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Selection and identification of single domain antibody fragments from camel heavy-chain antibodies

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Abstract Functional heavy-chain γ-immunoglobulins lacking light chains occur naturally in *Camelidae*. We now show the feasibility of immunising a dromedary, cloning the repertoire of the variable domains of its heavy-chain antibodies and panning, leading to the successful identification of minimum sized antigen binders. The recombinant binders are expressed well in *E. coli*, extremely stable, highly soluble, and react specifically and with high affinity to the antigens. This approach can be viewed as a general route to obtain small binders with favourable characteristics and valuable perspectives as modular building blocks to manufacture multispecific or multifunctional chimaeric proteins.

Key words: Antibody; Phage display; Single domain antibody fragment; VH; Camel

1. Introduction

The Fv containing the two variable domains (VH+VL) of immunoglobulins and engineered fragments thereof (e.g. single chain Fv (scFv) [1] or the disulphide stabilised Fv (dsFv) [2]) have been considered as the smallest antibody fragments with retention of the full antigen binding capacity [3,4]. PCR and the development of powerful panning techniques led to the generation of large libraries of scFvs from which several binders could be selected successfully [5–7]. This strategy to obtain binders for a large number of antigens was a major breakthrough in molecular biology. However, the application of the technique is not straightforward [3]. The cloning of two correctly spliced gene fragments (VH+VL) is a difficult step, generating a representative library is tedious, and the genetic constructs are often unstable in the bacterial host. Also, the expression yield, stability and/or functionality of scFv or dsFv often turn out to be problematic [8].

The work of Ward et al. [9] indicated that binders could be readily selected from a pool of cloned spleen VHs from an immunised mouse. These isolated VH domains are expected to bind antigen in absence of VLs. This approach avoids the introduction of a peptide linker used in the scFv constructs which might create additional problems (e.g. reduced affinity, aggregation or proteolytic cleavage) [10]. However, the insolubility of the isolated VH domains expressed in bacteria, and their reduced antigen binding affinity relative to the original VH-VL combination, posed serious limitations for their use. Reshaping the 'VL side' of the human VH to mimic the variable domain of camel heavy-chain immunoglobulins solved the solubility problem [11]. The camelisation of the human VH was based on the knowledge that *Camelidae* produce a substantial proportion of their functional immunoglobulins as homodimers of heavy chains, lacking light chains [12]. The variable domains of these heavy-chain antibodies are distinguishable from the classical VH domains because they consistently carry important substitutions of otherwise conserved amino acids located in the region which is normally covered by the VL [13,14]. To distinguish the classical VH domain from the variable domains of the camel heavy-chain antibodies we refer to the latter as V_HH.

Randomisation of the third hypervariable loop, the most important loop for antigen binding, on a 'camelised human VH' led to a synthetic library from which several hapten binders were selected [15]. Unfortunately, no useful binders against proteins (HIV regulatory protein rev, lysozyme) could be retrieved from the synthetic camelised human VH library.

To avoid this shortcoming, we immunised a camel with two model antigens and generated a V_HH library displayed on phage particles. The camel single domain V_HH harbours the original, intact antigen binding site [4] and is expected to react specifically and with high affinity to the antigen so that further affinity and specificity improvement is unnecessary. Indeed stable, soluble, specific antigen binders with valuable structural properties [4,14], and good affinities (in the range of 0.2×10^8 to 2×10^8 M^{-1}) were identified from this library.

2. Materials and methods

2.1. Camel immunisation

The serum of dromedary (*Camelus dromedarius*) was shown to be non-reacting with tetanus toxoid or lysozyme. This dromedary was injected with tetanus toxoid (100 μg) and lysozyme (1 mg) according to standard immunisation protocols. The blood of the immunised animal was collected and the peripheral blood lymphocytes were prepared with Lymphoprep (Nycomed) and stored at −80°C until further use.

