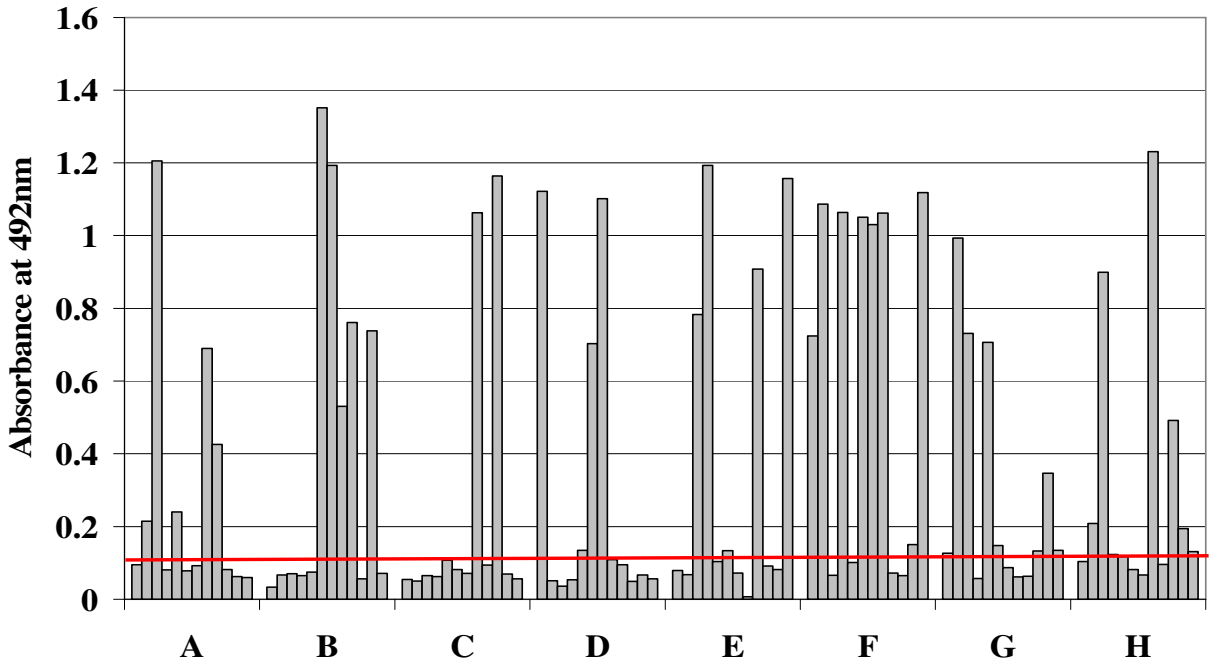


Figure 2.3 A monoclonal phage ELISA of randomly picked mB4 phage clones from the third round of panning against a milk powder control.

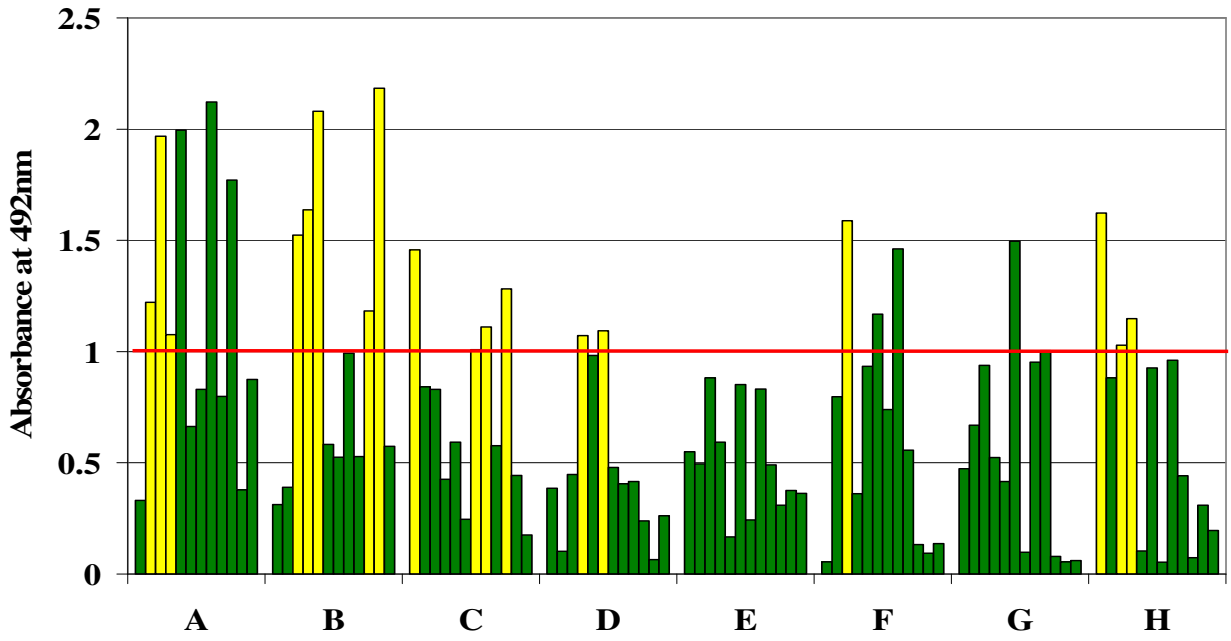


Figure 2.4 A monoclonal phage ELISA of randomly picked mB4 phage clones from the first round of panning reacting with the 16 kDa antigen. The yellow bars represent the clones that were sequenced. Milk powder control not shown.

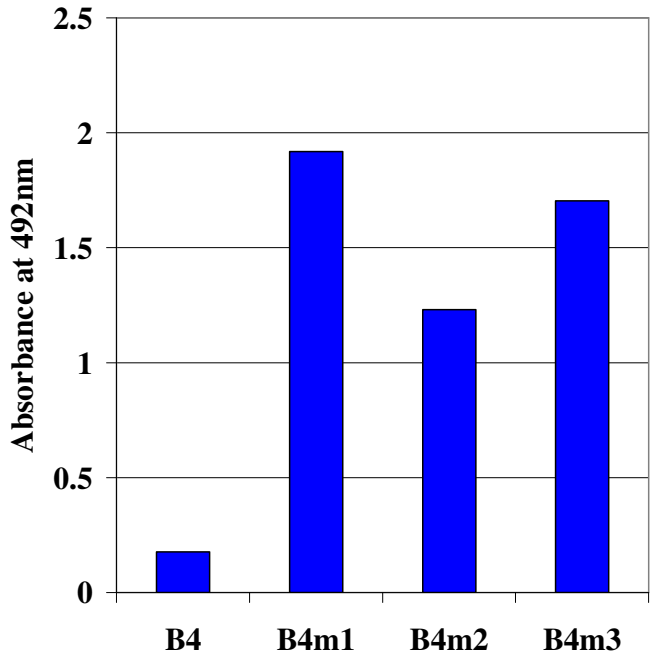


Figure 2.5 ELISA showing parent and mutant scFvs binding to the 16 kDa antigen from the mB4 library.

The comparison of the ELISA signals between the parent and mutant scFvs was based purely on whether there was an increase in the observed ELISA signals after mutagenesis of the parent scFv genes. An absorbance of more than 0.5 was taken as improvement in binding for all mB4 mutant scFvs. Since diversity was lost in the third round of panning of the mB4 clones, only a single round of panning was conducted for the mB8 and mD4 libraries. The monoclonal phage ELISA gave scFv binders with low signals from the mB8 library (Figure 2.6). These were grown in scFv format and twenty scFvs were selected which had signals greater than 0.255, the original signal of the parent scFv B8 (Figure 2.7). These were sequenced and five unique scFvs B8m1, B8m2, B8m3, B8m4 and B8m5 were obtained which had ELISA signals 3.3, 1.5, 3.6, 1.7 and 2.3 times higher than parent B8 (Figure 2.8). An absorbance of more than 0.8 was taken as improvement in binding for all mB8 mutant scFvs. It was not surprising that a large number of binders had high ELISA signals in the mD4 library in phage format (Figure 2.6). Since the parent phage antibody D4 had the highest signal to begin with it was difficult to find binders which had as high a signal as that of the parent antibody in phage displayed format (Figure 2.1). However when the same phage antibodies were grown in scFv format there was a drastic decline in the signal for the mD4 mutant scFvs (Figure 2.7). Most scFv clones had lost activity. Although there were three which

had signals higher than the original parent scFv, their signals were less than 0.5 and did not warrant further investigation.

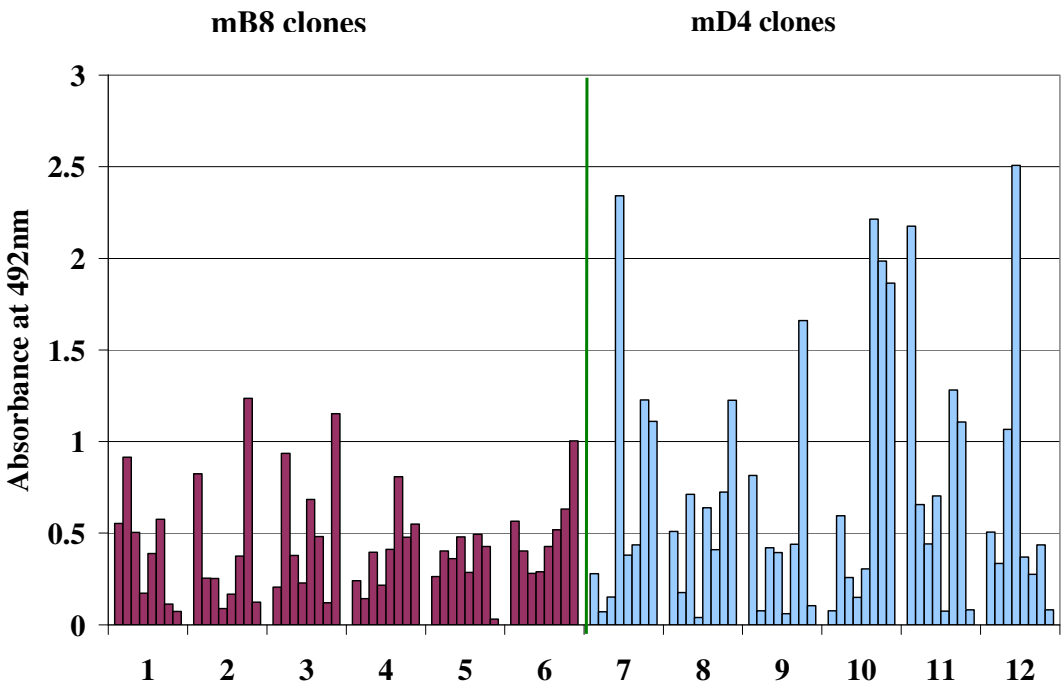


Figure 2.6 A monoclonal phage ELISA of randomly picked mB8 and mD4 phage clones from the first round of panning reacting with the 16 kDa antigen.

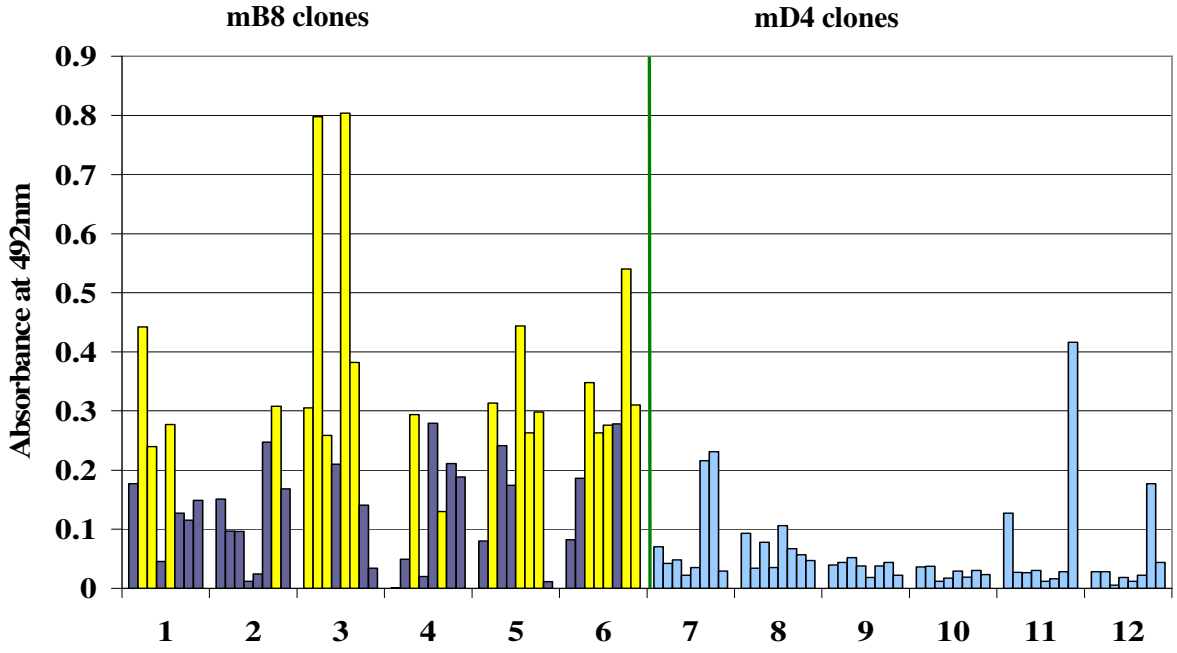


Figure 2.7 A monoclonal scFv ELISA of randomly picked mB8 and mD4 scFv clones from the first round of panning reacting with the 16 kDa. The yellow bars represent the clones that were sequenced.

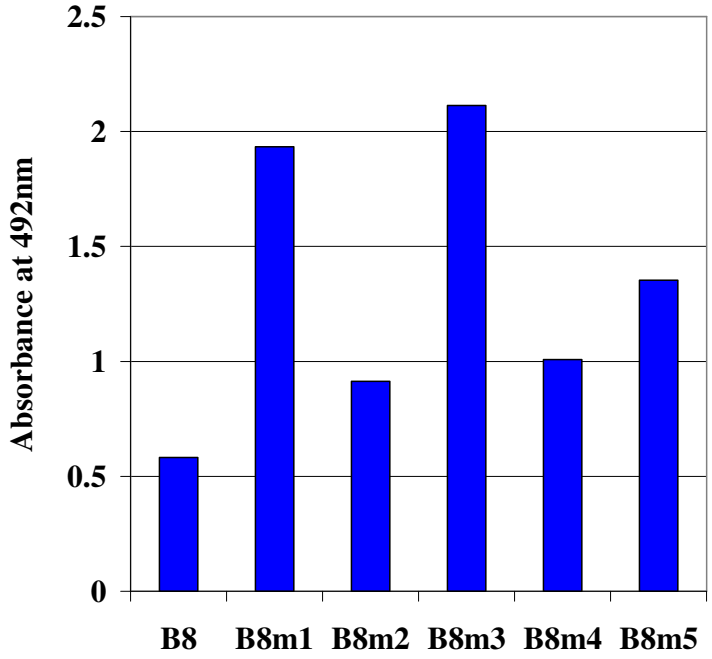


Figure 2.8 ELISA showing parent and mutant clones in scFv format binding to the 16 kDa antigen from mB8 library.

