

Research Article

# Camelid V<sub>H</sub>H affinity ligands enable separation of closely related biopharmaceuticals

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Interest in new and diverse classes of molecules such as recombinant toxins, enzymes, and blood factors continues to grow for use as biotherapeutics. Compared to monoclonal antibodies, these novel drugs typically lack a commercially available affinity chromatography option, which leads to greater process complexity, longer development timelines, and poor platformability. To date, for both monoclonal antibodies and novel molecules, affinity chromatography has been mostly reserved for separation of process-related impurities such as host cell proteins and DNA. Reports of affinity purification of closely related product variants and modified forms are much rarer. In this work we describe custom affinity chromatography development using camelid V<sub>H</sub>H antibody fragments as “tunable” immunoaffinity ligands for separation of product-related impurities. One example demonstrates high selectivity for a recombinant immunotoxin where no binding was observed for an undesired deamidated species. Also discussed is affinity purification of a coagulation factor through specific recognition of the gamma-carboxylglutamic acid domain.

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## 1 Introduction

The development of a downstream process, meeting the objectives set for product purity and yield, can be challenging when dealing with recombinant proteins sourced from complex mixtures containing high levels of host cell related and product-related impurities. Conventional techniques for protein separation that are based on differences in physical properties like charge, hydrophobicity

and size often requires multiple chromatography steps to reach the desired purity level which could hamper an economically feasible process depending on the loss of product at each step [1]. Most ideally an efficient and highly specific capture step is incorporated to limit the number of process steps and achieve predefined product specifications with adequate yields. For immunoglobulin G based therapeutics this is accomplished by protein A chromatography which, since its introduction in the 1970s, has evolved into a well-established affinity purification platform combining efficient clearance of the bulk impurities as well as concentration of the target product [2, 3].

To seek similar advantages of a highly specific capture step for a broader range of biopharmaceuticals, a unique class of antibody based affinity ligands has been explored over the last decade and has matured into a robust technology for generating chromatography matrices compatible with bioprocess manufacturing [4–6]. These affinity ligands consist of single monomeric variable domains of camelid heavy-chain only antibodies (V<sub>H</sub>Hs) that can efficiently be expressed at large scale in baker's yeast free of

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**Abbreviations:** AEC, anion exchange chromatography; CCCB, clarified cell culture broth; cGMP, current good manufacturing practice; CHO, Chinese hamster ovary; DBC, dynamic binding capacity; Gla, gamma-carboxylglutamic acid; HPLC, high performance liquid chromatography; IgG, immunoglobulin G; mAb, monoclonal antibody; PBS, phosphate buffered saline; PE, *Pseudomonas* exotoxin; QHP, Q Sepharose High Performance; QFF, Q Sepharose Fast Flow; RP, reversed phase; SEC, size exclusion chromatography; V<sub>H</sub>H, camelid heavy-chain only antibody variable domain

animal derived components [7, 8]. Although less than one tenth of the size of a conventional four-chain antibody, single  $V_HH$  domains can provide similar target specificity and affinity in addition to a more favorable physicochemical stability to suit chromatographic processes [9, 10]. These intrinsic properties have enabled the introduction of  $V_HH$  based affinity matrices that demonstrate protein A like features for HCP, DNA and viral clearance for a variety of biopharmaceuticals including recombinant proteins such as Factor VIII [5, 6, 11] and follicle stimulating hormone [12], adeno-associated viruses for gene therapies [13, 14], and human antibodies and antibody Fab fragments not covered by protein A [15–17]. Its implementation as a highly specific capture step in cGMP manufacturing processes for recombinant proteins [5, 6] further supports the advantages of  $V_HH$  based affinity chromatography in downstream process development of biopharmaceuticals.

Although separation from host cell related contaminants has been addressed, a more challenging objective for target selectivity arises when posttranslational modifications or harsh process conditions result in closely related, but less biologically active, forms of the biopharmaceutical in the product stream [18, 19]. In this work we have explored the capability of the  $V_HH$  affinity technology to generate selectivity for the most desired and biologically active forms of prothrombin and an immunotoxin fusion protein.

Human coagulation Factor II, or prothrombin, is the precursor of the protease thrombin that plays a key role in blood coagulation. Prothrombin is a single-chain glycoprotein with a molecular weight of 72 kDa that consists of several structural and functional domains. It is initially synthesized in the liver as a preproprotein with an amino-terminal signal sequence directing it to the endoplasmic reticulum. In the endoplasmic reticulum, the signal peptide is proteolytically removed, and the remaining pro-form of the molecule is recognized by the enzyme gamma-glutamyl carboxylase that catalyzes the carboxylation of ten gamma-carboxylglutamic acid (Gla) residues in the amino-terminal portion (referred to as the Gla-domain) of the molecule in a process that requires reduced vitamin K, oxygen and carbon dioxide [20–22]. This essential posttranslational modification confers metal-chelating properties on prothrombin: the Gla-domain can bind  $Ca^{+2}$  ions, and then mediates the interaction with phospholipid membranes that is required for activity. For the recombinantly expressed prothrombin molecule, a direct correlation was demonstrated between the level of carboxylated glutamate residues and bioactivity [18].

Moxetumomab pasudotox (m. pasudotox) is a 63.4 kDa recombinant immunotoxin composed of the  $V_H$  and  $V_L$  portions of an anti-CD22 antibody connected by a disulfide bond and fused to a truncated form of *Pseudomonas* exotoxin (PE38) by a peptide bond to  $V_H$ . M. pasudotox is

currently in clinical trials for the treatment of B-cell malignancies [23, 24]. The immunoglobulin variable domain is composed of affinity matured  $V_H$  and  $V_L$  chains of an anti-CD22 monoclonal antibody, while PE38 contains the PE toxin domains II and III. Domain II has translocation activity while domain III catalyzes the ADP-ribosylation of elongation factor 2, leading to the inhibition of protein synthesis and cellular death [25, 26]. Deamidation of the asparagine located on the toxin portion of the molecule has been shown to result in a loss of bioactivity [19, 27], and thus must be controlled during the manufacturing process.