2.2. mRNA isolation, and PCR amplification

mRNA from approximately 10^7 peripheral blood lymphocytes was isolated and used as template for cDNA synthesis (Micro-FastTrack Kit and cDNA Cycle Kit, Invitrogen). The 5′ part of the immunoglobulin heavy chains was amplified by PCR with two gene-specific primers: VHBACKA6: 5′-GAT GTG CAG CTG CAG GCG TCT GG(A\G) GGA GG-3′ and CH2FORTA4: 5′-CGC CAT CAA GGT

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Abbreviations: CDR, complementarity determining region; CRAbs, chelating recombinant antibodies; Fab, antigen binding fragment of an antibody; Fv, heterodimer of VH and VL; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; VH, heavy-chain variable domain; V_HH, variable domain of heavy-chain antibodies; VL, light-chain variable domain. Substitutions are denoted by the wild type amino acid followed by the residue number and the new amino acid

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ACC AGT TGA-3′. From these PCR products we reamplified the V_HH gene with VHBACKA4: 5′-CAT GCC ATG ACT CGC GGC CCA GCC GGC CAT GGC CGA (G\T)GT (G\C)CA GCT-3′ and VHFOR36: 5′-GG ACT AGT GCG GCC GCG TGA GGA GAC GGT GAC CTG-3′ containing *Sfi*I and *Not*I restriction enzymes sites (underlined). The resulting PCR fragments of 450–520 bp were purified from agarose by Geneclean (Bio 101, Inc.), digested with *Sfi*I and *Not*I, and purified again by Geneclean.

2.3. Library construction

The vector (pHEN1) [16] was modified by introducing a new cloning site to insert the camel V_HH gene and by substitution of the c-myc tag with the decapeptide HA-tag [17] (referred to as pHEN4). The pHEN4 phagemid (10 μg), digested with *Sfi*I and *Not*I and gel-purified, was ligated overnight at 16°C with T4 DNA ligase (Boehringer Mannheim) to the PCR fragments (5 μg).

The ligated material was transformed into ultracompetent *XL2-Blue MRF′* cells (Stratagene), and the cells were plated on LB-ampicillin agar plates. The colonies were scraped from the plates with 2×TY-ampicillin, and stored in 2×TY, 1% glucose, 50% glycerol at −80°C until further use. *E. coli XL1-Blue MRF′* cells were made electrocompetent and transformed with a second batch of ligated material to generate the second library.

2.4. Preparation of phage and selection of tetanus toxoid or lysozyme binders

Approximately 10^9 cells were grown to mid-logarithmic phase before infection with M13K07. Virions were prepared as described by Kang et al. [18], and used for panning at a titre of 10^{13}/ml. For panning, we coated the microtitre wells (Nunc, Maxisorp) overnight at 4°C with 100 μl tetanus toxoid (0.25 mg/ml in PBS). For lysozyme coating was performed at 3 mg/ml for the first round and at 1 mg/ml for subsequent pannings. The wells were blocked (PBS+1% casein) at 37°C for 1 h. The adsorption of virions on immobilised antigen and the washing and elution of binders with 50 mM diethylamine were carried out according to Marks et al. [19]. Phages eluted after each round of panning were added to exponentially growing *TG1* cells [16] and plated on LB-ampicillin. The enrichment was evaluated by testing 96 individual clones by ELISA (coating solutions of tetanus toxoid or lysozyme at 5 μg/ml). The presence of the virion binding was revealed by anti-M13/Horse Radish Peroxidase conjugate (Pharmacia).

2.5. Preparation of soluble V_HH fragments

The vector DNA from selected positive clones was transformed into the non-suppressor strain of *E. coli* (*WK6*). The *WK6* cells harbouring the recombinant phagemids were grown at 37°C in 300 ml TB-ampicillin, 1% glucose in culture flasks until OD_{550} 0.5. The cells were washed and resuspended in 300 ml TB-ampicillin, induced with IPTG (1 mM), and incubated overnight at 28°C. The periplasmic proteins were extracted according to Skerra et al. [20]. The periplasmic extracts were concentrated ten-fold by ultrafiltration (Millipore, cut-off 5000 Da). The concentrated extracts of the two tetanus toxoid binders (cAb-TT1 or cAb-TT2) were separated by gel filtration on Superdex-75 (Pharmacia) in PBS. One of the lysozyme binders (cAb-Lys3) was purified by affinity chromatography on a lysozyme-Sepharose column. After washing with PBS, the bound single domain antibody fragments were eluted with 50 mM diethylamine, neutralised and subsequently passed over Superdex-75 (Pharmacia) in PBS buffer. The second lysozyme binder (cAb-Lys2) was purified by a protein A-Sepharose affinity chromatography followed by gel filtration.

2.6. Determination of affinity, specificity and epitope mapping

Affinity was determined by competitive ELISA [21]. The bound camel V_HH fragment was detected with a mouse antibody specific for the Hemagglutinin Tag (clone 16B12, BAbCO, California) or with polyclonal rabbit anti-camel IgG antibodies.