2.3.3 Sequence analysis of mutant scFvs

A comparison of the mutant scFv genes their respective parent scFv genes showed that random mutagenesis did indeed occur. After selection scFvs with one to six and two to thirteen nucleotide mutations were obtained for the mutated B4 and B8 libraries respectively. All mutations were found to be point mutations and none occurred in a common position in the three mB4 mutant scFvs. A single amino acid exchange in B4m1 from asparagine (N) to serine (S) in the heavy chain CDR1 was observed (Figure 2.9). B4m3 had three amino acid exchanges from glutamine (Q) to arginine (R), aspartic acid (D) to glycine (G) and threonine (T) to alanine (A) in the heavy chain framework, light chain CDR2 and light chain framework respectively. B4m2 had three amino acid exchanges from T to A, lysine (K) to R, and K to R in the heavy chain framework, heavy chain CDR2 and light chain framework respectively. In addition, B4m3 had one and B4m2 had two silent mutations (nucleotide sequence not shown).

Sequence analysis of the B8 mutant scFvs showed that in the five scFv clones there were a total of 24 nucleotide substitutions. Both B8m1 and B8m3 had a mutation at nucleotide 339 resulting

in an amino acid exchange from N to K in the heavy chain CDR3 (Figure 2.10). B8m4 and B8m5 also had a common mutation at nucleotide 635 resulting in an exchange from N to S in the light chain framework. One more amino acid exchange occurred in B8m1 from valine (V) to G in the heavy chain CDR3. B8m2 had one amino acid exchange from isoleucine (I) to T in the heavy chain framework region. B8m3 had another amino acid exchange from proline (P) to S in the heavy chain framework. B8m4 had three additional amino acid exchanges from T to A, K to R, in the heavy chain framework, Y to histidine (H) in the light chain CDR1 and V to A in the light chain framework. The other amino acid exchange in B8m5 occurred in the light chain framework from leucine (L) to P. In addition B4m2 had two silent mutations.

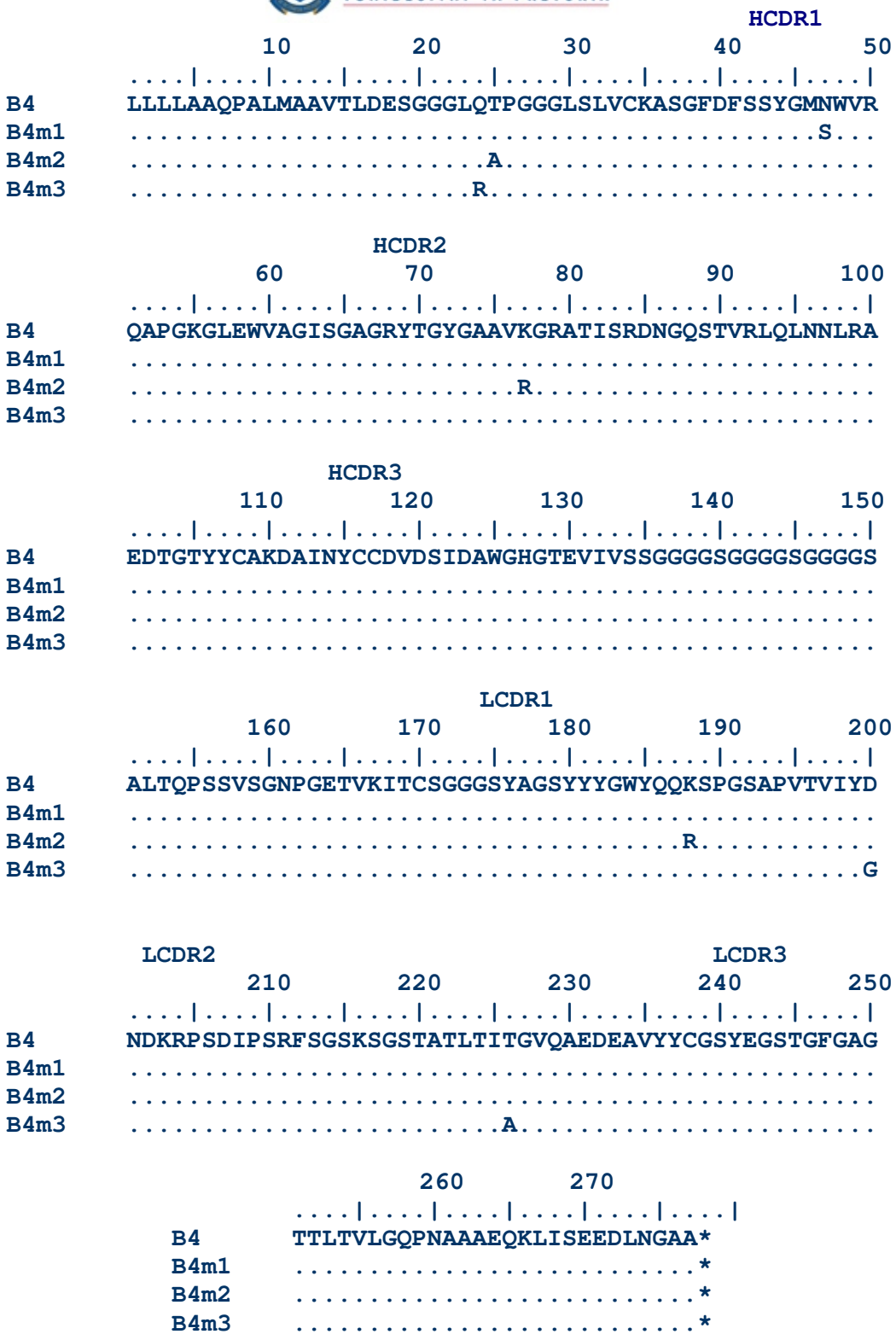


Figure 2.9 Amino acid sequence alignment showing the changes in the scFv genes compared to parent scFv B4. Dots indicate amino acids identical to that of the parent scFv B4. The highlighted areas represent the CDRs.

```
                                                                HCDR1
                10        20        30        40        50
       ....|....|....|....|....|....|....|....|....|....|
B8     LLLLAAQPALMAAVTLDESGGGLQTPGGALSLVCKASGFTFSDYGMGWVR
B8m1   ..................................................
B8m2   ..................................................
B8m3   ..................................................
B8m4   ......................A.........R.................
B8m5   ....................P.............................

                    HCDR2
               60        70        80        90        100
       ....|....|....|....|....|....|....|....|....|....|
B8     QAPGKGLEWVAGIGNTGSWTAYGAAVKGRVTISRDNGQSTVRLQLNNLRA
B8m1   ..................................................
B8m2   ..................................................
B8m3   ..S...............................................
B8m4   ..................................................
B8m5   ..................................................

                   HCDR3
              110       120       130       140       150
       ....|....|....|....|....|....|....|....|....|....|
B8     EDTATYYCAKTTNCRSGYSCGAYADEIDAWGHGTEVIVSSGGGGSGGGGS
B8m1   ............K.....................................
B8m2   ....................................T.............
B8m3   ............K.....................................
B8m4   ..................................................
B8m5   ..................................................

                                  LCDR1
              160       170       180       190       200
       ....|....|....|....|....|....|....|....|....|....|
B8     GGGGSALTQPSSVSASPGETVKITCSGGGTYAGSYYYGWYQQKAPGSAPV
B8m1   ............G.....................................
B8m2   ..................................................
B8m3   ..................................................
B8m4   ................S.................H.............
B8m5   ..................................................

   LCDR2                                          LCDR3
              210       220       230       240       250
       ....|....|....|....|....|....|....|....|....|....|
B8     TVIYDNNKRPSNIPSRFSGSLSGSTNTLTITGVQAEDEAVYFCGTWEGIF
B8m1   ..................................................
B8m2   ..................................................
B8m3   ..................................................
B8m4   ..........S...........A.............A.........
B8m5   ..........S...........................

              260       270       280
       ....|....|....|....|....|....|....|
B8     GAGTTLTVLGQPNAAAEQKLISEEDLNGAA*
B8m1   ............................*
B8m2   ............................*
B8m3   ............................*
B8m4   ............................*
B8m5   ............................*
```

Figure 2.10 Amino acid sequence alignment showing the changes in the scFv genes compared to parent scFv B8. Dots indicate amino acids identical to that of the parent scFv B8. The highlighted areas represent the CDRs.

2.3.4 Mutant binding analyses by ELISA.

The binding characteristics of the mutant scFvs were tested in ELISA. A normal ELISA with one hour incubation times shows the binding characteristics of B4 and B8 (Figure 2.11). B4m1, B4m2, and B4m3 had ELISA signals 10.8, 6.9 and 9.6 times that of the parent antibody B4. B8m1, B8m2, B8m3, B8m4 and B8m5 had ELISA signals 3.3, 1.5, 3.6, 1.7 and 2.3 times higher than parent B8.

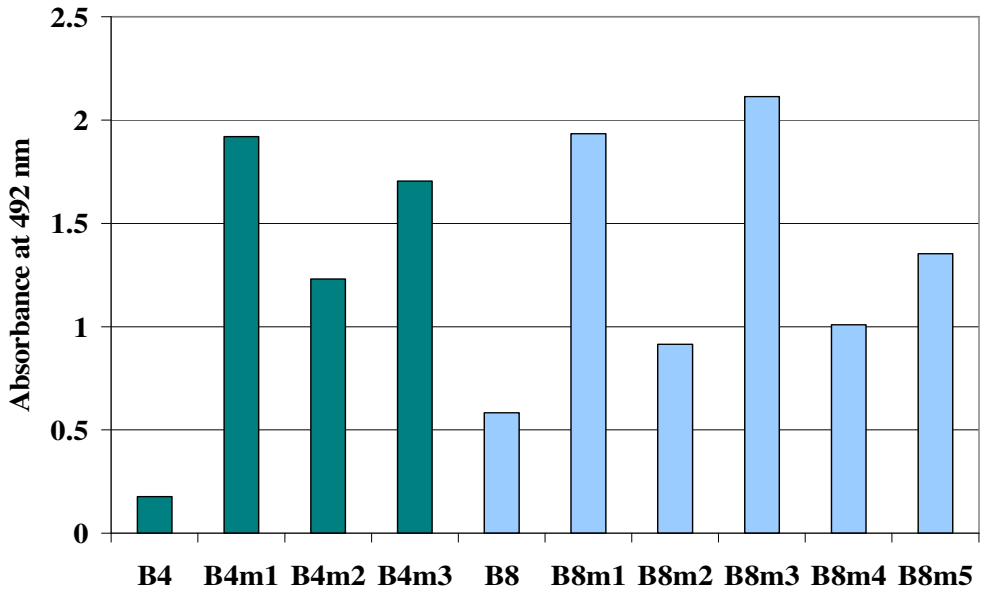


Figure 2.11 An ELISA showing the binding of mB4 and mB8 mutant scFvs to the 16 kDa antigen.

An ELISA with a short incubation time of 10 minutes was done to see how fast the scFvs can bind to antigen. An ELISA with stringent washing conditions was conducted to determine the strength of binding. The scFvs were stored at different temperatures over two weeks and then tested in ELISA to see if the scFvs were still viable. The scFvs were isolated from the periplasmic space and diluted in order to obtain a higher yield of scFv. The results of the short ELISA show that B4m3 bound quickly and stronger to the antigen than the other scFvs (Figure 2.12). When comparing the results with that of a normal ELISA (Figure 2.11) B4m1 and B4m2 emerge as poor performers, as the signal in ELISA declines after the short incubation and stringent washes. B4m3 again was the only scFv that maintained its binding ability after storage at 4°C for 2 weeks (Figure 2.13). All B4 mutant scFvs were active after storage at -20°C, though

there was a drop in signal for B4m1 when compared to the normal ELISA. The parent completely lost activity after storage at both the temperatures.

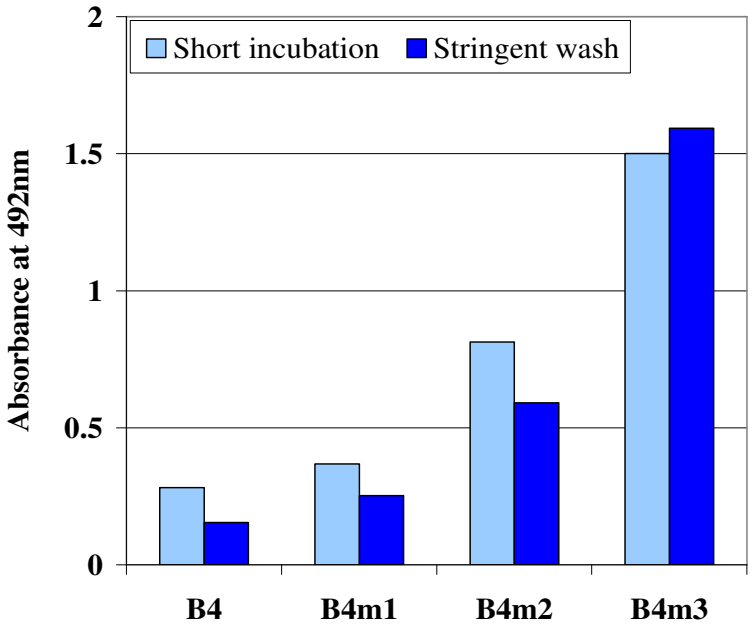


Figure 2.12 An ELISA showing the binding of mB4 mutant scFvs to the 16 kDa antigen using different ELISA conditions.

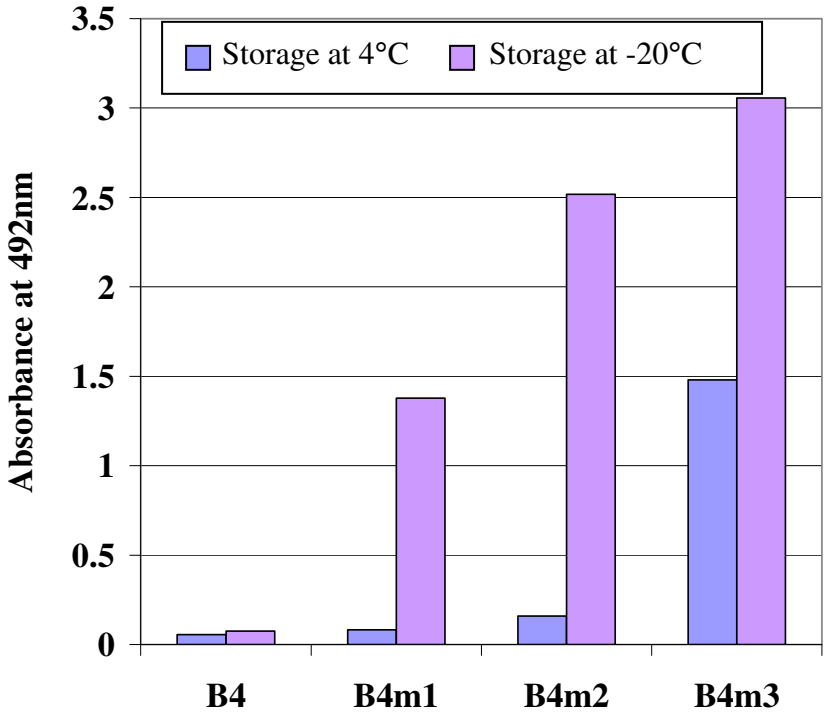


Figure 2.13 An ELISA showing the binding of mB4 mutant scFvs to 16 kDa antigen after storage for two weeks at different temperatures.