In this work, we describe two examples of  $V_HH$  affinity chromatography ligands capable of separating closely related product forms. In the first example, a ligand selective for the calcium bound form of prothrombin is examined. When calcium is present, the ligand tightly binds prothrombin. When calcium is removed using EDTA, the bound prothrombin can be gently eluted from the affinity matrix. Purification with the prothrombin selective resin was shown to increase the level of fully carboxylated variants. In the second example, we present a camelid affinity matrix capable of distinguishing between the deamidated and amidated forms of an immunotoxin. Results of a two column affinity purification process are compared against a conventional purification process for the immunotoxin.

## 2 Materials and methods

### 2.1 Buffer reagents and protein preparations

Chemicals used for buffer preparation were obtained from Sigma (St. Louis, MO, USA) and JT Baker (Phillipsburg, NJ, USA). Recombinant human prothrombin (rh coagulation Factor II) was expressed in suspension-adapted CHO cells co-expressing two proteins required for post-translational modification, gamma-glutamyl carboxylase and vitamin K-oxidoreductase, and purified according to the method described by Wendeler et al. [18]. M. pasudotox was produced in *E. coli* according to the methods described by Linke et al. [19, 27].

### 2.2 Chromatography columns and instrumentation

Chromatography experiments were conducted using an ÄKTA Explorer 100 from GE Healthcare (Piscataway, NJ, USA). For all chromatography experiments, 0.66 cm diameter Benchmark columns from Kinesis (West Berlin, NJ, USA) with nominal bed heights of 10–20 cm were used unless otherwise noted. Toyopearl Phenyl-650M (Phenyl 650M) was obtained from Tosoh Biosciences (King of Prussia, PA, USA) and Q Sepharose Fast Flow (QFF) and Q Sepharose High Performance (QHP) were obtained from GE Healthcare.

### 2.3 Llama immunization and library construction

For the construction of  $V_{\text{H}}\text{H}$  phage display libraries, a llama was immunized intramuscularly three times with three-week intervals as previously described [28] using 200  $\mu\text{g}$  purified, amidated *m. pasudotox* per immunization. One week after the second and third injections, peripheral blood lymphocytes were isolated for RNA extraction and preparation of cDNA as described by Adams et al. [29]. The gene fragments encoding the  $V_{\text{H}}\text{H}$  fragments were amplified by PCR and the resulting fragments of about 350 bp in size were cloned in a phagemid vector and used for transformation to the *E. coli* strain TG1 [29]. The same approach was taken for the construction of the anti-prothrombin  $V_{\text{H}}\text{H}$  phage display library, except a llama was immunized with purified, fully gamma-carboxylated human plasma-derived prothrombin from Haematologic Technologies Inc. (Essex Junction, VT, USA).

### 2.4 Selection and screening of $V_{\text{H}}\text{H}$ fragments.

Phage display was performed to enrich for target specific  $V_{\text{H}}\text{H}$  fragments followed by a screening of  $V_{\text{H}}\text{H}$  supernatants derived from isolated single clones in ELISA as described by Adams et al. [30]. In short, Maxisorp<sup>®</sup> 96-well plates were pre-coated overnight at 4°C with 10 or 1  $\mu\text{g}/\text{mL}$  *m. pasudotox* (100  $\mu\text{L}/\text{well}$ ) and subsequently blocked with 200  $\mu\text{L}/\text{well}$  of 2% w/v dried skimmed milk, (Protifar; Nutricia Medical, Dublin, Ireland) in phosphate buffered saline, pH 7.4 (2% P-PBS) for 1 h at RT.  $V_{\text{H}}\text{H}$  phages from the libraries generated after the second and third llama injection with *m. pasudotox* were diluted ten times in 1% w/v Protifar, PBS, 0.05% v/v Tween-20 (1% P-PBST) and incubated for 1 h at RT on the plates. After vigorously washing of the wells with PBST the residually bound  $V_{\text{H}}\text{H}$  phages were eluted following a rescue of the phagemids by infection of TG1 cells. Single TG1 clones from the anti-*m. pasudotox* enriched libraries were isolated (2  $\times$  95 clones in two microtiter plates) and used for production of soluble  $V_{\text{H}}\text{H}$  fragments equipped with a C-terminal c-Myc tag and a biotinylation sequence [31] to facilitate binding analysis in both Direct and Capture ELISA, respectively. The same approach was taken for the selection and screening of the anti-prothrombin  $V_{\text{H}}\text{H}$  fragments, except that the plates were coated with purified, fully gamma-carboxylated human plasma-derived prothrombin (Haematologic Technologies Inc.).

#### 2.4.1 Direct ELISA

Maxisorp<sup>®</sup> 96-well plates were coated with purified *m. pasudotox* (with either the amidated form or with a  $\approx$ 80% deamidated form at 5  $\mu\text{g}/\text{mL}$  in PBS (100  $\mu\text{L}/\text{well}$ ) and incubated for 1 h at RT followed by a 1 h blocking step with 2% P-PBS (200  $\mu\text{L}/\text{well}$ ). Crude *E. coli* supernatants with soluble  $V_{\text{H}}\text{H}$  fragments were diluted five times in 1% P-PBST and incubated on the coated plates (100  $\mu\text{L}/\text{well}$ )

for 1 h at RT followed by a washing step using PBST. All following incubations were carried out at 100  $\mu\text{L}/\text{well}$  for 1 h at RT. The bound  $V_{\text{H}}\text{H}$  fragments were detected using a mouse anti c-Myc monoclonal antibody (Thermo Fisher Scientific) at 0.5  $\mu\text{g}/\text{mL}$  in 1% P-PBST followed by incubation with a goat anti-mouse-IgG horseradish peroxidase conjugate (Bio-Rad Laboratories) diluted 1:2000 in 1% P-PBST. After washing of the plates, a color reaction was allowed using a 3,3',5,5'-Tetramethylbenzidine/Ureaperoxide solution (OptEIA<sup>™</sup>; Becton Dickinson, Franklin Lakes, NJ, USA) at 100  $\mu\text{L}/\text{well}$ , which was stopped after 5–10 min by addition of 50  $\mu\text{L}/\text{well}$  1 M H<sub>2</sub>SO<sub>4</sub>. Plates were measured in a plate reader at 450 nm. The same approach was taken for the anti-prothrombin  $V_{\text{H}}\text{H}$  fragments, except that the plates were coated with purified, fully gamma-carboxylated human plasma-derived prothrombin (Haematologic Technologies Inc.).