Specificity of V_HH fragments was examined by ELISA [22]. The non-related immunogens included casein, globular part of histone H5, BSA, RNase T1, Arcelin-5, concanavalin A, phytohaemagglutinin, *Pseudomonas aeroginosa* lipopolysaccharides, tetanus toxoid (for lysozyme binders) and lysozyme (for tetanus toxoid binders).

Binding to individual or overlapping epitopes was assessed according to Friguet et al. [22].

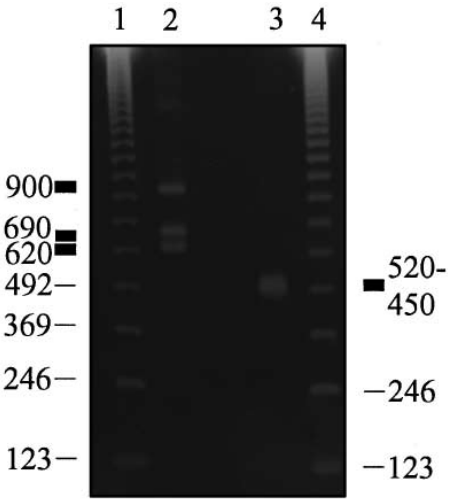

Fig. 1. Agarose gel electrophoresis of V_HH amplification products with VHBACKA6 and VHFORTA4 on camel lymphocyte cDNA (lane 2), and with VHBACKA4 and VHFOR36 on DNA of lane 2 (lane 3). Lanes 1 and 4: 123 bp ladder size marker. The sizes (in bp) are indicated for the marker (thin lines) and PCR products (thick lines).

3. Results

3.1. PCR amplification of camel V_HHs

We used 10^7 peripheral blood lymphocytes from a dromedary immunised simultaneously with tetanus toxoid and lysozyme, to extract mRNA from which cDNA was synthesised. The VH and V_HH regions were amplified by nested PCR. In the first PCR, the VHBACKA6 primer is based on available protein sequence information and on the camel V_HH clone cVH2 [13]. This primer is predicted to anneal to codons 1 to 10 of the camel V_HH of subgroup III, the only family reported to be present in camels [13]. The CH2FORTA4 primer hybridises in the CH2 region of camel g-immunoglobulin mRNA [12]. The resulting PCR fragments have distinct sizes around 620, 690 and 900 bp (Fig. 1). We infer that these PCR products are derived from the homodimer heavy-chain antibodies with short and long hinges and from the heavy chains of the classical heterotetramer immunoglobulins [12]. In a second PCR, we reamplified the V_H and V_HH part of these fragments with a VHBACKA4 primer containing a SfiI and a VHFOR36 primer containing a *Not*I site for cloning purposes. The VHFOR36 primer anneals to the framework 4 region. This nested PCR generated a single broad band between 450–520 bp in length (Fig. 1).

3.2. Camel VH/V_HH library

After *Sfi*I and *Not*I digestion, we ligated the purified PCR fragments into pHEN4 (a modified pHEN1 vector [16]), between the PelB leader signal and the gene III. The ligated DNA material was used to transform *E. coli XL2 Blue MRF′* cells, and approximately 500 000 individual recombinant clones were obtained. A second batch of ligated material was transformed in electrocompetent *E. coli XL1 Blue MRF′* cells. The second library contained 10^7 individual clones. Colony screening by PCR showed that 90% of the clones contained a plasmid with an insert of the expected size for a camel V_HH gene. The heterogeneity of the individual clones from the libraries was checked by sequencing the CDR3 part

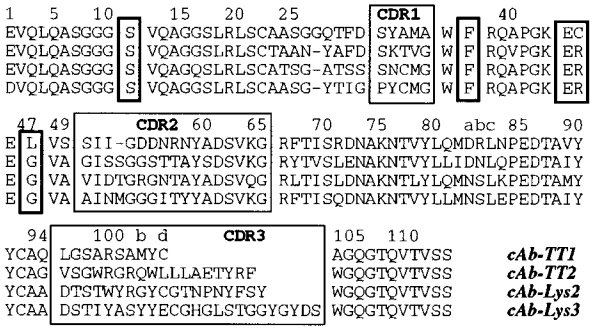

Fig. 2. Amino acid sequences of tetanus toxoid (cAb-TT1 and cAb-TT2) and lysozyme binders (cAb-Lys2 and cAb-Lys3). The Kabat et al. [23] numbering is used, the specific V_HH amino acids are thick-lined boxed, the CDRs are thin-lined boxed.

of the cloned insert. Each clone contained a different CDR3 sequence.

