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Journal of Chromatography B, 774 (2002) 1–15

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Rational combinatorial chemistry-based selection, synthesis and evaluation of an affinity adsorbent for recombinant human clotting factor VII

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Received 27 February 2001; received in revised form 8 February 2002; accepted 12 February 2002

Abstract

The selection, synthesis and chromatographic evaluation of a synthetic affinity adsorbent for human recombinant factor VIIa is described. The requirement for a metal ion-dependent immunoadsorbent step in the purification of the recombinant human clotting factor, FVIIa, has been obviated by using the X-ray crystallographic structure of the complex of tissue factor (TF) and Factor VIIa and has directed our combinatorial approach to select, synthesise and evaluate a rationally-selected affinity adsorbent from a limited library of putative ligands. The selected and optimised ligand comprises a triazine scaffold bis-substituted with 3-aminobenzoic acid and has been shown to bind selectively to FVIIa in a Ca^{2+}-dependent manner. The adsorbent purifies FVIIa to almost identical purity (>99%), yield (99%), activation/degradation profile and impurity content (~1000 ppm) as the current immunoadsorption process, while displaying a 10-fold higher static capacity and substantially higher reusability and durability.

Keywords: Affinity ligands; Combinatorial chemistry; Immunoadsorbent; Recombinant human clotting factor VII

1. Introduction

The ability to produce pure, safe and efficacious pharmaceutical proteins from recombinant expression systems is an on-going challenge for the biopharmaceutical industry. The impact of genomics, safety and revised legislative issues is pressurizing the industry into adopting high throughput, cost-effective and flexible manufacturing processes based on new technology. It is anticipated that highly selective techniques such as affinity chromatography will play an increasing role in bioprocessing, since, not only do they satisfy the requirement for the ultra-high resolution of the target protein from other host proteins present in the expression system, but they also provide in-built quality assurance by removing bioactive contaminants such as DNA, endotoxins, viruses and post-translational isoforms [1]. Unfortunately, many of the current highly selective affinity adsorbents based on natural biological