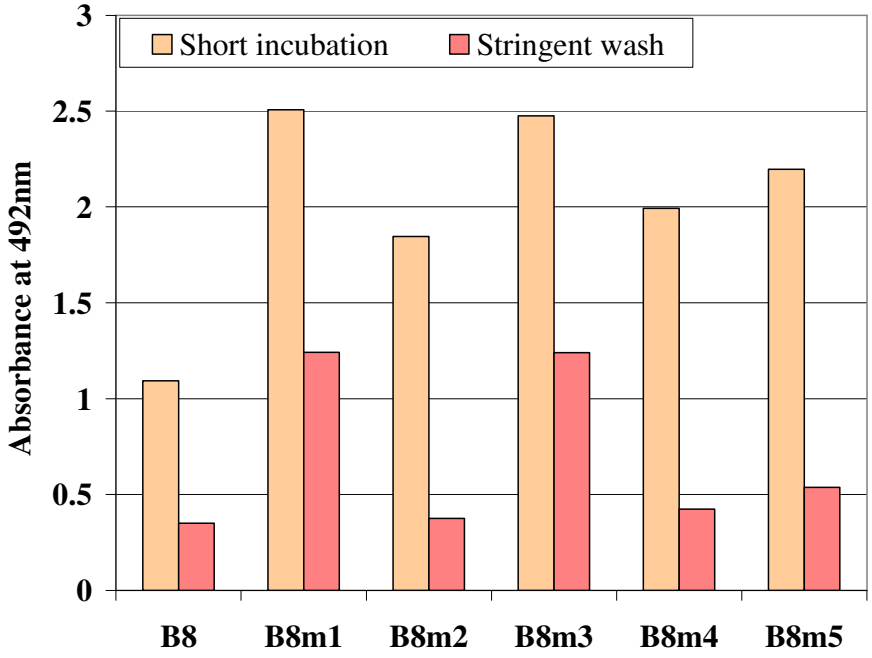


Figure 2.14 An ELISA showing the binding of mB8 mutant scFvs to the 16 kDa antigen using different ELISA conditions.

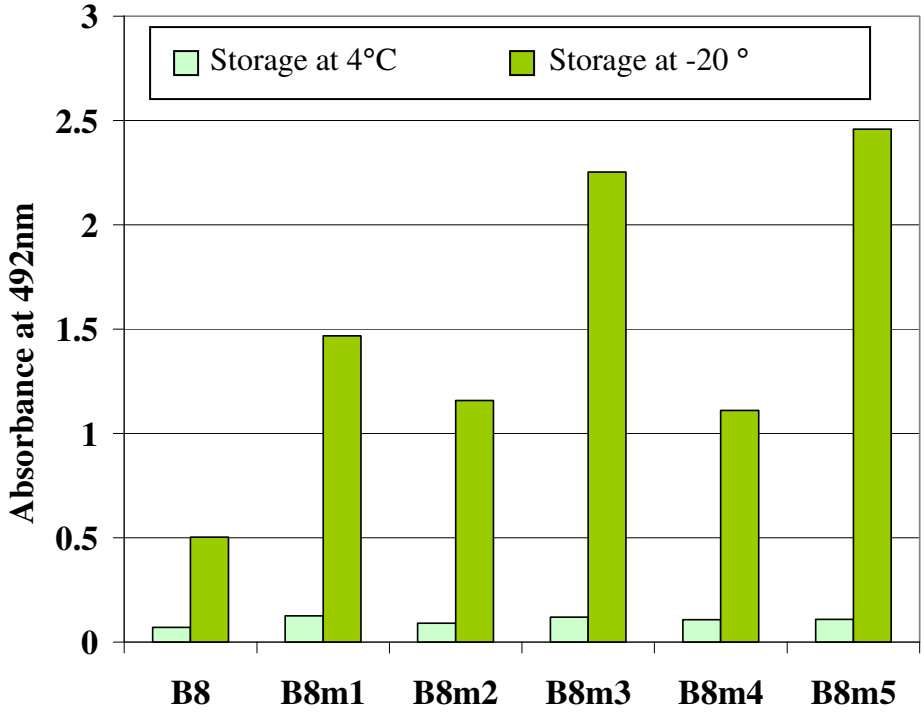


Figure 2.15 An ELISA showing the binding of mB8 mutant scFvs to the 16 kDa antigen after storage for two weeks at different temperatures.

All the mB8 mutant scFvs including the parent bound quickly to the antigen. While scFvs B8m1 and B8m3 remained bound after stringent washing conditions there was a drop in ELISA signal for all scFvs when compared to the normal ELISA (Figure 2.14). All B8 mutant scFvs retained their ability to bind to the antigen after storage at -20°C when compared to a normal ELISA (Figure 2.15). All mutant scFvs had lost activity after storage at 4°C for 2 weeks.

2.3.5 Mutant binding analyses by SPR

SPR shows the binding kinetics of molecules in real time, while the ELISA shows binding at equilibrium. SPR confirmed the ELISA results (Figure 2.12). All three B4 mutant scFvs gave a higher response than B4 with B4m3 showing a six-fold increase (Figure 2.16). In addition, the binding had not reached equilibrium after seventy seconds which was the case with B4 and the other two mutants. The most marked difference is the slower off rate of B4m3. After the wash step at the same flow rate as the injection, 75% of the B4m3 scFvs remained bound to the protein whereas B4, B4m1 and B4m2 were released rapidly and had 15-30% of the original signal left. The remaining signal of B4m3 was 18-fold that of the parent antibody. This confirmed the ELISA that with extended wash steps the scFv will remain bound.

Of all the B8 mutant scFvs, only B8m1 and B8m3 showed consistent results in ELISAs. So only these were analysed by SPR. Both bound fairly quickly to the antigen and the binding had reached equilibrium after fifty seconds (Figure 2.17). After the wash step at the same flow rate as the injection, 73-76% of both scFvs were released rapidly and only 24-27% of the original signal remained. This confirms the ELISA results that these scFvs behave in a similar manner (Figure 2.14), but proves that B8m1 has slightly better binding characteristics.

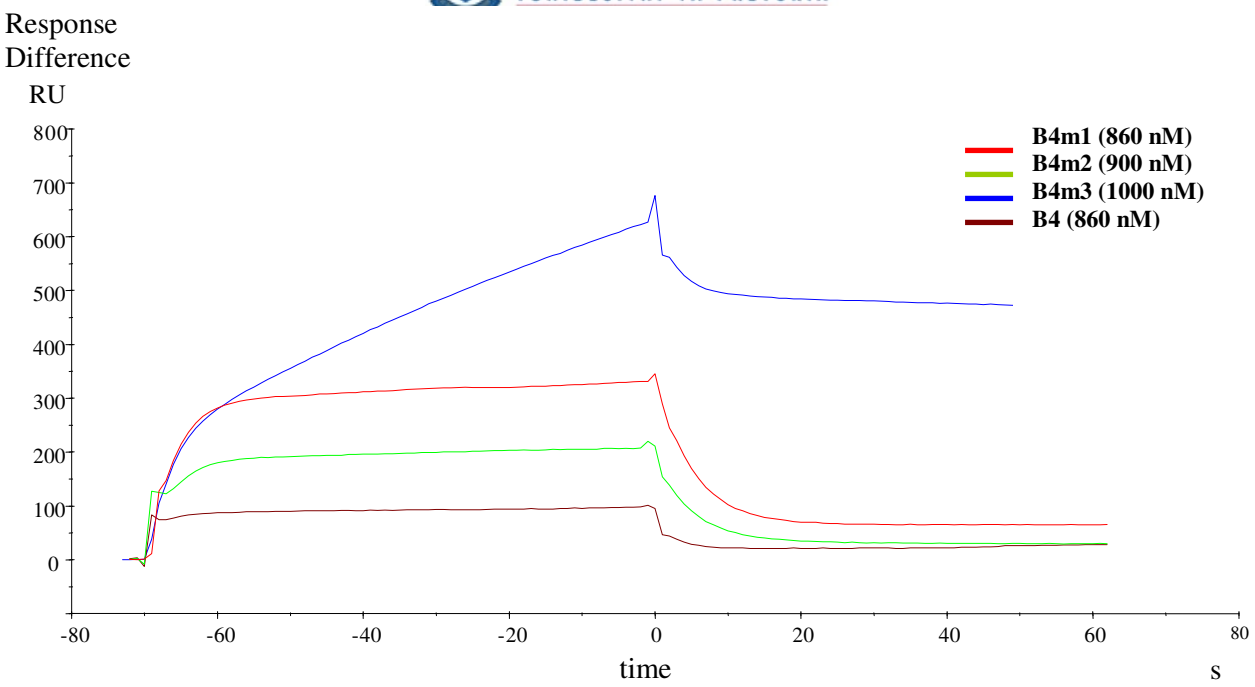


Figure 2.16 Sensogram of purified scFvs injected over the *M. tuberculosis* 16 kDa protein immobilised on the surface of a CM5 biacore chip.

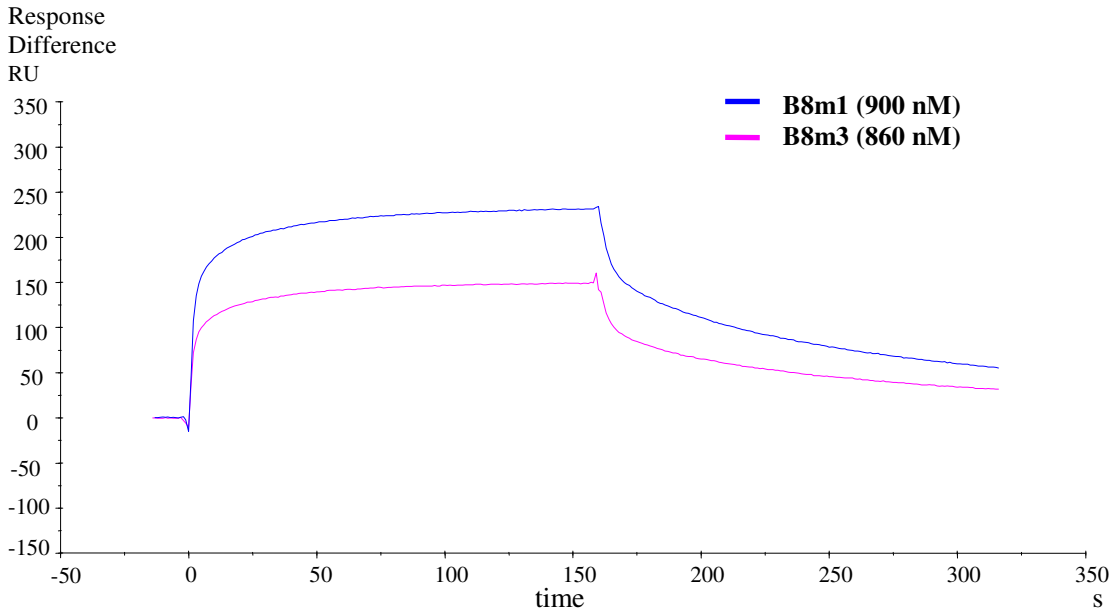


Figure 2.17 Sensogram of purified mB8 scFvs injected over the *M. tuberculosis* 16 kDa protein immobilised on the surface of a CM5 biacore chip.

2.3.6 SDS–PAGE and immunoblot

ScFvs in culture supernatant were tested by immunoblot to determine the amount of scFvs produced after mutagenesis. ScFvs induced from a 2ml culture supernatant were tested. The immunoblot showed that B4m1 had increased expression levels when compared to the parent antibody at the same induction volume (Figure 2.18). This scFv only had a single amino acid exchange yet it was enough to increase protein yield. There was no increase in expression for the mutant B8 scFvs when compared to the parent antibody (data not shown).

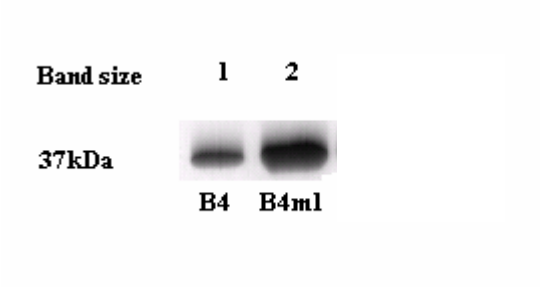


Figure 2.18 Immunoblot of anti-c-myc tag antibody 9E10 reacting with the scFvs on the blot. Lanes: (1) B4; (2) B4m1.

2.4 Discussion

The main advantage of random mutagenesis as a strategy for affinity maturation is that a large number of mutant clones can be readily generated. A major drawback is that only very few of those will maintain their antigen binding capability (Miyazaki *et al*., 1999). Conditions that allowed for a relatively low frequency of mutation were used. After selection, mutants with between one and three amino acid exchanges for the B4 mutants and between two and seven for the B8 mutants were obtained. Libraries with a low frequency of mutation have been favoured over libraries with high frequencies as they mimic the natural process of evolution more closely. Libraries with a high mutation frequency are likely to yield a large number of non-functional clones (Daugherty *et al*., 2000).

Figures 2.9 and 2.10 show the amino acid exchanges that occurred in the course of the affinity maturation of the anti-16 kDa antibodies. Exchanges in both the CDR and framework regions

involved mutations mostly to basic residues (arginine, lysine and histidine) in the CDRs and non-polar (alanine, glycine) and small polar (serine) in the framework. There was a high incidence in the framework of exchanges to alanine, with exchanges from threonine accounting for three out five of these. The substitution of large residues with a small alanine is characteristic of mutations associated with conformational flexibility (Villa and Kauvar, 1994; David *et al*., 2007). There were also five exchanges to serine with three of those from asparagine and two from proline. This could be because codons associated with serine have been identified as having the highest mutability rates (Shapiro *et al*., 1999). Also, proline has a high conformational rigidity thus it will tend to be replaced in favour of flexibility. There are three exchanges from lysine to arginine, two of those in the same position in different scFvs. Although these changes are conservative, arginine is larger than lysine. It might thus be capable of mediating a wider array of intermolecular interactions (Villar and Kauvar, 1994; Birtalan *et al*., 2008). The most important observation however is the exchange at amino acid position 25 from threonine to alanine in B4m2 and B8m4. This might be the first indication of an unambiguous preference for an alanine residue at that position for chicken scFvs. Furthermore, the best scFvs from the mB8 library had a common amino acid exchange at position 113 from asparagine to lysine. The exchanges from asparagine to serine at position 212 in B8m4 and B8m5 give further insight to residue preferences on these scFvs. The reason that there are more exchanges in the frameworks than in the CDRs is probably because the scFvs had already been exposed to the antigen, therefore the specificity determining residues in the CDR were already present (Padlan *et al*., 1995). The exchanges in the framework are probably more concerned with structural changes i.e. flexibility, structure preservation, folding and stability all of which directly influence affinity (Jung *et al*., 2001).