3.3. Phage selection

The small libraries was panned separately for the presence of tetanus toxoid or lysozyme binders. A total of approximately 3×10^{11} phages were used in each round of panning. For tetanus toxoid, we eluted 2×10^5, 1.5×10^6, 1.7×10^6, and 1.2×10^7 phages after the first, second, third, and fourth round of panning, respectively. The phage titre of the non-specific binders from wells without antigen remained constant ($4–6\times10^4$).

To evaluate the enrichment during the pannings with tetanus toxoid, we prepared virions from 96 randomly chosen colonies after each round of panning and tested their binding to tetanus toxoid. In the original library we found 1 clone out of 96 bound to the immobilised tetanus toxoid. This number was increased to 11, 48, 80 and 95 after the first, second, third and fourth round of panning, respectively.

A DNA fragment of V_HH size was amplified by PCR on twenty individual positive clones from the final round of panning. According to small migration differences on acrylamide gels the inserts were grouped into two classes. Sequencing confirmed the presence of two different clones. The DNA phagemids of these clones were referred to pHEN4-αTT1 and pHEN4-αTT2, (BCCM/LMBP; accession number LMBP3247).

Surprisingly, no lysozyme binder was extracted from this small library. The possibility remains that the size or complexity of the smaller library was insufficient. Indeed, following the same procedure, we succeeded in selecting two lysozyme binders from the second library of 10^7 individual clones. These DNA phagemids were named pHEN4-Lys2 and pHEN4-Lys3. Immobilising the C-fragment of tetanus toxin during the pannings, lead to the isolated of the pHEN4-αTT1 from this library. No other binders to the C-fragment of tetanus toxin were obtained.

3.4. V_HH sequences

The amino acid sequences of all four binders have the crucial substitutions Leu11Ser, Val37Phe, Gly44Glu, Leu45Arg (or Cys), and Trp47Gly (or Leu) (Fig. 2) (Kabat numbering [23]). These substitutions of otherwise conserved amino acids are hallmarks of V_HHs originating from heavy-chain homodimer immunoglobulins [13]. The sequence information of the two tetanus toxoid and the two lysozyme binders reveals that different V_HH germline genes were used. Furthermore, the CDR3's with lengths of 10, 18, 19 and 24 amino acids, do not share any sequence similarity. Three of the four clones contain a cysteine in the CDR3 which could possibly form a disulphide bond with a second cysteine located in the CDR1, or with a second cysteine at position 45 in the case of the anti-tetanus toxoid V_HH fragment of pHEN4-αTT1. We inserted a position 26a (Fig. 2) to improve the sequence alignment of this clone with other VHs.

3.5. Production of soluble, monomeric camel V_HHs

The four DNA phagemids of the isolated clones were transformed into *WK6* cells. These cells are unable to suppress the amber stop codon between the cloned V_HH and the gene III, and will produce soluble V_HH fragments upon induction with