#### 2.4.2 Capture ELISA

Maxisorp<sup>®</sup> 96-well plates were coated overnight at 4°C with purified streptavidin (Thermo Fisher Scientific) at 1  $\mu\text{g}/\text{mL}$  in PBS (100  $\mu\text{L}/\text{well}$ ) followed by a blocking step with 2% P-PBS as previously described. Crude *E. coli* supernatants with soluble  $V_{\text{H}}\text{H}$  fragments were diluted 10 times in 1% P-PBST and allowed to bind to the streptavidin coated plates for 1 h at RT (100  $\mu\text{L}/\text{well}$ ). All following incubations were carried out at 100  $\mu\text{L}/\text{well}$  for 1 h at RT. In order to analyze the binding domain recognized on the *m. pasudotox* target, the  $V_{\text{H}}\text{H}$  functionalized plates were incubated with either 5  $\mu\text{g}/\text{mL}$  purified amidated *m. pasudotox*, 1  $\mu\text{g}/\text{mL}$  anti-CD22 monoclonal mouse IgG (Abcam, Cambridge, MA, USA), or 5  $\mu\text{g}/\text{mL}$  polyclonal mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) in 1% P-PBST. For detection of bound *m. pasudotox*, polyclonal rabbit anti-Pseudomonas exotoxin A antibodies (Sigma Aldrich, St. Louis, MO, USA) were incubated at 1:2000 dilution in 1% P-PBST followed by an incubation with a swine anti rabbit-horseradish peroxidase conjugate (Dako, Carpinteria, CA, USA) diluted 1:4000 in 1% P-PBST. Detection of bound mouse IgG antibodies was achieved by incubation with a goat anti-mouse-horseradish peroxidase conjugate (Bio-Rad Laboratories) diluted 1:2500 in 1% P-PBST. After washing of the plates a color reaction was carried out and quantified using a plate reader at 450 nm as previously described. Capture ELISA was only used for screening anti-*m. pasudotox*  $V_{\text{H}}\text{H}$  fragments.

### 2.5 On column activation of prothrombin

For analyzing on column activation of prothrombin by Factor Xa, the anti-prothrombin  $V_{\text{H}}\text{H}$  affinity resin was packed in a Tricorn 5/20 column (0.5 cm  $\times$  2 cm; 0.4 mL). The column was equilibrated with PBS, pH 7.4 followed by a sample load of 1.5 mL of 1 mg/mL prothrombin. After washing out the unbound fraction, 450  $\mu\text{L}$  of PBS, pH 7.4 containing

20 µg (~24 units) Factor Xa (Haematologic Technologies Inc.) was applied and allowed to incubate for 1 h at 37°C. After this activation step the column was washed with PBS, pH 7.4 and released prothrombin fragments were collected. Subsequently, residually bound prothrombin products were eluted from the column with PBS, pH 2.0. The collected fractions were immediately adjusted to neutral pH by adding one tenth volume of 1 M Tris buffer, pH 8.0. The samples were diluted with a non-reducing sample buffer (Tris/Glycine) and loaded onto Novex 4–20% Tris-Glycine gradient gels (Thermo Fisher Scientific) and stained with Coomassie Brilliant Blue or further analyzed in a Western blot using either a mouse anti-human prothrombin specific antibody (R&D Systems, Minneapolis, MN, USA) or a sheep anti-human thrombin antibody (Haematologic Technologies Inc.) for detection.

## 2.6 Influence of EDTA and calcium on prothrombin binding

Maxisorp® flat-bottom, 96-well plates (Thermo Fisher Scientific) were coated with anti-prothrombin V<sub>H</sub>H fragment at 2 µg/mL in PBS, pH 7.4 at 100 µL/well for 1 h at room temperature (RT). The plates were washed, and then subsequently blocked with 2% P-PBS. The blocked plates were incubated for 1 h at room temperature with a dilution series of recombinant prothrombin in PBS, pH 7.4 with or without addition of 5 mM EDTA (100 µL/well), followed by a wash using PBS, 0.05% v/v Tween-20 (PBST). All following incubations were carried out at 100 µL/well for 1 h at room temperature. Bound protein was detected using a mouse anti-prothrombin antibody (R&D Systems), diluted 1:2000 in 1% P-PBST. After a washing step, the plate was incubated with a secondary goat anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad Laboratories, Hercules, CA, USA) diluted 1:2000 in 1% P-PBST followed by a washing step. A color reaction was carried out and quantified using a plate reader at 450 nm as previously described.

For analyzing the effect of EDTA and calcium chloride on the binding properties of the anti-prothrombin V<sub>H</sub>H affinity resin, 100 µL of resin was applied to each of the spin columns (MoBiTec GmbH, Goettingen, Germany) and washed five times with running buffer (200 µL of 20 mM Tris, 150 mM NaCl, pH 7.0) and then with 200 µL of binding buffer followed by a spin for 30 s at 2000 rpm for each wash. 100 µg of recombinant prothrombin in 250 µL of binding buffer was applied to each spin column and allowed to bind for 1 h at room temperature. For this step different binding buffers were tested, i.e. 20 mM Tris, 150 mM NaCl, pH 7.0 with addition of CaCl<sub>2</sub> (5, 25 or 125 mM) or EDTA (5, 25 or 125 mM). After this step the flow-through (non-bound) fractions were collected following five times wash with 200 µL of running buffer. The bound prothrombin was then eluted with 100 mM Glycine, pH 2.0 and immediately adjusted to neutral pH by

adding one tenth volume of 1 M Tris, pH 8.0. The collected samples were analyzed by SDS-PAGE as previously described.