The scFv B4m1 had an increase in the amount of scFvs it expressed (Figure 2.18). This scFv had a single nucleotide transition from A to G resulting in an amino acid exchange in the heavy chain CDR1 from asparagine to serine, both of which have polar side chains. Asparagine to serine substitutions have been found to be tolerated in v-domains and generally do not result in the destruction of the structural integrity of the protein (Weis *et al*., 2007). They may in fact increase the stability of scFvs (Brockmann *et al*., 2005). B4m1 was the only scFv selected from the third round of panning. It was selected after a stringent panning procedure, but when tested in an ELISA with a short incubation time and stringent washing conditions it did not perform well (Figure 2.12). This may suggest that it grows better than the other two scFvs (specify which

scFvs). This theory was supported by the immunoblot results which showed that the amount of scFvs it produced was more than the parent (Figure 2.18).

The binding kinetics of the mutants were determined using ELISA and SPR. The results showed that B4m3 had the highest affinity for the antigen and a reduced off rate compared to the other B4 mutant scFvs. These results were supported by the short incubation and stringent wash ELISAs where B4m3 maintained its binding after an increased number of washes at higher temperatures. B8m1 and B8m3 also showed improved affinity compared to the parent antibody in both ELISA and SPR.

For potential use of the scFvs as diagnostic reagents it is important to know if they are stable or not. All B4 and B8 mutants were kept at 4°C and -20°C for two weeks. Of all the mutant scFvs, B4m3 was the only one that was stable after storage at 4°C. All were found to be still active after two weeks of storage at -20°C except the B4 parent antibody.

Certain factors can influence the outcome of randomly mutated immunoglobulin genes *in vitro*. These include the fidelity of the DNA polymerase, PCR components and conditions of amplification, antigen selected mutational hot spots (Ho *et al*., 2005; Yau *et al*., 2005), the tendency with which each CDR readily mutates (Jolly *et al*., 1996) and the intrinsic capacity of different amino acids for facilitating binding affinity and specificity (Padlan, 1995; Birtalan *et al*., 2008). For the purposes of creating a database of mutations, knowledge of amounts of and types of polymerase induced mutation within the DNA is essential since mutations can result from the inherent bias of *Taq* DNA polymerase. *Taq* DNA polymerase has been said to favour transitional mutations over transversions i.e. mutations that interchange within purines (A to G) and pyrimidines (C to T); (Keohavong and Thilly, 1989). The reaction conditions i.e. temperature, dNTP and salt concentration and DNA template used will also influence the error rates and the kinds of mutations (Ling *et al*., 1991; Cadwell and Joyce, 1994; Fromant *et al*., 1995). All the mutations in the B4 mutant scFvs were transitions. Out of a total of eleven base substitutions, nine were A to G and two were T to C. This result is consistent with the mutational bias calculated for the Diversify® Mutagenesis kit that the higher the mutations per gene the higher the bias towards mutational transitions. A frequently used approach for calculating the mutational bias is to consider the ratio of transitions over transversions (Ts/Tv). Interestingly for the B8

mutant scFvs both transition and transversion mutations were found. The transversions accounted for the majority of the mutations which were made up of interchanges between C and G, A and C, A and T and T and G. Out of a total of twenty-four base substitutions only ten were transitions. Although the bias might appear reduced, it is important to note that all the transitions were A.T → G.C and not G.C → A.T. It has been suggested that decreasing the dGTP and dATP concentrations can minimize this bias (Fromant *et al*., 1995). A number of other methods have been suggested to decrease the mutational bias. These include performing sequential PCRs and purifying products by gel electrophoresis or diluting the products before doing the next PCR. Addition of a 2-hydroxy-dATP, increasing the concentration of the *Taq* and increasing the extension time have also been suggested (Cadwell and Joyce, 1992; Shafikhani *et al*., 1997; Kamiya *et al*., 2004). With this knowledge protocols can be optimised in order to obtain truly random PCRs with a reduced mutational bias and an adequate frequency.

The antibody recognition sites of antibodies are formed from six complementarity-determining regions. It has been shown in human and mouse antibodies that within each CDR there are amino acid positions that are more variable than others. Studies on *in vivo* somatic hypermutation have shown that CDRs contain mutational hotspots which are the naturally occurring preferred sites for mutations (Abraham *et al*., 2004; Ho *et al*., 2005). The role of the AGY/RGYW hotspots in *in vitro* affinity maturation has been established (Ho *et al*., 2005; Yau *et al*., 2005). An analysis of the AGY/RGYW motifs of the anti- *M. tuberculosis* chicken scFvs has revealed that the light chain CDR1 of B4 contains six AGY/RGYW motifs while B8 contains three. This is the highest number compared to the other CDRs with GGCA occurring in all CDRs except the heavy chain CDR3 and light chain CDR2 for both B4 and B8. Although the heavy chain CDR3 is thought to be the most diverse and most important for antigen recognition, it has been found that the majority of mutations that favour affinity maturation occur in the heavy chain CDR1 (Padlan, 1994; Jolly *et al*., 1996; Birtalan *et al*., 2008). In this study it was found that the light chain CDR2 had the most mutations in general. This was found to be surprising as this CDR contained no AGY/RGYW motifs. According to Wilson and Stanfield, (1994) the light chain CDR2 generally has less frequent interaction with the antigen (Wilson and Stanfield, 1994; Yang *et al*., 1995). It is possible that these mutations were more concerned with structure and stability of the scFvs than with their antibody-antigen interactions. In addition, the most improved scFv (B4m3) had a mutation from asparagine to glycine in the light chain CDR2. The possibility of hotspots is supported by mutations in the same position 339 and 635 in the heavy chain CDR3 and light

chain CDR2 respectively. The former induced an exchange from A to C in B8m1 and B8m3 and the latter an exchange from A to G in B8m4 and B8m5 at nucleotide level. A further observation is that while the mutations that occurred in B4 were to some extent evenly spread over the length of the V domains, in B8 mutants there was a tendency of the mutations to be clustered within a few bases of each other. Whether this pattern is an indication of preferred residues in anti-16 kDa scFvs after selection or is caused by the mutational frequency of the *Taq* DNA polymerase or hotspots is unclear.

This study has shown that the mutations in the chicken scFvs can improve affinity, expression and stability. It is also known that these types of mutations do not interfere with the ability of the antibody to fold into a functional scFv. More studies using directed mutagenesis at the residues which have shown improvement will confirm if those residues are essential for chicken scFv binding, stability or yield, which in turn would make it theoretically possible to tailor scFvs with desirable properties.

Chapter 3

The role of linker length in multimerisation of chicken scFvs

3.1 Introduction

ScFvs with different types of linkers have been constructed from Fab fragments of mouse antibodies. These were found to have the binding specificity and affinity similar to that of the parent antibody (Bird *et al*., 1988; Huston *et al*., 1988). Shortening the length of the linker joining the V_H and V_L domains of scFvs promotes multimerisation in mouse and human scFvs. This increases the number of binding sites available for antigen binding per molecule thus increasing the avidity or functional affinity of the scFvs (Kortt *et al*., 1997). Shortening the linker to less than 12 amino acid residues causes the pairing of complementary V_H and V_L from two adjacent scFvs thus forming bivalent molecules called diabodies (Holliger *et al*., 1993). Further shortening of the linker can result in interactions between three or four scFvs, forming trimers and tetramers which are called triabodies and tetrabodies (Kortt *et al*., 1997). The increase in size and avidity has the advantage of increased tumor localization and retention in cancer therapy, reduced clearance rate and improved imaging when compared to their smaller monomeric counterparts (Nielsen *et al*., 2000; Power *et al*., 2000; Wu *et al*., 1996).

To investigate the effects of shortening the linker (Gly_4Ser_3) of chicken anti-16 kDa scFvs, two scFv constructs were made; one with a short linker and one without a linker. The scFvs B4, B4m3, and B8m3 were chosen to be used as models for this study. The unmutated B4 was chosen because it was the weakest binder while B4m3 and B8m3 (best mutant binders) were chosen to see if their binding can be further improved. The linker sequence of the scFvs was shortened to one glycine residue using the strategy depicted in Figure 3.1 and also removed by directly joining V_H to V_L. ScFvs with a single residue linker have been shown to form exclusively tetramers, while those without a linker have been shown to form trimers for human and mouse scFvs (Iliades *et al*., 1997; Atwell *et al*., 1999; Le Gall *et al*., 1999).

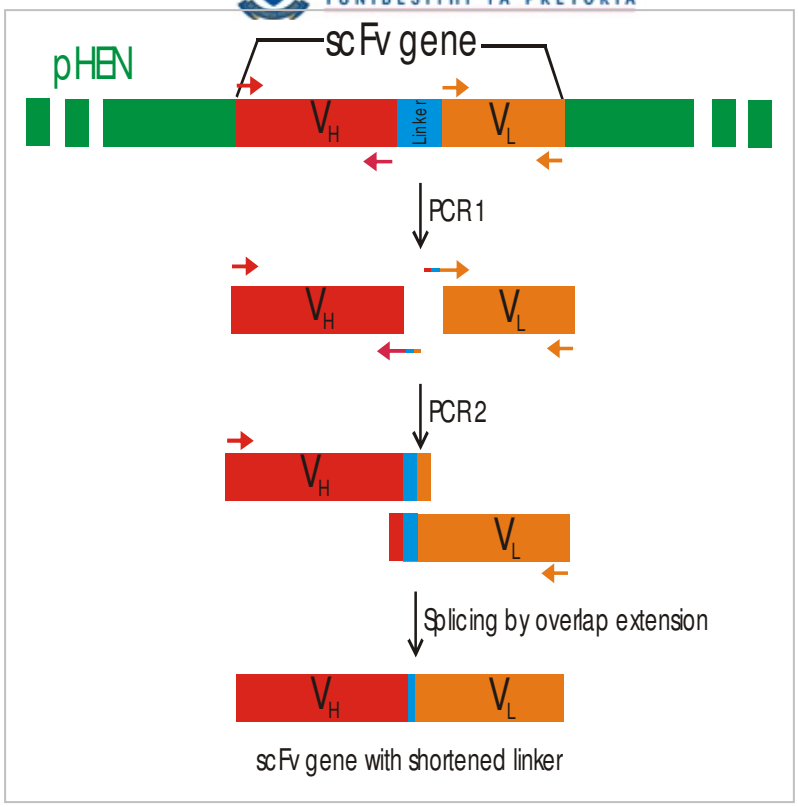


Figure 3.1 A schematic diagram showing how the linker was shortened. The chicken scFv gene consisted of V_H domain (red) and a V_L domain (orange) joined by a 15 amino acid linker (blue) in the vector pHEN1 (green). The arrows represent the primers.

3.2 Materials and methods

3.2.1 Shortening and removing the linker

To shorten the length of the linker to a single glycine residue the V_H and V_L domains of B4, B4m3 and B8m3 scFvs were amplified by PCR separately using 2.5 U of Faststart High Fidelity enzyme (Roche) in a 50 µl reaction. For all PCR reactions 1 ng of scFv DNA and 0.4 pmol/µl of each primer were used. The primer pairs Sfi1L (2.2.2), Hend1 (Table 2) and LStart1 (Table 2), LCNOT1 (2.2.2) were used for the heavy and light chain reactions respectively. The reaction was allowed to proceed for 30 cycles of 94ºC for 15 s, 55ºC for 15 s and 72ºC for 30 s. An initial denaturing step of 94°C and a final extension step of 4 minutes was conducted for all PCRs. HyperLadder (Bioline, London, UK) DNA standards were used to determine the size of the PCR

products. The PCR products were electroporated in a 1.5% Ethidium Bromide (EtBr) agarose gel using 1x Tris-acetate-EDTA (TEA) buffer (see Appendix) at 100 V for 20 minutes using TaKaRa Recochips (TaKaRa, Kyoto, Japan) as per manufacture's instructions. To add overlapping sequences, the recovered products were used in the second PCR using the primer pairs SfI1L, HendG1 for the heavy chain and LStart G1, LCNOT1 for the light chain reaction (Table 2). Thermal cycling conditions were the same as above for the heavy chain reaction and the melting temperature (Tm) was 47ºC for the light chain reaction. Joining of the V_H and V_L (each 100 ng) was carried out using splicing by overlap extension (SOE); (Horton *et al*., 1989). The reaction was allowed to proceed for 15 cycles of 95ºC for 30 s, 47ºC for 30 s and 72ºC for 30 s. Amplification of the joined genes after SOE was performed using the primers Sfi1L and LCNOT1. The reaction was allowed to proceed for 25 cycles of 95ºC for 30 s, 60ºC for 30 s and 72ºC for 30 s.

To remove the linker, the primers SfI1L and Hend 2 were used for the heavy reaction. For the light chain reaction the primers LStart1 and LCNOT1 were used. For the second PCR that adds overlapping sequences, the primer pairs SfI1L, Hend 2-SOE (Tm 47ºC), LStart-SOE, LCNOT1 (Tm 55ºC) were used for the heavy and light chain reactions respectively (Table 2). Joining and amplification of V_H and V_L were performed as above except with a Tm of 65°C. The PCR products were purified using a PCR purification kit (QIAGEN). The cloning of the shortened and no-linker linker constructs into the vector pHEN1 and transformation of *E. coli* was performed as described previously (van Wyngaardt *et al*., 2004). To confirm that the correct constructs were made DNA was extracted and sequenced as before (2.2.3).

Table 2: Nucleotide sequences of DNA primers used in the shortening and removal of the linker

Primer	Sequence
Hend1	5' CCG GAG GAG ACG ATG ACT TCG G 3'
Lstart1	5' GCG CTG ACT CAG CCG TCC TCG G 3'
HendG1	5' CGG CTG AGT CAG CGC TCC GGA GGA GAC GA 3'
LstartG1	5' TCG TCT CCT CCG GAG CGC TGA CTC AGC CG 3'
Hend2	5' ACG ATG ACT TCG GTC CCG TGG 3'
Hend2-SOE	5' CGG CTG AGT CAG CGC TAC GAT GAC TTC GGT C 3'
Lstart-SOE	5' CCG AAG TCA TCG TAG CGC TGA CTC AGC CG 3'

3.2.2 ELISA

The scFvs were grown to a 2 ml final culture volume as described before (2.2.5). A maxisorp immunoplate was coated with 10 µg/ml of 16 kDa antigen overnight at 4°C. The plate was blocked with 2% BSA-PBS, incubated for one hour at 37°C then washed 3 times with 0.05% Tween-PBS. The engineered scFvs in the supernatant were diluted 1:1 with 4% BSA-PBS then incubated at 37°C for one hour, thereafter detected with 9E10 as described before (2.2.6).

3.2.3 Size exclusion chromatography

To determine the level of multibody formation, purified scFvs were separated by gel filtration using a Superdex 75 HR10/30 (Amersham Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Samples were compared with gel filtration molecular mass markers (Blue Dextran, 2000 kDa; Amylase, 200 kDa, Alcohol dehydrogenase, 150 kDa; Albumin, 66 kDa; Cytochrome c, 12.4 kDa) (Sigma, Missouri, USA) to estimate the size of the molecules. The scFvs were purified by J. Frischmuth (National Bioproducts Institute) and the HPLC was conducted and analysed by L. Primrose and A. Swemmer (Residue Laboratory, OVI).