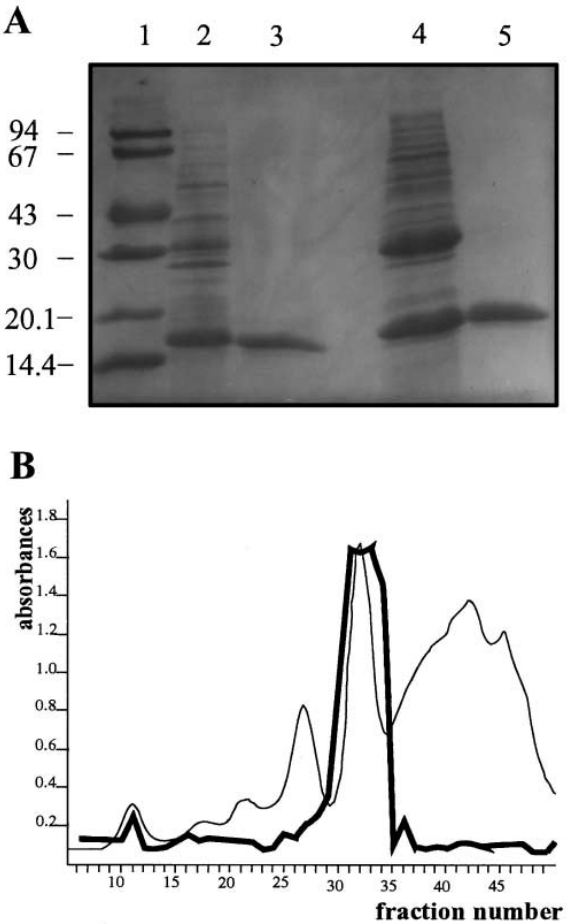

Fig. 3. A: SDS-PAGE of periplasmic extract from IPTG induced *WK6* cells harbouring pHEN4-αTT1 (lane 2), purified fraction of cAb-TT1 (lane 3), periplasmic extract of WK6 with pHEN4-αTT2 (lane 4) and purified fraction of cAb-TT2 (lane 5). Lane 1 is a MW marker (sizes in kDa are indicated). B: Gel filtration of the cAb-TT2 periplasmic extract followed by absorbance at 280 nm (thin line). The tetanus toxoid binding activity of each fraction was measured in ELISA (bold line). An absorbance around 0.1 at 405 nm was measured in wells without tetanus toxoid coating, or without addition of cAb-TT2. The fractions 30–35 contain the monomeric cAb-TT2, whereas fractions 26–29 contain the proteins with MW around 35 000.

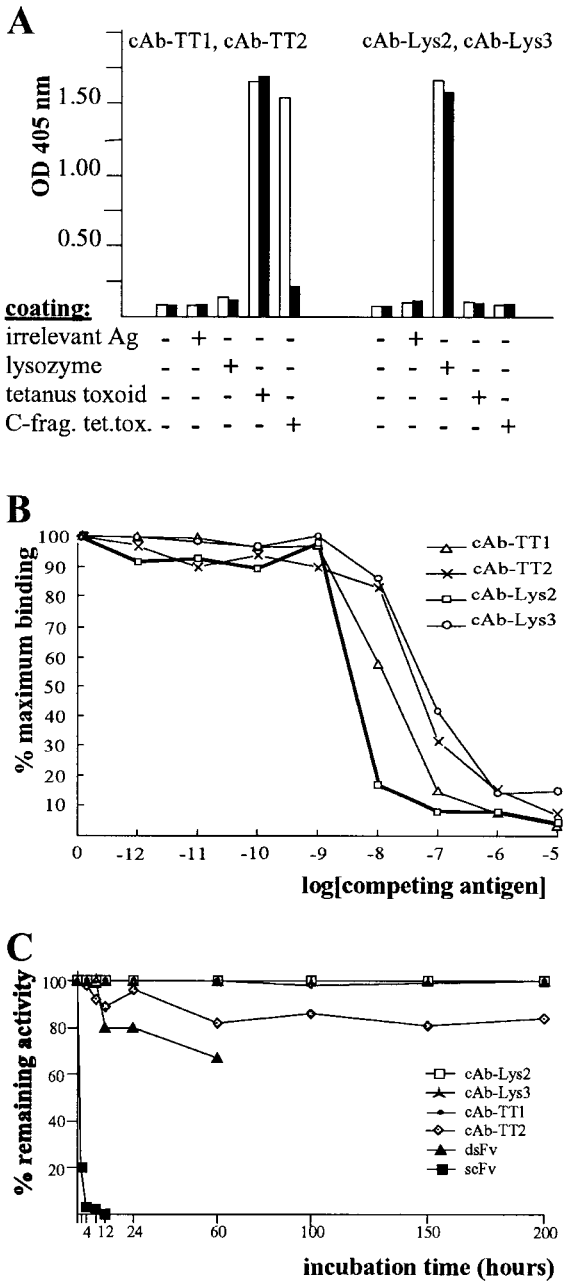

Fig. 4. A: Specificity of the soluble cAb-TT1 and cAb-TT2 (respectively white and black rectangles at the left), or cAb-Lys2 and cAb-Lys3 (white and black rectangles respectively at the right) as determined by ELISA. See Section 2 for a list of the 'unrelated antigens' used (differences among the different unrelated antigens were negligible). B: Affinity of different cAbs measured according to Friguet et al. [21]. C: Antigen binding activity of the different cAbs measured after up to 200 h incubation at 37°C. The experimental conditions were those reported by Reiter et al. [2], and for reference we included their data of the B3 scFv and dsFv.