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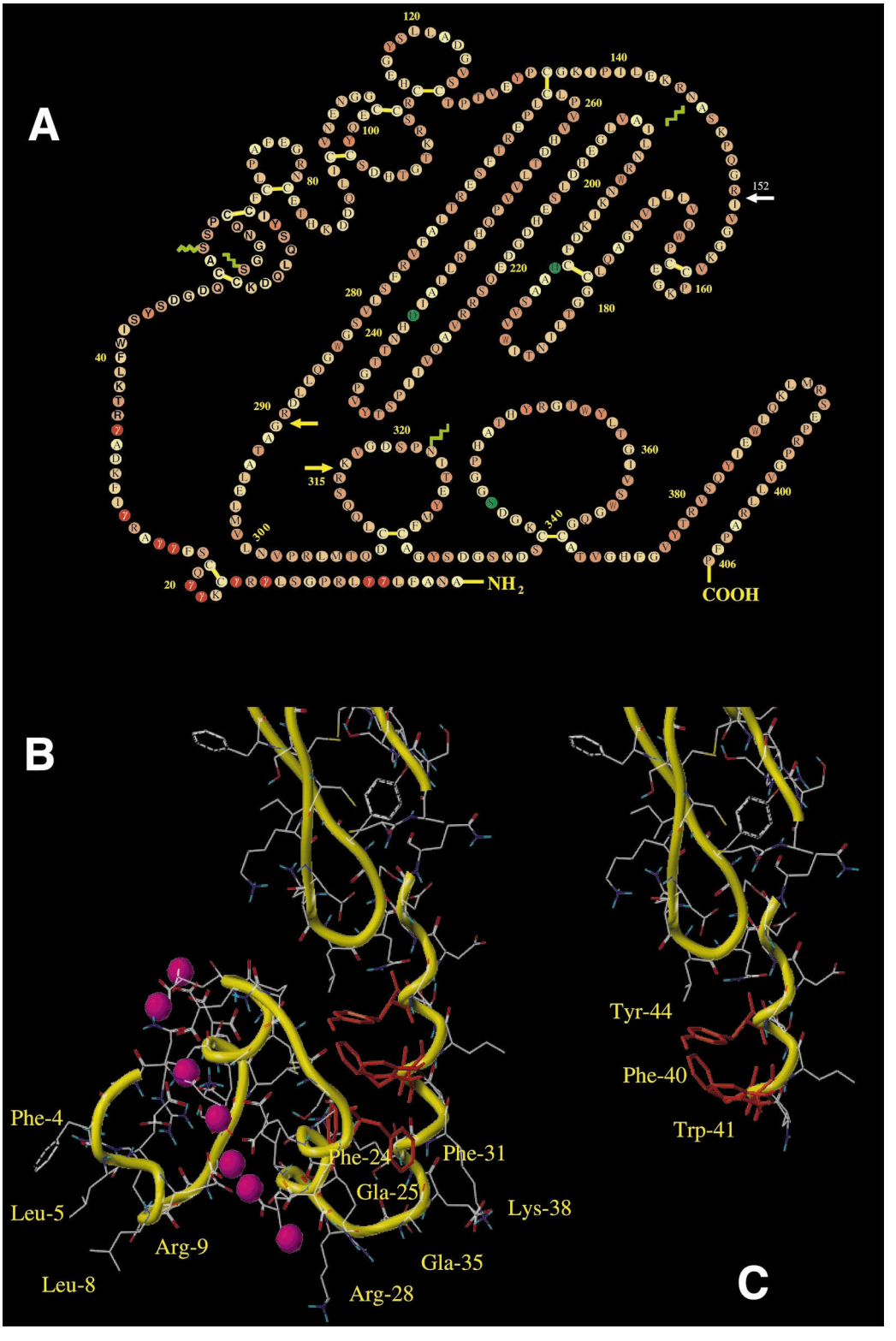
1570-0232/02/$ – see front matter
PII: S1570-0232(02)00103-4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ligands such as proteins and peptides are fragile, expensive and not readily amenable to scale-up and ruggedization for the harsh manufacturing environment of the biopharmaceutical industry. This is particularly true for immunoadsorbents based on monoclonal antibodies, which, although they can offer impressive and rational purifications, can suffer from high production costs, low capacities and are unable to withstand sterilization or harsh cleaning-in-place schedules [2].

High capacity synthetic adsorbents capable of withstanding the rigours of depyrogenation, sterilization and cleaning-in-place during multiple operational cycles, and which retain their ability to adsorb and elute native proteins in high yields have been created. De novo design strategies for generating affinity ligands are now available for target proteins where a suitable template structure is derived from known substrates [3] or complementary binding molecules [4] exist. However, selection of an appropriate target site and the design, synthesis and evaluation of a complementary affinity ligand is at best only a semi-rational process, since ligand immobilisation introduces numerous unknown factors. The affinity of the immobilised ligand for the complementary protein is determined partly by the chemical and spatial characteristics of the ligand per se and partly by the matrix, spacer and scaffold groups and the activation and coupling chemistries. Analytical studies between soluble ligands and target proteins do not fairly reflect the chemical, geometrical and steric constraints imposed by the complex three-dimensional matrix environment. Hence, more recently, a generic approach to the construction of affinity adsorbents which exploits the structural knowledge of the target protein per se in association with combinatorial ligand libraries and effective screening protocols has been developed [5–10].

In this report, this strategy has been applied to generate a robust synthetic affinity adsorbent which significantly exceeds the capacity and reusability of a monoclonal antibody immunoadsorbent currently used for the purification of a human recombinant clotting factor, Factor VIIa, whilst retaining its high selectivity for the target protein. Human factor VII (FVII) is a 50-kDa single-chain precursor glycoprotein which is converted to the two-chain FVIIa by cleavage of an internal peptide bond located at Arg-152:Ile-153 to generate a light chain (1–152) consisting of an N-terminal domain containing ten γ-carboxyglutamic acid (Gla) residues followed by a few aromatic residues (hydrophobic stack) and two epidermal growth factor (EGF)-like domains, and a heavy chain (153–406), containing the serine protease domain, held together by a single disulphide bridge between Cys-135 and Cys-262 (Fig. 1A). FVIIa, in the presence of Ca^{2+} and tissue factor (TF) initiates the extrinsic pathway of blood coagulation [11,12]; it has no measurable coagulant activity in the absence of calcium or tissue factor.

