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

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Abstract Functional heavy-chain γimmunoglobulins 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 chimeric proteins.

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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^{9} to 2×10^{9} 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

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ACC AGT TGA-3'. From these PCR products we reamplified the
VH gene with VHBACKA4: 5'-CAT CCT GGT ATG AGT GCC CCA GGC CC GCC CAT GGC CCA GGT GCT GTC CGG CTA GCT-3' and
VHFOR36: 5'-GG ACT AGT GCG GCC GCG TGA GGA GAC GAC GGT GAT CTG-3' containing SfiI and NotI restriction enzymes sites
(underlined). The resulting PCR fragments of 450-520 bp were
purified from agarose by GeneClean (Bio 101, Inc.), digested with SfiI and
NotI, 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 VH gene and by substitution of the c-myc tag
with the decapetide HA-tag [17] (referred to as pHEN4). The
cHEN4 phagemid (10 μg), digested with SfiI and NotI and gel-puri-
fied, 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 (Strategene), and the cells were plated on LB-ampicillin agar plates. The colonies were scraped from the plates with 2×TY-
ampicillin, and stored in 300 ml TB-ampicillin, induced with
5 μg/ml of tetanus toxoid or lysozyme at 5 μg/ml. The presence of the virion binding
was revealed by anti-M13/Horse Radish Peroxidase conjugate (Phar-
macia).

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

Approximately 10^9 cells were grown to mid-logarithmic phase be-
fore infection with M13KO7. Virions were prepared as described by Kanai et al. [18], and used for panning at a titre of 10^11/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 TGI 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 (Phar-
macia).

2.5. Preparation of soluble VH H 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-ampi-
cillin, 1% glucose in culture flasks until OD600 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 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 bind-
ers (cAb-Lys3) was purified by affinity chromatography on a lyso-
yzme-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 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 VH H 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 VH H fragments was examined by ELISA [22]. The
non-related immunogens included casein, globular part of histone
H5, BSA, RNase T1, Ascaris-S, concanavalin A, phoTohAaglulivi-
Hain, Pseudomonas aeroginosa lipopolysaccharides, tetanus toxoid
(for lysoyme binders) and lysozyme (for tetanus toxoid binders).

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

Approximately 3 x 10^{11} phages were used in each round of panning. The VgH heavy-chain sequences [12] were used to identify the VHHS immunoglobulins [13] as hallmarks of VHHS origin. Several substitutions, such as Leu11Ser, Trp47Gly (or Cys), and Thr12Val (or Cys) were obtained. The C-fragment of tetanus toxin were obtained from this library. No other binders to the C-fragment of tetanus toxoid were isolated during the pannings, leading to the isolation of the pHEN4-otTT1 and pHEN4-Lys3. Immobilising the C-fragment of tetanus toxoid in the original library we found 1 clone out of 96 bound to the immobilised tetanus toxoid. This number increased to 11, 48, 80 and 95 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 x 10^{10}).

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 or lysozyme. A total of approximately 3 x 10^{11} phages were used in each round of panning. For tetanus toxoid, we eluted 2 x 10^{9}, 1.5 x 10^{6}, 1.7 x 10^{6}, and 1.2 x 10^{10} 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 x 10^{10}).

The 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 VHH amino acids are thick-lined boxed, the CDRs are thin-lined boxed.

3.3. Phage selection

The small libraries was panned separately for the presence of tetanus toxoid or lysozyme binders. A total of approximately 3 x 10^{11} phages were used in each round of panning. The sequence information of the cloned insert. Each clone contained a different CDR3 sequence.

3.4. VgH sequences

The amino acid sequences of all four binders have the crucial substitutions Leu11Ser, Val37Phe, Gly44Glu, Leu45Arg (or Cys), and Trp47Gly (or Cys) (Fig. 2) (Kabat numbering [23]). These substitutions of otherwise conserved amino acids are hallmarks of VgHs originating from heavy-chain homodimer immunoglobulins [13]. The sequence information of the two tetanus toxoid and the two lysozyme binders reveals that different VgH germline genes were used. Furthermore, the CDR’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 VgH fragment of pHEN4-αTT1. The inserted a position 26α (Fig. 2) to improve the sequence alignment of this clone with other VHs.

3.5. Production of soluble, monomeric camel VgHs

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 VgH and the gene III, and will produce soluble VgH fragments upon induction with

![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. (2002) numbering is used, the specific VHH amino acids are thick-lined boxed, the CDRs are thin-lined boxed.](http://example.com/fig2)

![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 35000.](http://example.com/fig3)
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 16,000 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_{H}Hs 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_{400nm} = 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 $6 \times 10^7$ M$^{-1}$ and $3 \times 10^7$ M$^{-1}$ for the cAb-TT1 and cAb-TT2, respectively. The anti-lysozyme cAb-Lys2 and cAb-Lys3 have an affinity of $2 \times 10^7$ and $2 \times 10^8$ 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 functionality 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_{H}H 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 anti-dromedary IgG serum or with an anti-decapeptide tag antibody (data not shown).
this camel V\textsubscript{H}H 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 	extit{camelidae} sera [12] indicated that isolated camel V\textsubscript{H}Hs 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\textsubscript{H}H domains supported this view [13]. Indeed, 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\textsubscript{H}H) 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\textsubscript{H}H 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\textsubscript{H}Hs. Apparently different V\textsubscript{H}H 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 scFvs 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 Rieckmann [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\textsubscript{H}Hs. 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 disulphide 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\textsuperscript{9} M\textsuperscript{-1} compare favourably with those of human anti-tetanus toxoid Fab fragments (10\textsuperscript{7}–10\textsuperscript{9} M\textsuperscript{-1}) [17,26] or mouse anti-lysozyme scFvs D1.3 and HyHEL-10 (1.4×10\textsuperscript{8} and 3.0×10\textsuperscript{8} M\textsuperscript{-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\textsubscript{H}Hs 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 matured 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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