3.2.4 Surface plasmon resonance

SPR was conducted to compare the binding characteristics of B4 and B4sL as described previously (2.2.7). Dilutions of the scFvs B4 (670 nM) and B4sL (900 nM) were used.

3.3 Results

3.3.1 Sequence Analysis

The linker sequence joining the heavy and light chain variable domains of B4, B4m3 and B8m3 was shortened from $(Gly_4Ser)_3$ to only one glycine residue. The short linker scFvs were called B4sL, B4m3sL and B8m3sL respectively. Sequence analysis revealed that the linker had indeed been shortened (Figure 3.2). The scFv B4 where the linker was removed by directly joining the V_H to V_L was named B4-noLinker (Figure 3.2). Upon analyzing the sequence of B8m3sL it was found that one of the scFv clones had two amino acid substitutions at positions 137 and 157 from isoleucine to valine and leucine to glutamine respectively (Figure 3.3). This clone was named B8m3sL1 and it was decided that since these mutations were at close proximity to the linker sequence, the clone should be further analysed to study the effect of these, if any. The mutations were thought to be due to PCR error. The normal scFv clone with a shortened linker was named B8m3sL2 (Figure 3.3).

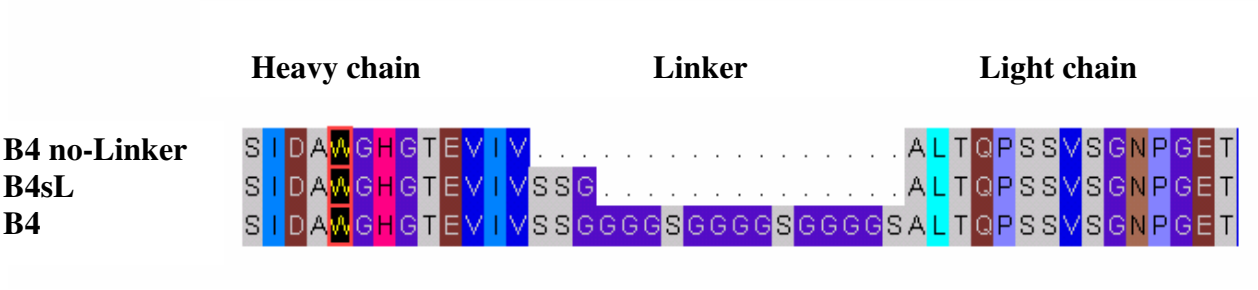


Figure 3.2 Amino acid sequence alignment depicting a short-linker scFv B4sL and an scFv without a linker (B4 no-Linker) compared to the parent scFv B4 with a 15 amino acid linker. Dots represent the nucleotides removed from the linker.

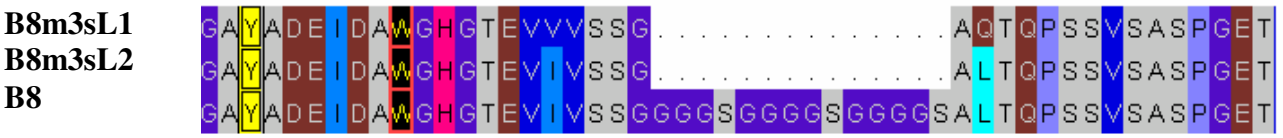


Figure 3.3 Amino acid sequence alignment depicting short-linker scFvs B8m3sL1 and B8m3sL2 compared to the parent scFv B8m3 with a 15 amino acid linker. Dots represent the nucleotides removed from the linker.

3.3.2 Analysis if short linker and no-linker constructs

The shortening of the linker resulted in an increase in the functional avidity of the antibody as was shown in ELISA (Figure 3.4). The signal was increased almost 14-fold when compared to that of the parent antibody B4. This was an indication that multibodies had been formed. Size exclusion chromatography showed that B4sL multimerized to form a 116 kDa tetramer (Figure 3.5). In the figure, B4m3 was used for comparison purposes as it occurred mainly as monomers with low levels of dimers, while B4 occurs as a mixture of monomers and dimers (not shown). Also, in all chromatographs in this chapter the x-axis is given as retention time rather than retention volume. The retention time is not an ideal parameter as these values change with the flow rate used. However, the size exclusion experiments and analyses in this chapter were offered as a service with no control over the execution, analyses and the results returned.

No reaction in ELISA was obtained for B4-noLinker thus it was not used for size exclusion chromatography and no other constructs like this were made. The binding kinetics of the scFv constructs was examined by surface plasmon resonance (Figure 3.6). As expected, the tetrameric scFv (B4sL) with its higher valency performed better than its monomeric parent. The association kinetics obtained for the tetrameric molecule showed a gradual increase as compared to the fast on rate of the parent. The binding of the parent to the immobilised antigen reached equilibrium in less than 10 seconds while the tetramer reached its equilibrium only after 70 seconds. The off rate of the tetramer was slower than that of the parent, after 140 seconds a greater amount still remained antigen bound as compared to the parent. The slower on and off rates observed are due to multiplicity of binding.

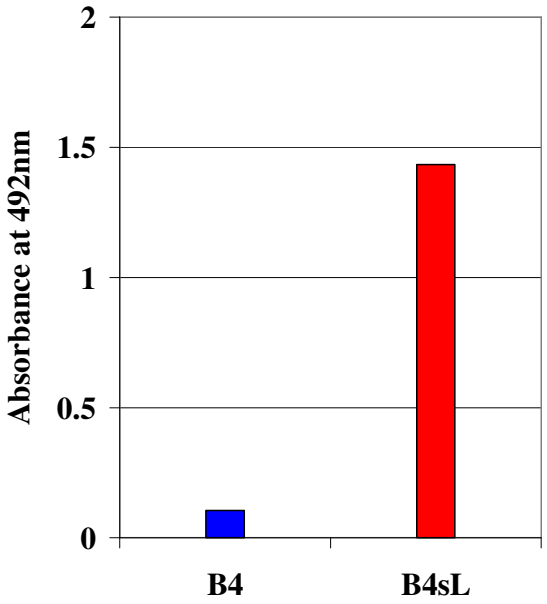


Figure 3.4 ELISA showing the difference in binding to the 16 kDa antigen of a 15 amino acid linker scFv B4 compared to B4sL with a single amino acid residue linker.

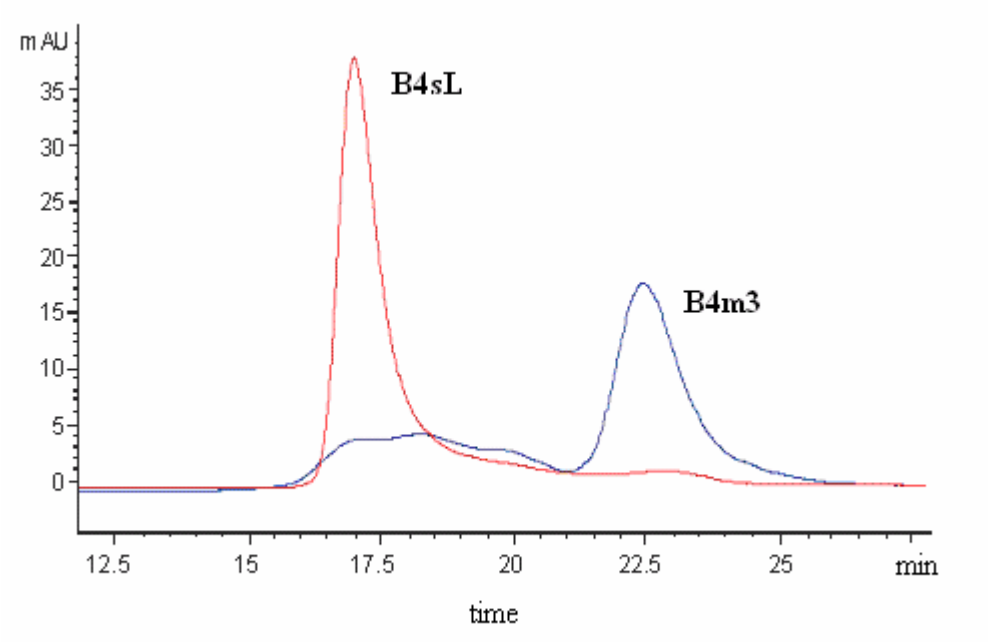


Figure 3.5 Size exclusion chromatograph depicting [1]B4m3 as a monomer (29-31 kDa), and B4sL as a tetramer (116 kDa).
[1]B4m3 is used for illustration purposes to depict the chromatograph of a monomer

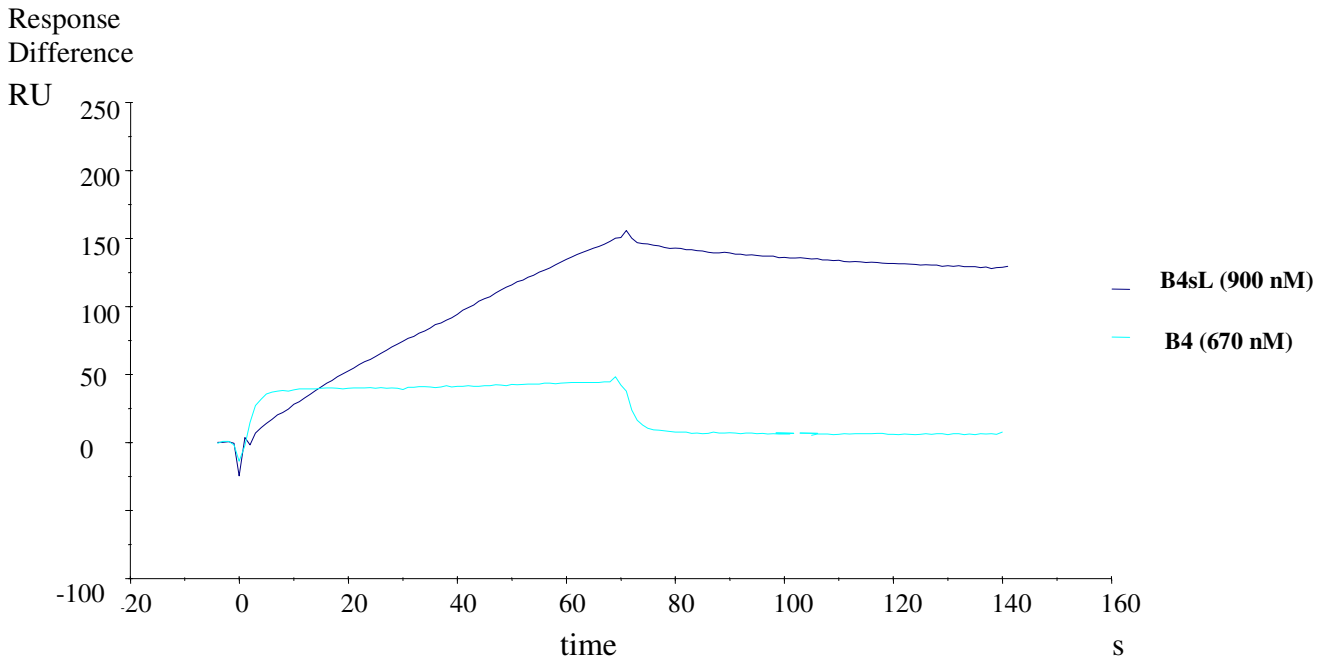


Figure 3.6 Sensogram of purified B4 and B4sL scFvs injected over the *M. tuberculosis* 16 kDa protein immobilised on the surface of a CM5 biacore chip.

There was a slight increase in signal obtained in ELISA when the linker of the mutant scFv B4m3 was shortened (Figure 3.7). Size exclusion chromatography showed that its short-linker clone B4m3sL predominantly formed tetramers (Figure 3.7). SPR was not conducted for B4m3sL as the antigen was depleted. As discussed in chapter 2, B4m3 was found to consistently give high ELISA signals, thus it is difficult to improve an already good antibody. However these results did show that reducing the linker to one glycine residue did not have a detrimental effect on the ability of the chicken scFv to bind to its target antigen. Note that the ELISA signals are very high; perhaps if the scFvs were diluted there would have been a difference in the results obtained.

The ELISA signals obtained for B8m3sL1 and B8m3sL2 were lower than that of the parent antibody, but the scFvs were still active (Figure 3.8). B8m3 occurs mostly as a monomer (29-31 kDa) with a mixture of dimers (54-55.9 kDa) and trimers (81-83.9 kDa) in lesser amounts (Figure 3.9). B8m3sL1 formed a mixture of trimers and tetramers (91.8-92.5 kDa), with slightly more tetramers than trimers. B8m3sL2 formed slightly more trimers than tetramers.

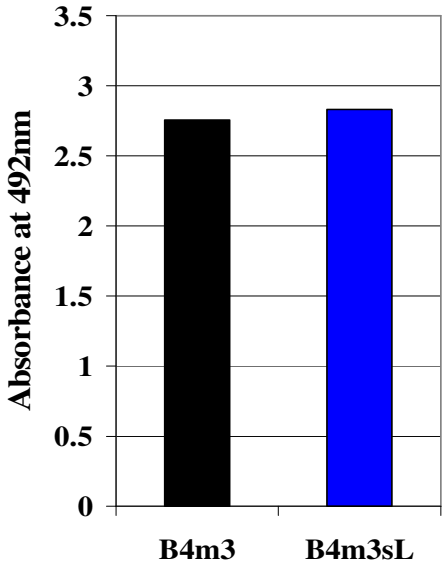


Figure 3.7 ELISA of short linker B4m3sL compared to the parent scFv B4m3 binding to the 16 kDa antigen.

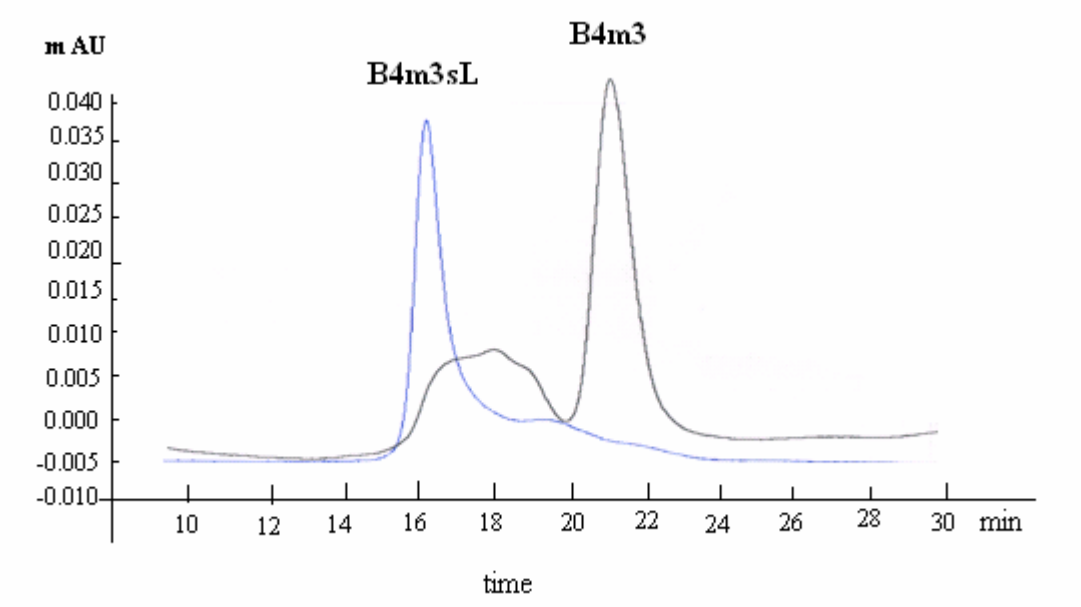


Figure 3.8 Size exclusion chromatograph depicting the scFv B4m3 as a monomer (29-31 kDa) and B4m3sL as a tetramer.