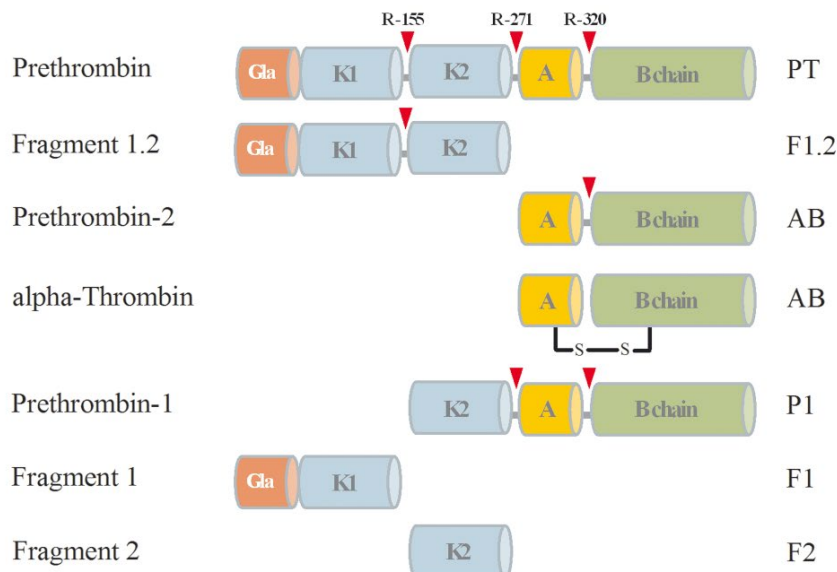
## 2.7 Screening of prototype anti-m. pasudotox V<sub>H</sub>H affinity resins

In order to obtain sufficient V<sub>H</sub>H material for preparing prototype affinity resins, the anti-m. pasudotox V<sub>H</sub>H fragments (named Exotox-V<sub>H</sub>H) were re-cloned into a yeast expression vector and produced in the yeast strain *Saccharomyces cerevisiae* as previously described [30]. V<sub>H</sub>H production was carried out at shake-flask scale followed by purification from the extracellular medium using cation-exchange chromatography. The purified V<sub>H</sub>H fragments were directly dialyzed into coupling buffer (0.1 M HEPES, 0.5 M NaCl, pH 8) and randomly coupled to NHS-Activated Sepharose 4 Fast Flow (GE Healthcare) according to the manufacturer's protocol with an applied ligand concentration of 10 mg V<sub>H</sub>H per mL matrix. The coupling was performed overnight at 4°C on a roller bank. After coupling, the remaining active groups of the resin were blocked by adding 1 M Tris pH 8.0 and incubated for 1 h, and then washed by alternating high and low pH to remove any unbound V<sub>H</sub>H ligand. Each prototype Exotox-V<sub>H</sub>H resin was packed in a Tricorn 5/20 (0.4 mL) column and equilibrated with PBS, pH 7.4 running buffer using an ÄKTA Explorer chromatography system (GE Healthcare). Each prototype affinity column was then analyzed for elution efficiency as follows. A 1 mg/mL stock solution of purified, amidated m. pasudotox was diluted 1:1 with running buffer and then 8 mL (i.e. 4 mg m. pasudotox) was loaded onto the column at 150 cm/h. Columns were washed with 10 column volumes (CVs) running buffer followed by a primary elution with 15 CVs of either of the following elution conditions (all in a 20 mM Bis-Tris, pH 7 buffer matrix): 2 M MgCl<sub>2</sub> (elution condition 1, EC-1), 1 M NaCl, 50% v/v propylene glycol (EC-2), or 0.8 M arginine (EC-3). After a wash with 15 CVs of running buffer the columns were stripped with 15 CVs of 0.1 M glycine pH 2 to remove any residually bound m. pasudotox. From each of the resulting chromatograms, the surface area (mAU\*mL) of the primary elution (EC) and strip (S) peak was determined and elution efficiency was calculated as a ratio of surface area of the elution peak (EC) divided by total surface area of the material bound to the column (EC+S).

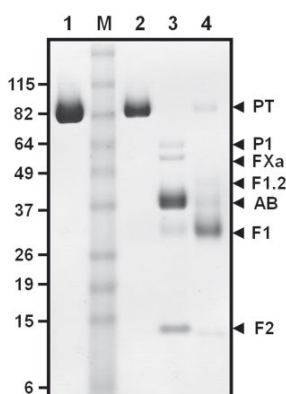
## 2.8 V<sub>H</sub>H matrix evaluation

For prothrombin affinity chromatography, the V<sub>H</sub>H affinity resin was equilibrated with 20 mM Tris, 150 mM NaCl, 10 mM CaCl<sub>2</sub>, pH 7.0. Clarified cell culture broth (CCCB) was spiked with calcium chloride to a final concentration of 10 mM and loaded onto the equilibrated column. The column was washed with 20 mM Tris, 150 mM NaCl, 1.25 mM CaCl<sub>2</sub>, pH 7.0, followed by elution with 20 mM

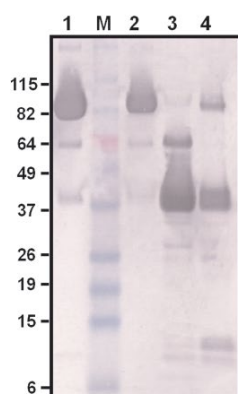
(A)



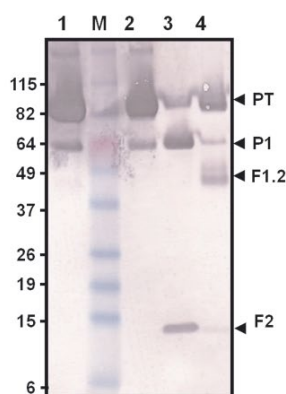
(B)



(C)



(D)



**Figure 1.** Determination of the binding region recognized by the anti-prothrombin  $V_HH$  ligand. (A) Illustration of prothrombin and corresponding fragment structures upon activation through its potential cleavage sites. Fractions from on-column activation of prothrombin were analyzed by (B) SDS-PAGE, (C) Western blot using an anti-thrombin specific antibody, and (D) an anti-prothrombin specific antibody. Lane 1: prothrombin load; lane M: BenchMark™ pre-stained protein marker; lane 2: prothrombin  $V_HH$  non-bound fraction (overloaded), lane 3: released prothrombin fragments upon Factor Xa activation, 4: strip fraction of residually bound prothrombin and fragments thereof.