IPTG. The camel single domain V_HH binders are referred to as cAb.

The expressed proteins were extracted from the periplasmic space and visualised by Coomassie blue staining on SDS-PAGE. Protein bands with apparent MW between 16000 to 18000 were clearly present in the induced *E. coli* cultures containing the recombinant DNA (Fig. 3A). The same proteins were detected by Western blot using a specific rabbit anti-dromedary IgG serum or with an anti-decapeptide tag antibody (data not shown).

The expressed cAbs constitute already a large part of the proteins present in the periplasmic extract. Further enrichment of the soluble cAb-TT1 or cAb-TT2 was achieved by gel filtration on Superdex-75. The single peak containing the anti-tetanus toxoid activity eluted at the expected MW of 16000 indicating that the protein behaves as a monomer and does not dimerise in solution (Fig. 3B).

The anti-lysozyme cAbs were produced with a similar yield. Here the purification was performed by affinity chromatography on protein A-Sepharose for the cAb-Lys2 and on a lysozyme-Sepharose column for the cAb-Lys3. Of all the identified cAbs, only the cAb-Lys2 binds to the protein A-Sepharose column. This is somewhat surprising as most mammalian VHs of family III, to which the camel V_HHs belong, are recognised by protein A [24]. The purification of the cAb-Lys2 on the lysozyme-Sepharose affinity chromatography was abandoned as it eluted from the column only under harsh conditions.

The fractions containing the pure cAbs were pooled. From the absorption measurement at 280 nm, a yield in the range of 3 to 6 mg of purified protein per litre of bacterial culture ($OD_{600nm} = 12$) was calculated. The purified protein was concentrated by ultrafiltration up to 10 mg/ml in PBS or water without any sign of aggregation.

3.6. *Specificity, affinity and stability*

The specificity of the soluble cAb-TT1, cAb-TT2 and of cAb-Lys2, cAb-Lys3 was suggested from the ELISA experiments in which binding was competed with free tetanus toxoid or free lysozyme. Also, no cross-reactivity could be detected in a direct ELISA experiment with several other unrelated immobilised antigens (Fig. 4A).

The affinity constant, measured accurately by ELISA [21], was found to be 6×10^7 M^{-1} and 3×10^7 M^{-1} for the cAb-TT1 and cAb-TT2, respectively. The anti-lysozyme cAb-Lys2 and cAb-Lys3 have an affinity of 2×10^7 and 2×10^8 M^{-1}, respectively (Fig. 4B).

To test the stability of the cAbs, we followed the procedure of Reiter et al. [2] in which the remaining antigen binding activity of the purified antibody fragments was determined after different incubation times in PBS at 37°C. These authors analysed the B3 scFv and the B3 dsFv over an incubation period of 60 h. This particular scFv lost all its funtionality after 24 h, whereas the dsFv retained 60% of its maximal antigen-binding capacity after 60 h of incubation [2]. In contrast, under these conditions the purified camel V_HH fragments retained between 80 and 100% of their original antigen binding activity (Fig. 4C). Moreover, the incubation could be prolonged for up to 200 h without further reducing the lysozyme or tetanus toxoid binding. Therefore the superposition of the data reported by Reiter et al. [2] for the B3 scFv and the B3 dsFv on our cAb data (Fig. 4C) proves that for these conditions the stability of all the cAbs is largely superior to that of the B3 scFv or dsFv [2].

3.7. *Epitope mapping*

Tetanus toxin consists of three domains, named A, B, and C [25]. We showed by ELISA that the cAb-TT1 extract binds equally well both to the complete tetanus toxoid and to the recombinant C fragment (Fig. 4A). Therefore, the epitope of

this camel V_HH is present on the C fragment. In contrast, the cAb-TT2 extract binds to the complete tetanus toxoid, but not to the recombinant C fragment, suggesting that cAb-TT2 recognises an epitope located on the A or B domain (Fig. 4A). An ELISA additivity experiment [22] confirmed that separate epitopes on the tetanus toxoid are recognised (additivity index of 43%).

The anti-lysozyme-specific cAbs were also subjected to an ELISA additivity test. Here the measured additivity index was 58%, which is decisive for binding to two different epitopes on lysozyme [22].