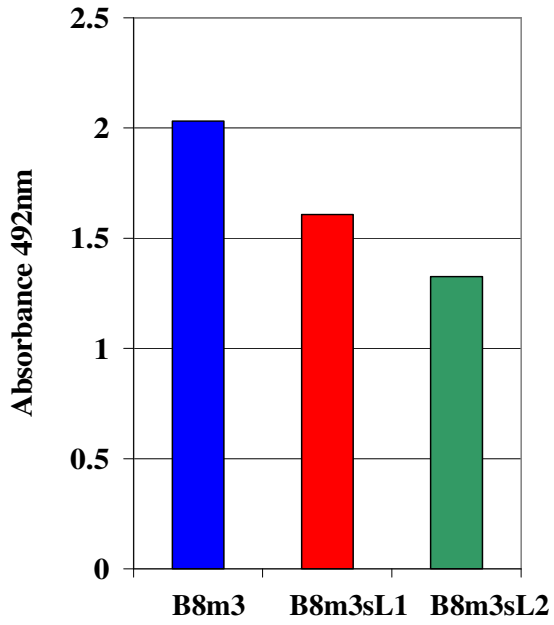


Figure 3.9 ELISA of B8m3 and its short linker scFvs B8m3sL1 and B8m3sL2 binding to the 16 kDa antigen.

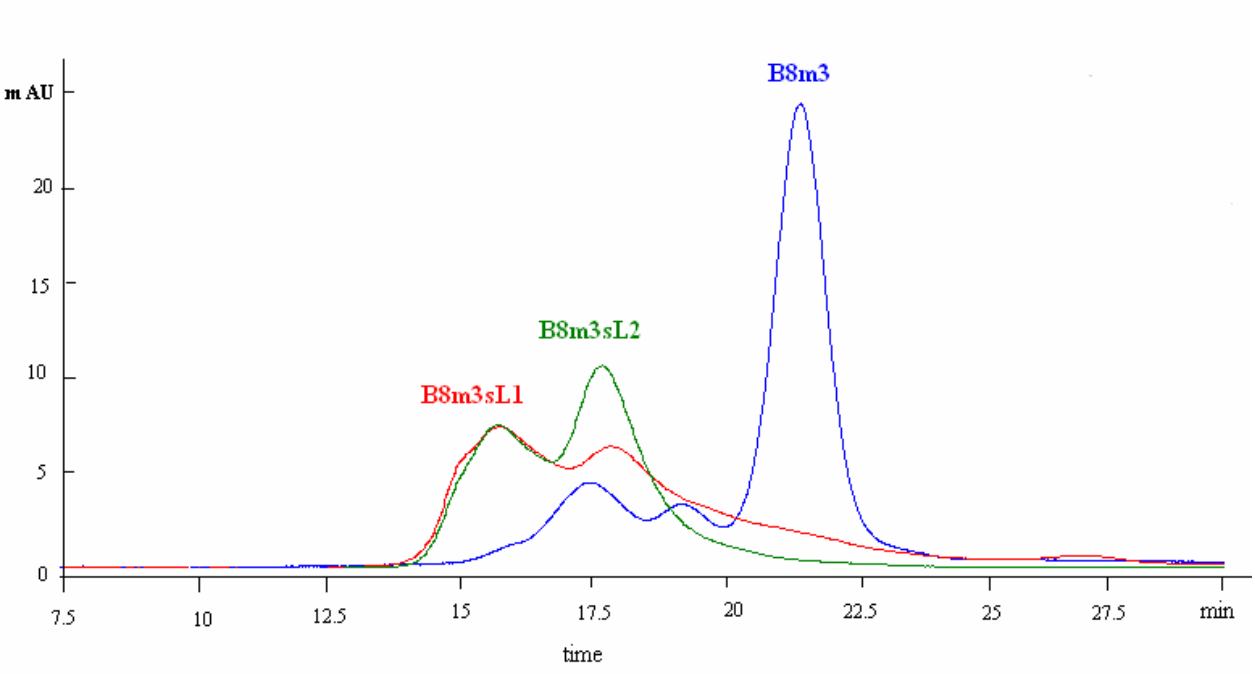


Figure 3.10 Size exclusion chromatographs depicting the scFv B8m3 as monomers, dimers (54-55.9 kDa) and trimers (81-83.9 kDa). ScFvs B8m3sL1 and B8m3sL2 are trimers and tetramers (91.8-92.5 kDa).

3.4 Discussion

Nkuku® chicken scFvs with a 15 amino acid linker $(Gly_4Ser)_3$ were found to occur mostly as monomers and a mixture of lesser amounts of multibodies. Shortening the linker of a weak binder significantly improved its signal in ELISA. Size exclusion chromatography showed that trimers and tetramers were formed as a result. The length of the scFvs B4, B4m3 and B8m2 linkers was shortened to a single glycine residue in the orientation V_H-V_L resulting in the formation of tetramers for B4sL and B4m3sL while B8m3sL1 and B8m3sL2 formed a mixture of trimers and tetramers. The formation of trimers from a linker with a single glycine residue has been reported previously for mouse scFvs (Atwell *et al*., 1999). Others found that when they reduced the linker to a single residue, the scFvs formed a mixture of dimers, trimers and tetramers. However, in their case, the linker was made up of a serine residue and the domains were arranged in a V_L-V_H orientation. The formation of a tetramer in a V_H-V_L position was described, but again the linker was made up of a serine residue (Malby *et al*., 1993; Le Gall *et al*., 1999). These results indicate that different scFvs and different linker compositions show different propensities to form dimers, trimers or tetramers. This is unlike the strict transitions observed when the linker was reduced to one glycine residue for an anti-neuraminidase antibody NC10 (Atwell *et al*., 1999).

In murine and chicken derived scFvs, the amino acids VSS are the last residues at the C- terminus of the heavy chain (Huston *et al*., 1988, Holliger *et al*., 1993, van Wyngaardt *et al*., 2004). Linker dependent multimerisation of murine scFvs has been studied in detail. It has been found that by directly joining the last residue of the heavy chain V_HS^{113} to the first residue of the light chain V_LD^1 a mixture of trimers and tetramers was obtained (Arndt *et al*., 2004). When joining V_HS^{113} to V_LD^1 formation of 40% dimers and 60% trimers was observed (Le Gall *et al*., 2004). Another study reported an exclusive trimer formation by fusing V_HS^{112} to V_LD^1 and an exclusive tetramer conformation when V_HS^{113} was ligated to V_LD^1 (Le Gall *et al*., 1999). In this present study, both the terminal serine residues of the heavy chain were removed and the light chain was directly joined to valine (Figure 3.2). The effect of this on scFvs was investigated by ELISA and no functional scFvs were found. This supports the theory that linker dependent oligomerisation of scFvs may be affected by the sequence of the variable domains (Arndt *et al*., 2004). Removal of the terminal serine residues might have prevented correct alignment of the variable domains of the multibodies and the orientation of the antigen binding sites. Therefore if multiple binding is

not sterically possible there will likely be no gain in functional affinity (Lawrence *et al*., 1998). This study has shown that removing the terminal serine residues is detrimental to binding of chicken scFvs to their antigen. In future the linker will be removed without removing the terminal serine residues of the heavy chain and the effect of this on multimerisation will be studied

The SPR analysis was only conducted for B4sL due to a limited availability of antigen. However the data obtained were sufficient to show that shortening the linker does reduce the off-rate. Affinity constants (SPR) were not determined since most of the scFvs occurred as multibodies and thus the 1:1 Langmuir model could not be used. This is because the results obtained for SPR analyses of multibodies is influenced by the multiplicity of binding, amount of antigen immobilized on the biosensor chip, the multibody alignment and its orientation with respect to the exposure of Fv portions to the immobilized antigen (Illiades *et al*., 1997). Nevertheless the SPR data can still be used as a convenient way to rank scFvs by comparing the apparent dissociation rates.

As mentioned above, scFvs with a single residue linker and those without a linker, in either V_H-linker-V_L or V_L-linker-V_H have been produced by various research groups. All the above scFvs were of either human or mouse origin. Lee *et al*, (2007) created a chicken scFv library with a seven residue linker but they did not report whether they found multimers. Furthermore, when they compared the binding in ELISA of scFvs from a short linker library with those from a long linker library (18 linker residues) they found that the scFvs bound equally well.

This study has shown that shortening the linker of chicken scFvs to one glycine residue always results in the formation of multibodies, but it is not in all instances that the ELISA signal is increased. Completely removing the linker was also shown to be detrimental to binding in ELISA. This is, however the first report that shortening the linker of chicken scFvs to one glycine residue in a V_H-V_L orientation can result in the formation of trimers and tetramers. Tetramers derived from the unmutated scFv showed the greatest improvement in ELISA. Furthermore it was noted that it is easier to improve the avidity of an scFv that performs poorly in ELISA than of that which is already a good binder.

Chapter 4

ScFv-alkaline phosphatase fusion proteins

4.1 Introduction

The pHEN1 vector was used to construct the *Nkuku*® library. It is a good cloning and expression vector but it only contains the c-myc tag for detection and purification of scFvs. The c-myc tag binds to an anti-c-myc tag monoclonal antibody 9E10 during purification. For large scale purification use of 9E10 is expensive as a large amount is needed thus it became necessary to explore alternative purification systems with alternative tags. The pSANG 14-3F vector was explored as an alternative as it contains a 6x His-tag for purification using metal affinity chromatography. It also allows for the fusion of the scFv to bacterial alkaline phosphatase (AP) which occurs naturally as a dimer (Martin *et al*., 2006). This forms a molecule presenting two scFvs with an advantage of increased avidity (Kerschbaumer *et al*., 1996; Griep *et al*., 1999). In addition to that, the alkaline phosphatase allows direct detection of scFv binding with the substrate thus removing the need for secondary reagents in ELISA.

A gene encoding the recombinant chicken scFv F10 that reacts with high affinity to the VP7 antigen of bluetongue virus was cloned into the pSANG 14-3F vector. A primer was used to add the *NcoI* site to the coding region for the scFv by PCR followed by expression in *E. coli* cells using an auto-induction medium. Dimeric fusion proteins were created consisting of scFv fused to the N-terminus of alkaline phosphatase and a 6x His-tag to the C-terminus of the alkaline phosphatase gene for easy purification using metal affinity chromatography (Figure 4.1).

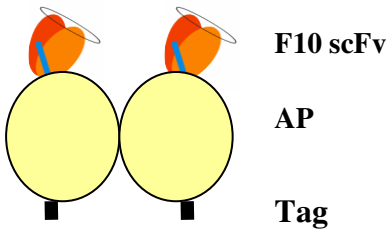


Figure 4.1 Schematic representation of the of the F10-AP fusion protein. The blue line represents the linker and the circled part is the paratope. The image was adapted from that of Furuta *et al*., 1998.

4.2 Materials and methods

4.2.1. Antigens, scFvs and vector

The F10 scFv (Genbank AY631243), purified VP7 antigen and the VP7 capturing antibody anti-BTV rabbit IgG were provided by Mr. Wouter van Wyngaardt. The pSANG 14-3F vector was provided courtesy of D. Martin from the Sanger Institute (Martin *et al*., 2006). Agar stabs of the vector were sub-cultured by streaking on Luria Broth (LB) agar plates (see Appendix) supplemented with 0.2% glucose and 50 µg/ml Kanamycin (Kan) (LB/Glu/Kan). The plates were incubated overnight at 37°C. A single colony was picked and inoculated into 5ml 0.2% LB/Glu/Kan medium (see Appendix) and incubated at 37°C for 8 hours shaking at 220 rpm. The starter culture was diluted $^{1}/_{500}$ into LB/Glu/Kan medium and grown at 37°C shaking overnight. The rest of the starter culture was used to make 15% glycerol stocks and stored at -70°C. The plasmid was purified from the cells using QIAquick plasmid purification kit and stored at -20°C.

4.2.2. Cloning scFv F10 into pSANG 14-3F vector

In order to be able to use the vector, an *NcoI* site had to be inserted into the coding region for the V_H domain of the scFv by PCR. The primer pair Hnco1 (Table 3) and LCNOT1 (2.2.2) were used to amplify the coding region for the F10 scFv out of the vector pHEN1. The PCR reaction consisted of 2.5 U of Faststart High Fidelity enzyme, 1 ng of scFv DNA and 0.4 pmol/µl of each primer in a total reaction of 50 µl. Supplementary PCR reagents were added according to the Faststart kit manufacturer's instructions. The reaction was allowed to proceed for 30 cycles of 94ºC for 15 s, 55ºC for 15 s and 72ºC for 30 s. The PCR products were extracted from a 1.5% EtBr agarose gel using TaKaRa Recochips as per manufacture's instructions. HyperLadder DNA standards were used to determine the size of the PCR products. To cut the insert at the *NcoI* and *NotI* sites 1 U of each restriction enzyme was added with 1 µg insert DNA in a total reaction of 25 µl and incubated at 37°C overnight. The vector (1 µg) was digested as above. The vector was dephosphorylated with 1 U Antarctic phosphatase (New England Biolabs, Massachusetts, USA) at 37°C for 20 minutes followed by incubation at 65°C for 20 minutes to deactivate the enzyme. The scFv gene was ligated into the vector with 1 U T4 DNA Ligase (Roche). OneShot® BL21 Star™ (DE3) chemically competent cells (Invitrogen) were transformed with 10 ng of the

ligations by heat shock at 42°C for 30 s (Inoue *et al*., 1990). Serial dilutions of 10^{-1}-10^{-4} were plated onto LB/Glu/Kan plates and incubated overnight at 30°C. To determine the presence of inserts by colony PCR, 10 colonies were picked randomly and resuspended in 20 µl distilled water (dH2O). These were streaked on LB/Glu/Kan plates for each colony picked to serve as a source of the scFv once the colony PCR results have been seen. The rest was boiled for 5 minutes and put on ice thereafter centrifuged for 5 minutes at 16 000 x g. The supernatant (8.5 µl) was used in a 25 µl PCR reaction comprising 12.5 µl Promega PCR Master Mix (Promega, California, USA), 0.4 pmol/µl primers OP52 and M13rev. The reaction was allowed to proceed for 25 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s. The PCR products were electroporated on a 1% EtBr agarose gel using 1x TAE buffer at 100 V for one hour. HyperLadder makers were used to determine the size of PCR products. To determine if the cloning was correct, DNA was isolated from single colonies grown up in 5ml LB/Glu/Kan at 30°C, shaking at 240 rpm by using a QIAprep Spin Miniprep plasmid purification kit. Sequencing primers T7promoter2 (Martin *et al*., 2006) and T7rev were used (Table 3). Automated sequencing was done by the Molecular Biology Division. Sequences were analysed using the BioEdit software package (Hall, 1999).