Tris, 150 mM NaCl, 25 mM EDTA, pH 7.0. The column was stripped with 100 mM glycine, pH 2.4. For anion exchange capture purification of prothrombin, conditions are described in Wendeler et al [18].

For m. pasudotox, Exotox- $V_HH$  affinity resins were evaluated as part of a two column purification process ( $V_HH$  affinity column followed by a Phenyl-650M column). The affinity columns were equilibrated with 50 mM phosphate, pH 7.4 and then loaded to 3.9 and 5.6 g/L resin for Exotox- $V_HH$ -4 and Exotox- $V_HH$ -8, respectively. After loading, the columns were washed with 50 mM phosphate, pH 7.4, and then eluted with 50 mM phosphate, 1 M NaCl, 50% v/v propylene glycol, pH 7.4. Product pool was then buffer exchanged into 20 mM phosphate, 200 mM NaCl, pH 7.4 by dialysis (10 kDa) and then diluted 1:1 with 20 mM phosphate, 1.2 M  $Na_2SO_4$ , pH 7.4 to prepare for Phenyl-650M chromatography. The Phenyl-650M

column was equilibrated with 20 mM phosphate, 0.6 M  $Na_2SO_4$ , pH 7.4 and then loaded to 5–10 g/L resin. The column was washed with equilibration buffer and then eluted in a linear gradient to 20 mM phosphate, pH 7.4 over 20 CVs. The final product pool was buffer exchanged into 25 mM phosphate, 4% w/v sucrose, 8% w/v glycine, pH 7.4. For comparison, a four column (non-affinity) purification process was used which has been described in Linke et al. [27].

## 2.9 Analytical methods

Prothrombin analytical methods to assess charge heterogeneity by anion exchange chromatography (AEC-HPLC), size heterogeneity by size exclusion chromatography (SEC-HPLC) and host cell protein (HCP) analysis have been described in detail in Wendeler et al. [18].

M. pasudotox analytical methods for purity including AEC-HPLC and SEC-HPLC as well as bioactivity have been described in detail in Linke et al. [27]. Methods for refold titer by AEC-HPLC, purity by reversed phase chromatography (RP-HPLC) and HCP analysis have been described in detail in Linke et al. [19].

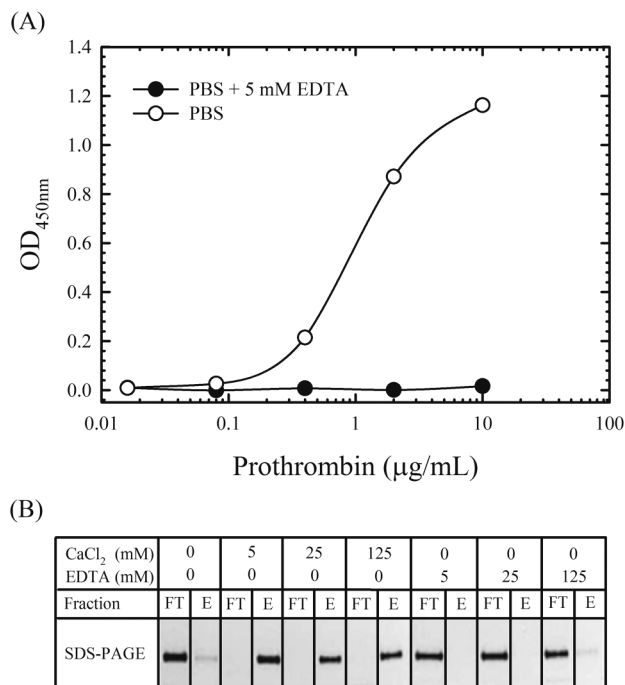
### 3 Results and discussion

#### 3.1 Prothrombin

##### 3.1.1 Binding characteristics of an anti-prothrombin $V_{H^H}$ affinity ligand

Figure 1A shows prothrombin and corresponding fragment structures upon activation through its potential cleavage sites. A camelid  $V_{H^H}$  affinity ligand that was developed for the specific capture of human prothrombin was analyzed in a chromatography set-up to determine the domain region recognized by this protease precursor molecule. For this purpose the corresponding  $V_{H^H}$  affinity resin was used to specifically capture the prothrombin target in a first step followed by an on-column activation step with Factor Xa to form active thrombin. By analyzing the distribution of the resulting prothrombin fragments, either still bound to the affinity resin or released upon activation, the potential binding region can be determined. The results displayed in Fig. 1 indicate that the epitope recognized resides on the fragment 1 region of prothrombin. Fragment 1 consists of the N-terminal Gla-domain and kringle domain 1 and is identified as smallest and most prominent residually bound prothrombin fragment in the affinity resin strip fraction. The prothrombin fragments lacking this fragment 1 region, like alpha-thrombin, prethrombin 1 and fragment 2, are released from the affinity resin upon activation with factor Xa, which further supports fragment 1 to be essential for binding to the anti-prothrombin  $V_{H^H}$  affinity ligand.