4. Discussion

The presence of naturally occurring and functional homodimer heavy-chain immunoglobulins in *camelidae* sera [12] indicated that isolated camel V_HHs would be more soluble than their mouse or human equivalents. The important amino acid substitutions of otherwise conserved amino acids in the 'former' VL and CH1 sides found in the camel heavy-chain V_HH domains supported this view [13]. Indeed, introducing these substitutions improved the folding and solubility of isolated human VHs [11,15].

In this study we tested the feasibility to identify single domain antibody fragments (V_HH) against two proteins, namely tetanus toxoid and lysozyme. The first antigen was chosen because it is a immunogenic protein [26] that is routinely used as a vaccine in humans. The results of the selected camel V_HH might therefore be compared to the human anti-tetanus toxoid Fabs [17,26]. The availability of abundant detailed structural knowledge of the anti-lysozyme antibodies [27] was the major reason for choosing the second antigen.

Libraries containing the variable region repertoire of heavy chains from immunised camel blood lymphocytes, were constructed. The libraries were panned separately against tetanus toxoid and lysozyme. After four rounds of panning with tetanus toxoid or lysozyme we retrieved each time two clones binding to different epitopes on the antigen. The amino acid sequences of all four binders are consistent with the camel V_HHs. Apparently different V_HH germline and D minigenes were used in the VDJ recombination. This indicates that a sufficiently large repertoire of minigenes lies at the origin of the camel heavy-chain antibody generation. We are therefore confident that other immunogens can also be used to select camel single domain binders.

The yield of the purified cAb-TT2 reaching 6 mg per litre of bacterial culture is on a molar basis comparable or even better than most scFv or Fab expressions obtained in *E. coli*. Since three of our four clones under study possess Gly47, it can be inferred from the work of Davies and Riechmann [15] that the expression yield might even increase by a Gly47Ile substitution. Furthermore, the solubility of cAb-TT2 at 10 mg/ml is certainly higher than that of isolated mouse VH fragments. We also indicated the strictly monomeric nature of the V_HHs. The yield and solubility are well within the range needed for most applications.

A striking feature of the cAbs is their capacity to withstand prolonged incubation at 37°C. cAb-TT2 is slightly less stable, possibly due to the absence of a disulphide bond between its CDRs. Indeed, the importance for stability of the extra disulfide bond in the camelised human VH fragments was shown by thermal denaturation [28].

The cAb affinities in the range of 0.2 to 2×10^8 M^{-1} compare favourably with those of human anti-tetanus toxoid Fab fragments (10^7-10^9 M^{-1}) [17,26] or mouse anti-lysozyme scFvs D1.3 and HyHEL-10 (1.4×10^6 and 3.0×10^8 M^{-1} respectively) [29]. Probably the high affinity and specificity of cAb for a large antigen comes predominantly from the long CDR3 loop which provides a sufficiently large antigen binding surface able to insert into clefts of the antigen. In the crystal structure of the cAb-Lys3::lysozyme complex [14] the CDR3 protrudes from the remaining antigen binding site and is indeed inserted in the cleft of the catalytic site of the enzyme. Consequently, the absence of the VL domain in camel heavy-chain antibodies does not compromise their affinity or specificity. Moreover, the generation of specific camel V_HHs might be an adequate strategy to develop enzyme or receptor inhibitors [4].

It might be argued that a library of a camelised human VH topped with a long synthetic CDR3 might be used to generate small molecular recognition units. This strategy is similar to the one we describe here and has the advantage that it bypasses the dromedary immunisation. However, it should be noted that these synthetic antibody fragments are not maturated in vivo like the natural camel antibodies, and so the camelised single domain VHs might require additional engineering, especially in the CDR1 and CDR2, to improve their affinities [29]. Secondly, although this method was successful for the generation of hapten binders, only poor binders to protein antigens could be retrieved from the synthetic library of a camelised human VH [15]. Possibly, the long and unconstrained synthetic CDR3 loop which becomes immobilised upon antigen interaction has a counterproductive entropic effect on binding. In contrast, the long CDR3 loop of the cAb-Lys3 showed considerable internal structure to stabilise and constrain the loop even in the uncomplexed form [14].

Considering the single domain nature of the cAbs, it should be straightforward to construct small bivalent or even multivalent binders, or CRAbs [30] in order to increase their avidity. Also, the generation of bispecific constructs can be envisaged to broaden the potential applications of camel single domain antibodies [31]. In all cases the resulting molecules will always be simpler, smaller, and probably more stable than the corresponding derivatives of scFvs or Fabs.

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