Table 3: Nucleotide sequences of DNA primers in cloning and sequencing F10-AP fusion proteins

Primer	**Sequence**
Hnco1	5’ CCA TGG CGG CCG TGA CGT TG 3’
T7promoter2	5’ GAT CGA GAT CTC GAT CCC GCG A 3’
T7rev	5’ TAGTTATTGCTCAGCGGTGG 3’

4.2.3. Expression and purification F10-AP fusion proteins

Upon sequence confirmation that the gene was correctly inserted into the vector, transformation was performed as above and a $^{1}/_{100}$ dilution of culture was made in 500 ml Overnight ExpressTM Instant TB Medium (Novagen, Darmstadt, Germany) supplemented with 1% glycerol and 50 µg/ml Kan and incubated for 48 hours at 30°C, 150 rpm. The F10-AP fusion protein was

extracted from the periplasm. Cells from the overnight culture were collected by centrifugation at 2000 x g for 15 minutes. The pellet was placed on ice and gently resuspended $^{1}/_{20}$th volume ice cold TES (see Appendix) containing Benzonase® Nuclease (Novagen), Lysozyme™ Bioprocessing reagent (Novagen) and Complete Mini EDTA-free protease inhibitors (PI); (Roche). The suspension was incubated on ice for 10 minutes then centrifuged at 6000 x g for 10 minutes at 4°C. The supernatant containing the F10-AP was transferred into a new tube. The remaining pellet was gently resuspended in ice cold 5 mM MgSO4/benzonase/lysozyme/PI. The suspension was incubated on ice and centrifuged at 6000 x g for 10 minutes at 4°C. The °supernatant was transferred into the tube containing the TES supernatant and mixed gently. The combined scFv-AP supernatants were centrifuged at 16000 x g for 20 minutes at 4°C. The supernatant was stored at 4°C and was of sufficient purity to be used in ELISA. The scFv-AP fusion protein extracts were also purified under native conditions using a Probond™ Purification System (Invitrogen) column as described by the manufacturer. The protein was dialyzed against 1x PBS at 4°C overnight using Slide-A-Lyzer 7K MWCO Dialysis Cassettes (Pierce) in order to remove the imidazole in the elution buffer. The PBS was changed four times during the dialyses. The protein was measured at 562 nm using the Bicichoninic Acid (BCA) Protein Assay Kit (Sigma) according to the manufacture's instructions. The dialysed protein was loaded onto an SDS-PAGE gel as described previously. Samples were compared with 5 µl of Benchmark™ His-tagged Protein Standards (Invitrogen). The gel was stained in Coomassie solution (see Appendix) and destained with 4% acetic acid. The protein was transferred onto a PDVF membrane overnight (Towbin *et al*., 1979). The membrane was developed in $^{1}/_{1000}$ HisDetector™ Nickel-HRP (KPL, Gaithersburg, USA) according to the manufacture's instructions.

4.2.4 ELISA

The F10-AP fusion proteins were characterised by ELISA (Fehrsen *et al*., 2005) with modifications. Instead of F10, F10-AP scFv (10 µg/ml) in 2% MP-PBS was added and incubated for one hour at 37°C, and the plate washed as described. Phosphatase substrate (50 µl) p-Nitrophenyl phosphate (Sigma) was added and left to stand for one hour at RT. The absorbance was measured at 405 nm. An ELISA of unpurified periplasmic extract was also conducted where F10-AP scFvs were diluted 1:1 in 4% MP-PBS followed by a $^{1}/_{125}$ serial dilution in 2% MP-PBS.

4.2.5 Size exclusion chromatography

To confirm the mass of the F10-AP fusion protein purified scFvs-AP proteins were separated by gel filtration using a Superdex 75 10/30 GL (Amersham Pharmacia, Uppsala, Sweden) on the AKTA Prime Plus (Amersham Biosciences) liquid chromatography system according to the manufacturer's instructions. Samples were compared with gel filtration molecular mass markers (Sigma) to estimate the size of the molecules. Sample fractions were collected in 1 ml eppendorf tubes and the presence of F10-AP scFv was tested by addition of 50 µl p-Nitrophenyl phosphate substrate and left to stand for one hour at RT. These experiments were conducted by Joy Sixholo (author).

4.3 Results

4.3.1. Cloning scFv into pSANG 14-3F vector

In order to be able to insert the scFv gene into the pSANG 14-3F vector an *NcoI* site has to be added to the 5' end of the coding region for the scFv F10 by PCR. A PCR product of ~800 bp in molecular mass on EtBr gel confirmed that the primer Hnco1 added an *NcoI* site to the scFv gene (Figure 4.2, Lane 2). F10 (without *NcoI* site) was amplified with the primers Sfi1L and LCNOT1 as a control (Figure 4.2, Lane 1). After the ligation was used to transform *E. coli* BL21 cells, ten colonies were picked randomly for colony PCR. The PCR showed four ~800 bp bands (1, 3, 5, and 8) representing scFv insert (Figure 4.3). Sequence analyses showed that the scFv had been inserted correctly into the pSANG 14-3F vector (data not shown). ScFv 3 was chosen to be expressed.

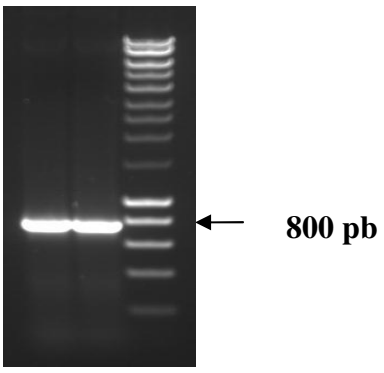


Figure 4.2 PCR after addition of *NcoI* site to F10. Lanes: (M) molecular mass markers; (1) F10; (2) F10 with *NcoI* site.

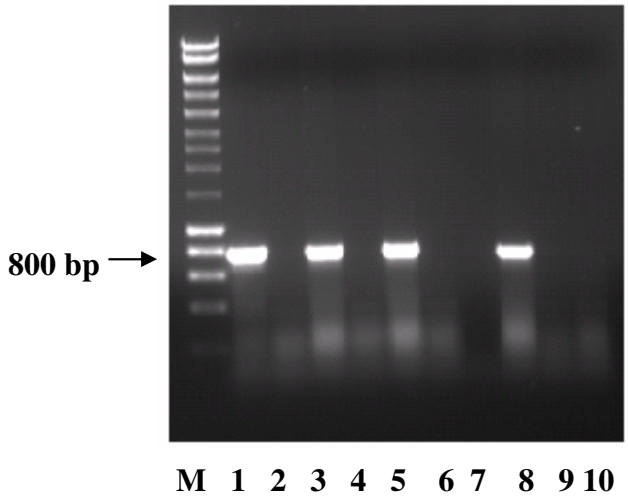


Figure 4.3 Colony PCR of F10-AP clones. Lanes: (M) molecular mass markers; (1-10) PCR products from randomly picked clones.

4.3.2. Expression and purification of F10-AP fusion proteins

ScFv 3 was used to induce protein expression and the protein purified using a nickel column. The purity of the F10-AP fusion protein was assessed by SDS-PAGE followed by Coomassie Blue staining (Figure 4.4). A band with an apparent molecular mass of 75 kDa was visible on the gel. This was confirmed to contain a histidine tag by a band of the same molecular mass on the western blot. This molecular mass is made up of the 45 kDa subunit of alkaline phosphatase plus the scFv which is about 30 kDa in size (Figure 4.1). The purified protein was found to contain other small proteins probably due to non-specific binding to the column. However, only a single band was seen on the western blot showing that only the scFv-AP fusion protein reacted with the HisDetector™ Nickel-HRP or the western blot was not sensitive enough to detect the other proteins as they occurred in lesser amounts (Figure 4.5). The flowthrough and wash samples did not show any binding to the nickel-HRP indicating that there was very little or no loss of product during purification. The fusion protein was expressed and a yield of 0.6mg of protein was obtained from a 500 ml culture.

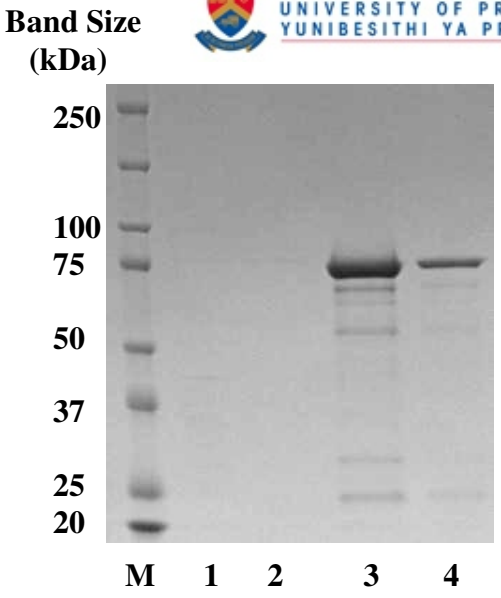


Figure 4.4 A Coomassie stained SDS-PAGE gel showing an affinity purified scFv-AP fusion protein. Lanes: (M) molecular mass markers; (1) first wash; (2) second wash; (3) first elution of purified protein; (4) second elution of purified protein.

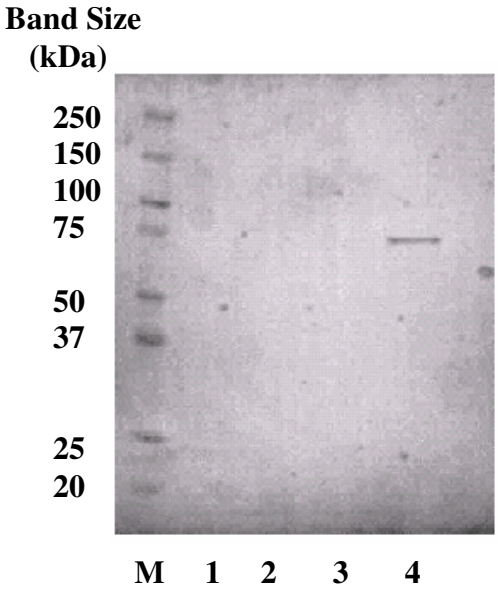


Figure 4.5 A western blot showing purified F10-AP fusion protein reacting with a nickel detector. Lanes: (M) molecular mass markers; (1) flowthrough; (2) first wash; (3) second wash; (4) purified protein.

4.3.3 ELISA

The ELISA confirmed that the F10-AP fusion exhibited both antigen binding and enzyme activity (Figure 4.6). Although purified protein was used in this ELISA, the purity of the periplasmic extract is also adequate for use in ELISA without further purification (Figure 4.7). The concentration of the periplasmic fraction was so high that it had to be diluted more than 3000-fold to get a signal of less than 1.0 in ELISA. Furthermore the fusion of the scFvs to bacterial alkaline phosphatase has allowed for direct detection thus eliminating the need for secondary reagents.

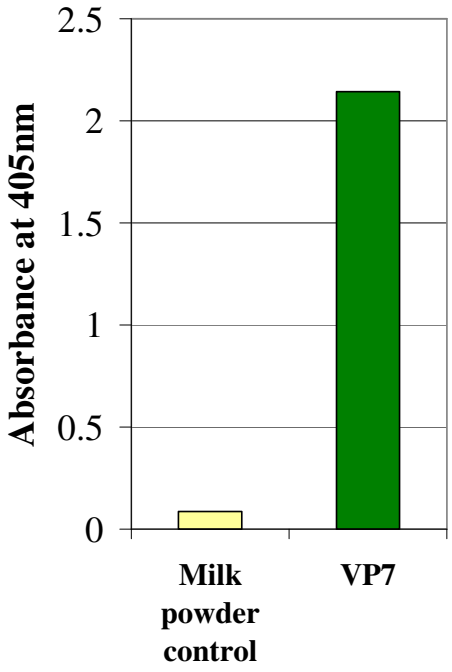


Figure 4.6 Sandwich ELISA of purified F10-AP scFv binding to VP7 antigen and milk powder control.

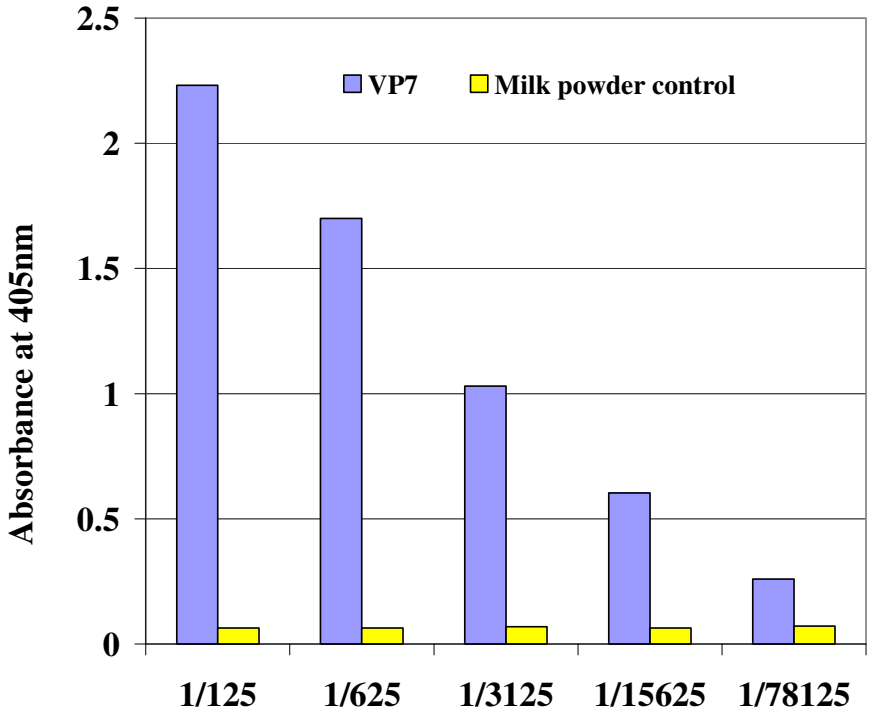


Figure 4.7 Sandwich ELISA of serial dilutions of unpurified F10-AP scFv binding to VP7 antigen and milk powder control.

4.3.4. Size exclusion chromatography

Size exclusion chromatography showed a 140.9 kDa protein confirming that fusion proteins had been formed (Figure 4.8). The R^2 value of the calibration curve for the size exclusion chromatograph depicted in Figure 4.8 was only 94% hence the difference in mass from the expected 150 kDa. Since the protein was not of 100% purity there were other peaks of a smaller size on the chromatograph which can also be seen on the Coomassie stain (Figure 4.4). The presence of F10-AP scFv was confirmed by a colour change resulting from addition of p-Nitrophenyl phosphate substrate in the peak (9.8ml) fraction. There was no colour change in non-peak fractions.