Since the carboxylated glutamic acid residues in Gla-domains can bind calcium ions and thereby induce conformational changes that are necessary for the Gla-domain to fold properly [32], the possible dependency of this conformation on the binding interaction between the  $V_{H^H}$  fragment and prothrombin was examined. Figure 2A shows a dose response curve of prothrombin under physiological conditions (PBS, pH 7.4) using the anti-prothrombin  $V_{H^H}$  as capture antibody in an ELISA set-up. When EDTA is added to the sample incubation buffer, in an attempt to alter the prothrombin conformation by removing calcium ions from its Gla-domain, the binding functionality of the  $V_{H^H}$  fragment is fully abolished. When analyzing the  $V_{H^H}$  affinity resin in a spin-column set-up, its preference for this calcium dependent conformation of prothrombin could be verified. The results as shown in Fig. 2B demonstrate that a lack of calcium ions in the binding buffer prohibits efficient binding of the prothrom-



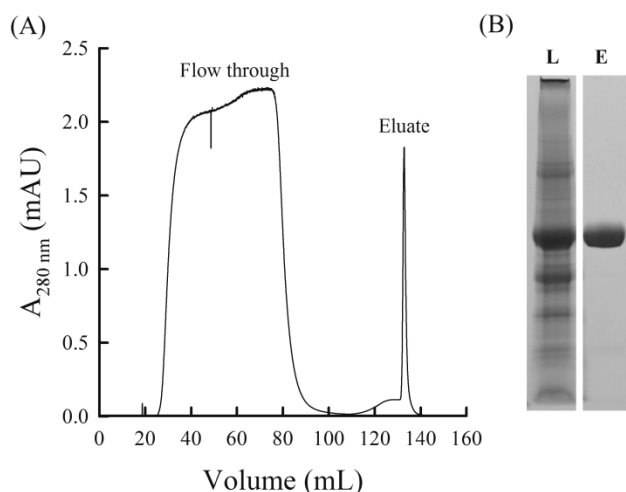
**Figure 2.** Effect of EDTA and calcium ions on the binding reactivity of the anti-prothrombin  $V_{H^H}$  ligand. **(A)** Capture ELISA showing binding in PBS and PBS + 5 mM EDTA. **(B)** SDS-PAGE of  $V_{H^H}$  spin column flow through (FT) and eluate (E) fractions at increasing levels of either calcium or EDTA in a Tris/NaCl buffer.

bin target to the affinity resin, which is further hampered by addition of EDTA. However, when 5 mM calcium chloride (lowest concentration tested) is added to the binding buffer no detectable prothrombin can be observed in the flow-through fraction showing complete capture of the prothrombin sample applied.

Recognition of a calcium dependent conformation implies the potential selectivity for highly gamma-carboxylated prothrombin, which is a more biologically active form. Furthermore, the mechanism of altering this conformation by EDTA in order to disrupt the binding interaction between prothrombin and this  $V_{H^H}$  fragment could be used as a mild elution condition in an affinity chromatography setting.

##### 3.1.2 Chromatographic behavior

Figure 3 shows a chromatogram from the  $V_{H^H}$  affinity purification of prothrombin and the corresponding SDS-PAGE analysis of fractions. After the addition of calcium chloride to a final concentration of 10 mM, prothrombin selectively binds to the resin from CCCB. After a wash step, prothrombin is efficiently eluted with a buffer containing 25 mM EDTA (Fig. 3a) and the corresponding gel image shows a highly purified eluate (Fig. 3b). It should be noted that the flow through fraction consists predominantly of undercarboxylated variants and host cell impurities; however, the crude nature and very low concentra-



**Figure 3.** Purification of recombinant prothrombin with  $V_HH$  affinity resin. **(A)** Chromatogram obtained from affinity chromatography where protein is bound to the column in the presence of 10 mM calcium chloride and eluted with 25 mM EDTA. **(B)** SDS-PAGE analysis of column load (L) and eluate (E) fractions.

tion of the flow through sample makes it difficult to quantify with AEC-HPLC or observe the band on a gel without a concentration step or gel overloading.

It was demonstrated previously that recombinant prothrombin expressed in the CHO system is not fully carboxylated, but rather it consists of a mixture of variants with a distribution of gamma-carboxylation levels in the Gla-domain [18]. For process development, charge heterogeneity can conveniently be monitored by AEC-HPLC, where the undercarboxylated variants are apparent as pre-peaks that elute before the fully carboxylated product. To evaluate the potential selectivity of the  $V_HH$  ligand towards protein variants with varying levels of carboxylation, material was deliberately generated under conditions that promote expression of undercarboxylated variants. Table 1 summarizes the purification of prothrombin from CCCB using a  $V_HH$  affinity resin or an anion exchange resin. When the CCCB was purified by conventional anion exchange chromatography using QFF, where both undercarboxylated and fully carboxylated variants bind and elute, pre-peak (or undercarboxylated variant) levels in the eluate were 30.5%. In contrast, for prothrombin eluted from the  $V_HH$  affinity column, where the undercarboxylated variants largely remain in the column flow through fraction, a significantly lower pre-peak content of 7.5% was measured, corresponding to a reduced content of undercarboxylated variants. This demonstrates that the  $V_HH$  affinity resin shows selectivity for the fully carboxylated form of prothrombin, allowing for efficient separation from less active product variants and the enrichment of highly carboxylated, and highly active, prothrombin. Analysis by SEC-HPLC showed that monomer purity after the anion exchange capture step is around 95%, and

**Table 1.** Purification of prothrombin by anion exchange or  $V_HH$  affinity chromatography.

Parameter	Units	CCCB	QFF	$V_HH$ affinity
HCP	ng/mg	1,453,955	19,336	3,109
AEC-HPLC pre-peak	%	–	30.5	7.5
SEC-HPLC monomer	%	–	94.9	97.6

greater than 97% after affinity capture. Moreover, HCP analysis revealed that the purification by  $V_HH$  affinity resin results in significantly lower host cell protein content in the eluate.