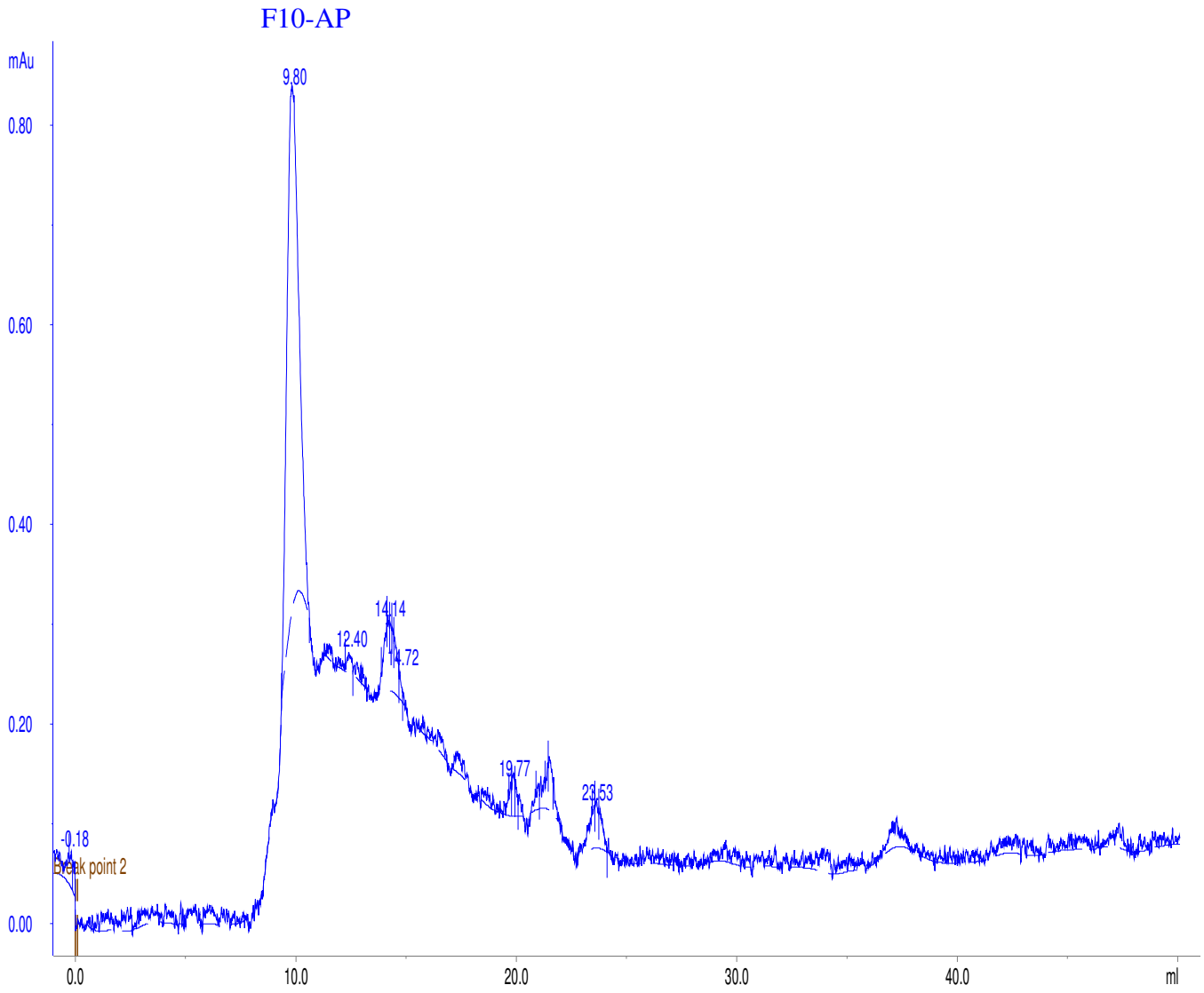


Figure 4.8 Size exclusion chromatograph depicting the F10-AP fusion protein as a dimer (140.9 kDa).

Table 4: Molecular mass of F10-AP and key molecular mass markers, their retention times and elution volumes

Protein	Molecular mass	Retention time	Elution volume (Ve)	(Ve/Vo)
Blue Dextran	2000	16.72	8.35	
Amylase	200	17.46	8.72	1.044
Alcohol dehydrogenase	150	18.23	9.1	1.090
F10-AP	140.9	19.63	9.8	1.174
BSA	66	19.88	9.94	1.190
Carbonic anhydrase	29	24.27	12.14	1.454
Cytochrome c	12.4	27.58	13.78	1.650

Vo = void volume
y = 2.5091x -0.1628
R^2 = 0.9375

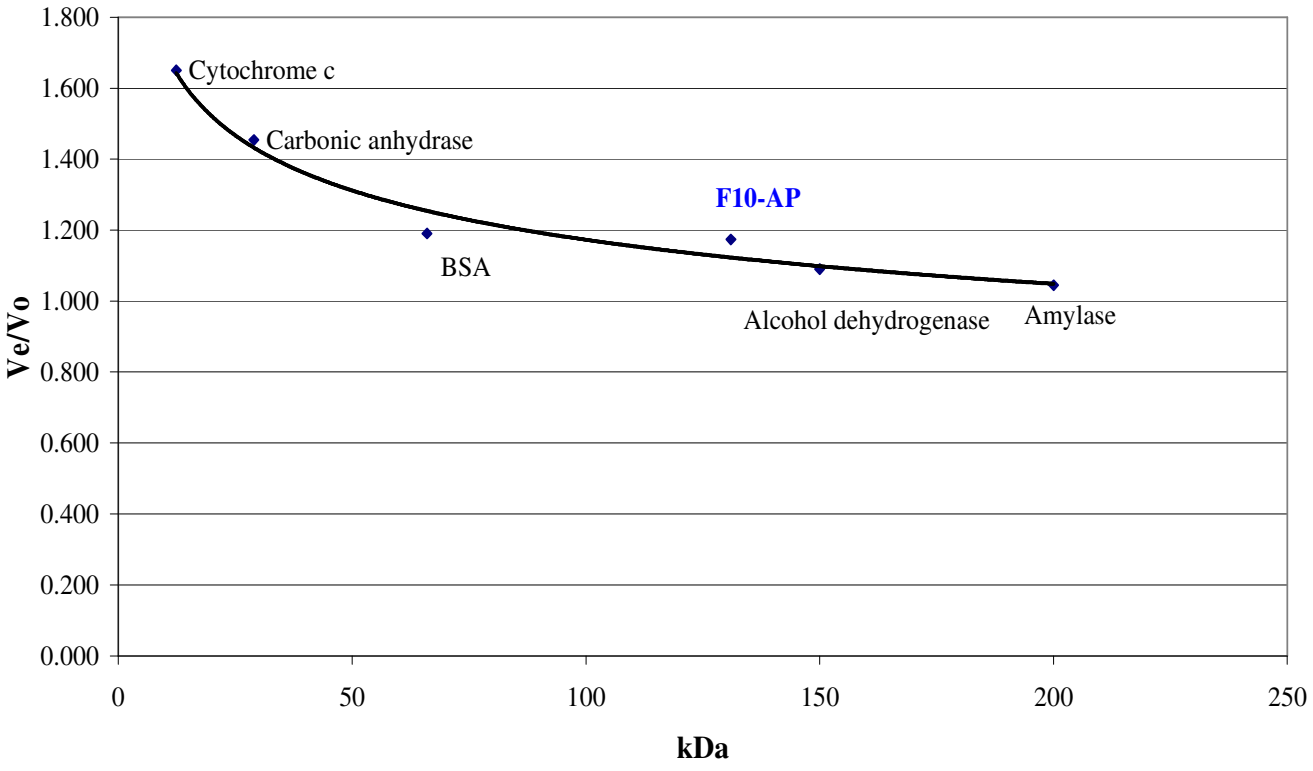
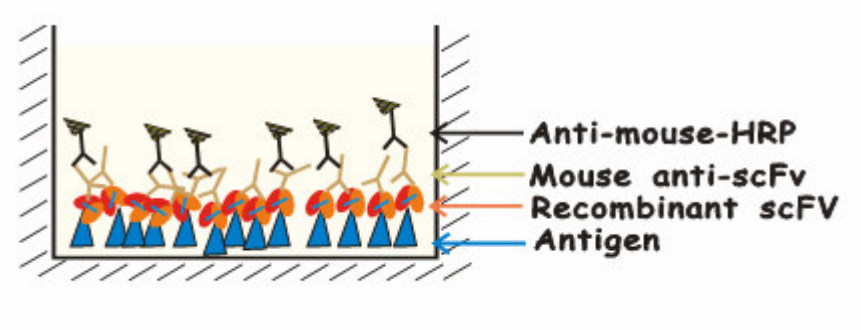


Figure 4.9 Calibration curve at a flow rate of 0.5ml/min obtained for the scFv F10-AP corresponding to Table 4.

4.4 Discussion

This study has shown that it is possible to transfer chicken scFv genes from the pHEN1 vector into the pSANG 14-3F vector resulting in a bi-functional chicken scFv-AP fusion protein that exhibits both alkaline phosphatase activity and specific antigen binding. Furthermore it was possible to directly detect the scFv-AP fusion protein in ELISA without the use of the anti-c-myc tag and rabbit anti-mouse monoclonal reagents. With this system higher avidity has been gained, and the cost and time it takes to do the ELISA has been reduced. A simple illustration demonstrates how elegant this system is.

A



B

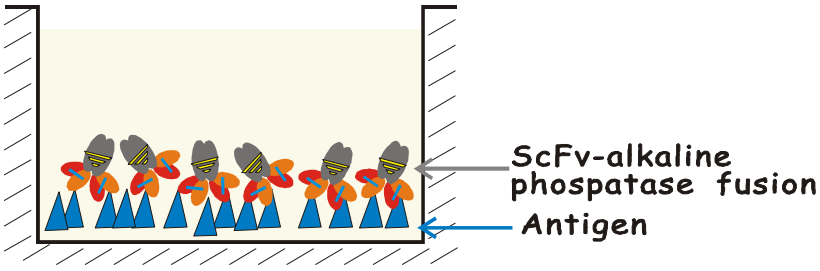


Figure 4.10 An scFv ELISA using 9E10 (A) compared to a scFv-AP direct ELISA (B). Image courtesy of Jeanni Fehrsen.

An ELISA using an unconjugated scFv with a c-myc tag would take four hours out of an eight hour working day using four reagents. The fusion of scFvs to AP cuts the above time required to do the ELISA to two hours.

The scFv-AP fusion proteins were expressed using an auto-induction medium which does not require the addition of IPTG or any other intervention to turn on recombinant protein expression. Auto-induction medium contains a mixture of glucose and lactose (Studier, 2005). The medium is balanced to allow the *E. coli* to reach high density and automatically induce protein production from functional lacY and lacZ promoters. The scFvs-AP fusions were purified in a single step via metal affinity chromatography. A protein yield of 0.6mg was obtained from a 500ml culture. However this was lower than yields of up to 10mg per litre of culture previously obtained when expressing F10 in pHEN1 vector (Personal communication, J. Fehrsen). Other authors with a similar expression system found that when incubation is prolonged for more than 48 hours the proteins are released into the culture supernatant thus eliminating the need for periplasmic extraction (Harper *et al*., 1997). Kerschbaumer *et al*, (1996) were able to obtain 10mg/l of fusion protein from the culture supernatant after a 36 hour incubation using pDAP2 while only 1mg/l from the periplasm was obtained. In the initial stages of the experiment protein expression was carried out over 16 hours. This yielded very low concentrations of protein (not shown), however when the culture was prolonged for 48 hours, protein yield was increased seven-fold. Only the periplasmic fraction of protein was analysed, perhaps the bulk of the protein was released into the supernatant as found by Kerschbaumer *et al*.

In this experiment the dimeric nature of AP was used to dimerise the scFv. Several mouse and human antibody fragments have been fused to AP using a similar approach (Wels *et al*., 1992; Ducancel *et al*., 1993; Harper 1997). The resultant bivalency has been reported to enhance the avidity of antibodies thus contributing to increased affinity for their antigens. VP7 has a single epitope for binding F10 (Fehrsen *et al*., 2005) thus the ELISA signals obtained are due to a 1:1 interaction between the scFv and the antigen. The bivalency of scFv-AP means that two VP7 proteins can bind on a single antibody thus increasing the functional affinity. Also the size of AP does not seem to interfere with scFv binding to antigen. This is the first report that chicken scFvs can be genetically fused to AP with a result of increased avidity.

Concluding remarks

This study has shown that chicken scFvs behave in much the same way as mammalian scFvs when subjected to low frequency random mutagenesis and shortening of the linker. Methods have now been established to improve chicken scFvs and they can be applied if any antibodies against other antigens that have poor characteristics are obtained. A database of the amino acid residues identified to be essential in the improvement of antibody characteristics is being compiled. Some residues will be important for antigen binding while others will be for structure, stability and expression. The effect of some mutations might be specific for binding to the 16 kDa antigen while others may apply for all chicken scFvs. More studies using directed mutagenesis at the residues which have shown to have an effect on improvement will confirm if those residues are essential for antibody-antigen binding, which in turn would make it possible to tailor scFvs with desirable properties.

Appendix

Antibiotics

Antibiotic	Stock solution	Working solution
Ampicillin	100 mg/ml	100 µg/ml
Kanamycin	50 mg/ml	50 µg/ml

Dissolve in dH_2O and filter sterilize with 0.2 µm filter. Aliquot and store at -20°C

Coomassie brilliant blue stain

Coomassie blue	1.25 g
Methanol	225 ml
Acetic acid	50 ml
dH_2O	225 ml

HBS-EP

HEPES pH7	410 mM
NaCl	150 mM
EDTA	3 mM
Surfactant P20	0.005%

Luria broth agar

Agar	13 g
Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
dH_2O	make up to 1 L

Mix well until a clear solution is obtained. Autoclave for 20 minutes at 1.3 kgf/cm^2

Luria broth

Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
dH_2O	make up to 1 L

Mix well until a clear solution is obtained. Autoclave for 20 minutes at 1.3 kgf/cm^2

10x SDS running buffer

Sodium dodecyl sulphate	10 g
Tris	30.3 g
Glycine	144.1
dH_2O	make up to 1 L

50 x TAE buffer

Tris	242 g in 600 ml dH_2O
Na2EDTA (pH 8.0)	0.5M in 100 ml
Glacial acetic acid	51.1 ml
dH_2O	male up to 1 L

TES

Tris-HCl, pH 8.0	30 mM
EDTA	1 mM
Sucrose	20 (w/v)

Bring to volume with dH2O

Filter-sterilise and store at 4 °C

Prior to use add:

Benzonase	25 U/ml
Lysozyme	1500 U/ml
Protease inhibitors	2 µl/ml

Towbin Buffer

Tris	4.53 g
Glycine	21.6 g
dH_2O	make up to 1.5 L

TYE Agar

Agar	15 g
NaCl	8 g
Tryptone	10 g
Yeast extract	5 g
dH_2O	make up to 1 L

Mix well until a clear solution is obtained. Autoclave for 20 minutes at 1.3 kgf/cm^2

Prior to use add:

Glucose	20%
Ampicillin	100 µg/ml

2XTY broth

Tryptone	16 g
Yeast extract	10 g
NaCl	5 g
dH_2O	make up to 1 L

Mix well until a clear solution is obtained. Autoclave for 20 minutes at 1.3 kgf/cm^2

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