## 3.2 Moxetumomab pasudotox

### 3.2.1 Screening and isolation of *m. pasudotox* $V_HH$ candidates

The use of  $V_HH$  affinity ligands with a preferred selectivity for the amidated, and biologically active, form of *m. pasudotox* was also explored. For this purpose a  $V_HH$  expression library was used that was derived from a llama immunized with a purified, and properly amidated, form of *m. pasudotox*. After target enrichment of the  $V_HH$  library in a standard phage panning procedure, single  $V_HH$  *E. coli* clones were isolated and subsequently tested in ELISA on expression of *m. pasudotox* binding  $V_HH$  fragments. In order to identify binders with potential isoform selectivity, the isolated  $V_HH$ s were tested in a direct ELISA on binding to both an amidated as well as a deamidated form of *m. pasudotox*. Since this immunotoxin consists of a mouse anti-CD22 Fv domain and a truncated form of the *Pseudomonas* exotoxin (PE38), capture ELISA was also used to determine which part of the fusion protein is recognized by the  $V_HH$  candidates. Table 2 summarizes the results of both ELISA screening assays and shows a total number of 43  $V_HH$  candidates capable of binding to the recombinant immunotoxin. Based on the ELISA binding profiles the  $V_HH$  candidates are divided into five different groups (A to E). The majority of the  $V_HH$  fragments (group A to C), bind to an epitope that resides on the PE38 exotoxin domain since no binding is observed with the anti-CD22 mouse mAb that possesses the same  $V_H$  and  $V_L$  domains (Fv) as displayed by *m. pasudotox*. The  $V_HH$  fragments binding to the Fv domain either reveal an anti-idiotypic selectivity due to a lack of binding to irrelevant polyclonal mouse IgG (group D) or binding to more common regions present on the variable domains of mouse antibodies (group E). By following this straight forward screening set-up, a significant number of  $V_HH$  fragments that bind to the PE38 exotoxin domain demonstrates a clear preference for the amidated form of *m. pasudotox* in direct ELISA (group C), whereas no discriminative binding is observed for the exotoxin binders belonging to group A and B. As expected, no preferred isoform selectivity is found by  $V_HH$ s targeting the Fv

**Table 2.** Screening results overview of isolated anti m. pasudotox V<sub>H</sub>H fragments

Binding group # of positives (unique V <sub>H</sub> Hs)	A 4 (3)	B 14 (11)	C 15 (5)	D 8 (2)	E 2 (2)
Direct ELISA <sup>a)</sup>					
m. pasudotox (amidated)	0.21 ± 0.14	1.39 ± 0.36	1.70 ± 0.13	1.69 ± 0.31	1.68 ± 0.07
m. pasudotox (deamidated)	0.20 ± 0.14	2.04 ± 0.37	0.22 ± 0.13	2.22 ± 0.28	2.37 ± 0.06
Capture ELISA <sup>a)</sup>					
m. pasudotox (amidated)	0.90 ± 0.14	1.02 ± 0.25	0.92 ± 0.27	1.15 ± 0.21	0.65 ± 0.06
mouse IgG: anti-CD22	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	2.08 ± 0.51	2.09 ± 0.29
mouse IgG: polyclonal	0.05 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	1.14 ± 0.02
No coat (control)	0.04 ± 0.00	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.00

a) Average OD<sub>450</sub> values of total number of positives per binding group.

domain. Despite this minor difference induced by deamidation of the asparagine residue located on the exotoxin domain it apparently alters the epitope recognized by the V<sub>H</sub>H fragments of group C resulting in a significantly reduced binding affinity. This deamidation event is known to lead to reduced biological activity in a cell-based bioassay [19, 27] which, as also described for other proteins like human αB-Crystallin [33], might be induced by a different conformation of this region compared to the properly amidated form of m. pasudotox. Therefore, the binding mechanism of the identified amidated selective V<sub>H</sub>H binders could be driven by a conformation dependency similar to the V<sub>H</sub>H binder found for the anti-prothrombin V<sub>H</sub>H affinity ligand.

This potential selectivity for the amidated form of m. pasudotox was further investigated in a chromatography setting. For an initial screen eight exotoxin reactive V<sub>H</sub>H fragments were selected and cloned into a yeast expression vector and produced in the yeast production strain *Saccharomyces cerevisiae* suitable for large scale production [7, 8]. This set of exotoxin binders included an equal number of non-discriminative V<sub>H</sub>H fragments from group B and those showing selectivity towards the amidated form of the exotoxin domain from group C. This selection of candidates was also based on the ability of the exotoxin binders to release the m. pasudotox target under neutral pH elution conditions as analyzed in capture ELISA (data not shown). After V<sub>H</sub>H production and purification, the

Exotox-V<sub>H</sub>H candidates were randomly coupled to NHS-Sepharose to serve as small-scale resin prototypes for functionality testing in chromatography. Table 3 summarizes the results of the prototype resin screening showing elution efficiency of three neutral pH elution conditions. Exotox-V<sub>H</sub>H candidates 4 and 8, which demonstrated efficient target release under mild elution conditions, were selected for further testing and a direct comparison with the optimized, non-affinity purification process that is currently used to manufacture m. pasudotox.

### 3.2.2 Chromatographic behavior

The manufacturing process of m. pasudotox currently consists of a refold step and four non-affinity chromatography steps and is capable of delivering highly purified material suitable for clinical trials [27]. Like many purification processes, the first two columns (anion exchange capture and hydroxyapatite) greatly reduce the process-related impurities such as HCP, DNA, and endotoxin. The final two columns (hydrophobic interaction and anion exchange polishing) are responsible for removing the product-related impurities such as fragments, aggregates, and charge isoforms. The most important of these product-related impurities is a deamidated variant that has been observed at levels of up to 40% and has been shown to be inactive [19]. The level of the deamidated variant can be monitored using AEC-HPLC where the deamidated product elutes as a pre-peak ahead of the amidated form. In order to remove the deamidated variant, which only differs by a single charge, anion exchange chromatography using OHP has been employed as the final chromatography step in the purification process. Due to the nature of this difficult separation, the column has to be operated at low loadings (<1 g/L) and eluted with a very shallow salt gradient, both of which have led to low yields (30–40%). Not surprisingly when the starting level of deamidated species is lower going into the final OHP column, the yields can be much higher (>80%).

V<sub>H</sub>H affinity resins capable of binding m. pasudotox were investigated in a two-column purification process (V<sub>H</sub>H affinity column followed by a Phenyl-650M column) to assess the opportunity to generate material with product

**Table 3.** Elution properties of m. pasudotox on V<sub>H</sub>H affinity resins

Binding group	B				C			
	2	3	4	5	6	7	8	
Exotox-V <sub>H</sub> H candidate <sup>a)</sup>								
Elution efficiency (%) <sup>b)</sup>	EC-1	39	68	0	27	12	89	94
	EC-2	42	77	93	4	58	74	72
	EC-3	21	31	2	18	38	66	74

a) No expression in yeast for Exotox-V<sub>H</sub>H-1; candidate excluded from resin testing.

b) Elution efficiency calculated as a ratio of surface area of the elution peak divided by total surface area of all bound peaks. Elution condition-1 (EC-1): 2 M MgCl<sub>2</sub>, EC-2: 50% v/v Propylene Glycol, 1 M NaCl, EC-3: 0.8 M Arginine, all in 20 mM bis-tris, pH 7.0.



**Table 4.** Comparison of the purification of *m. pasudotox* by a two-column ( $V_{\text{H}}\text{H}$  affinity) or four-column (non-affinity) process

Parameter	Units	4-column		2-column	
		Non-affinity	Exotox- $V_{\text{H}}\text{H}$ -4	Exotox- $V_{\text{H}}\text{H}$ -8	
Yield	mg/L <sup>a)</sup>	16	34	15	
HCP	ng/mg	6	< 3	22	
DNA	ng/mg	< 0.001	< 0.001	0.011	
Monomer	%	99.5	89.5	97.9	
Aggregate	%	0.5	2.3	2.1	
Fragment	%	2.5	10.2	2.0	
IEC pre-peak	%	18.4	17.5	2.9	
Bioactivity	%	78	68	108	

a) milligrams of purified, amidated product per liter of refold.

quality consistent with biopharmaceuticals for human use. Two  $V_{\text{H}}\text{H}$  resins were evaluated; one that selectively binds the amidated form of *m. pasudotox* (Exotox- $V_{\text{H}}\text{H}$ -8), and one that binds *m. pasudotox* but does not discriminate between the amidated and deamidated forms (Exotox- $V_{\text{H}}\text{H}$ -4). The two-column processes incorporating the  $V_{\text{H}}\text{H}$  affinity resins were run under non-optimized conditions and compared to a highly optimized four-column non affinity process. To test these process options, *m. pasudotox* was generated under refold conditions that resulted in high levels of aggregate and deamidated product.

Table 4 summarizes the result of the  $V_{\text{H}}\text{H}$  affinity process as well as the four-column non-affinity purification process. As can be seen in the table, all three process options had good clearance of HCP, DNA, and aggregates, and all but the Exotox- $V_{\text{H}}\text{H}$ -4 process showed good clearance of fragments. In the case of the Exotox- $V_{\text{H}}\text{H}$ -4 process, the elevated fragment content is interesting since the Phenyl-650M has been shown to have excellent fragment clearance in the four-column process, and also in the Exotox- $V_{\text{H}}\text{H}$ -8 process. It may be that the fragment in the Exotox- $V_{\text{H}}\text{H}$ -4 process is a fragment that contains the deamidation site, and may behave differently than the fragment that is typically observed throughout the four-column process. Comparing the AEC-HPLC pre-peak data in Table 4, the Exotox- $V_{\text{H}}\text{H}$ -8 process selectively removed the deamidated species while the Exotox- $V_{\text{H}}\text{H}$ -4 process and the four-column process did not. It should be noted that the OHP column in the four-column process was operated under conditions such that the chromatographic yield is high (i.e. the large pre-peak in the OHP elution peak was not selectively cut out of the product peak). The impact of removing the deamidated product during the purification process can ultimately be seen in the biological activity of the purified product, where the Exotox- $V_{\text{H}}\text{H}$ -8 process results in a much higher activity compared to the other two process options.

While the Exotox- $V_{\text{H}}\text{H}$ -8 process has the highest product purity, some discussion is needed when considering the process yields. At first glance it may seem like the Exotox- $V_{\text{H}}\text{H}$ -4 process is very high yielding, but this pro-

cess would need a third chromatography column to remove the deamidated species and possibly some additional peak cutting on the Phenyl-650M or a fourth column to remove the remaining fragment. In any event, the additional column would significantly lower yield, such that the two affinity process options may be very comparable in terms of yield (and purity after the additional chromatography step). When comparing the Exotox- $V_{\text{H}}\text{H}$ -8 process to the four-column process, yields seem comparable. However, if the OHP process was operated such that the deamidated pre-peak is removed (to match high purity of the Exotox- $V_{\text{H}}\text{H}$ -8 process) then the overall process yield would be significantly lower ( $\approx 7$  mg purified amidated product per L refold). Thus it seems that the Exotox- $V_{\text{H}}\text{H}$ -8 process, and possibly the Exotox- $V_{\text{H}}\text{H}$ -4 process, would be suitable options to replace the four-column, non-affinity process.

## 4 Conclusions

In this work, we describe two examples of  $V_{\text{H}}\text{H}$  affinity chromatography ligands capable of separating closely related product forms. These affinity ligands consist of single monomeric variable domains of camelid heavy-chain only antibodies, and were designed such that they preferentially bind the biologically active form of two different molecules, prothrombin and a recombinant immunotoxin. For prothrombin, a chromatographic stationary phase consisting of a ligand selective for the calcium bound form was developed, which allowed for strong binding of prothrombin in the presence of calcium, and gentle elution in the presence of EDTA. Under these conditions, the highly gamma-carboxylated (i.e. highly biologically active) recombinant prothrombin was purified from cell culture broth in a single step, and the affinity purification was shown to be more effective than conventional anion exchange chromatography for process- and product-related impurity removal. In a second example, a  $V_{\text{H}}\text{H}$  affinity resin was developed to selectively bind the amidated, and highly active, form of a recombinant immunotoxin. The ligand showed strong binding under physiological conditions and the immunotoxin was gently eluted in the presence of salt and propylene glycol. Under these conditions, the  $V_{\text{H}}\text{H}$  affinity purification compared favorably to a non-affinity purification process in terms of process yield and product quality. These two examples show the potential for  $V_{\text{H}}\text{H}$  camelid antibody affinity ligands to be developed and used in the purification of highly pure and highly active biological therapeutics. While this work describes two specific examples of product variants, there is potential to develop  $V_{\text{H}}\text{H}$  camelid antibody affinity ligands to selectively purify biopharmaceuticals from other types of closely related product variants, such as truncated variants or other modifications that result in a conformational change.

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