Recombinant FVII is currently obtained by culturing and transfection of BHK cells with a human FVII recombinant construct [13,14]. The crude cell culture medium is passed through a 0.65-μm filter and subjected to ion-exchange chromatography on a mono Q column using a $CaCl_2$-containing buffer as eluent, immunochromatography and a final polishing step on an ion-exchange column. FVII binds to the antibody only in the presence of Ca^{2+} and, like other Gla-containing proteins [15], can be eluted from the immunoadsorbent with buffers containing EDTA. The four-step purification protocol gives an overall yield of ~40% with the principal losses being attributable to removal of the Gla-domain (des-Gla-Factor VIIa) and spurious nicking of the heavy chain. A key aim of the present work was to alleviate regulatory concerns over the use of animal-derived monoclonal antibodies and replace this step in the

Fig. 1. Structure of human coagulation factor VII. (A) Primary sequence showing the principal domains, γ-carboxyglutamate (Gla) residues (Ⓨ; red), glycosylation sites (∿), activation (→; white) and cleavage (→; yellow) sites, disulphide bridges (©-©; yellow) and catalytic site residues (○; green). (B) Ribbon representation of the X-ray crystallographic structure of the Gla-domain (residues 1–45) and part of the EGF-1 domain of FVII showing the key residues in the ''hydrophobic stack'' (red), other key residues used in the affinity ligand design and the seven Ca^{2+} ions bound to Gla residues (dark magenta). (C) Structure of the N-terminal part of EGF-1 minus the Gla-domain showing retention of part of the hydrophobic stack but loss of the Ca^{2+}-binding sites.

protocol with a durable, sterilizable affinity adsorbent which retained the Ca^{2+}-dependent on–off selectivity of the original immunoadsorbent and produced equivalent product purity. Our rationale was to select a binding site on the Factor VIIa molecule which would allow a putative affinity ligand to bind FVII/VIIa, but not des-Gla-VIIa, in a Ca^{2+}-dependent fashion. The specification also required that the adsorbent bind 5 mg FVII/VIIa/ml gel in the pH range 5.5–9 and elute the protein in the absence of Ca^{2+} at a concentration of 2–5 mg/ml, a purity of >99% and with a yield of >95%. The adsorbent itself should be able to withstand in excess of 200 cycles of adsorption, elution, regeneration and equilibration and be tolerant to CIP/SIP (Cleaning-in-Place/Sterilization-in-Place) under relatively harsh conditions.

2. Experimental

2.1. Chemicals

1-Amino-6-naphthalene sulphonic acid was purchased from Lancaster Synthesis (Eastgate, Morecambe, UK) and 1-amino-7-naphthol, 2-amino-1-naphthol and 6-amino-1-naphthol from TCI Tokyo (Tokyo, Japan), while all other chemicals were of the highest grade available from Aldrich (Gillingham, UK). Calcium chloride and trizma base were purchased from Sigma (Poole, Dorset, UK). Coomassie Brilliant Blue G-250 was from Pierce Immunochemicals (Rockford, IL, USA). All solutions, washes and procedures that required water utilised high purity (18 MΩ/cm) water from an Elgastat system. Analytical TLC plates, coated with 0.25 mm silica gel containing UV 254 nm fluorescent indicator were purchased from Macherey-Nagel (Düren, Germany). Glass capillary tubes were hand drawn from pasteur pipettes supplied by John Poulten (Essex, UK).

2.2. Biochemicals

Recombinant factor VII (rFVIIa; M_r 50 kDa) and Gla-domainless FVII (des-Gla-FVIIa; M_r ~46 kDa) were kindly supplied by Novo Nordisk (Gentofte, Denmark). Samples were tested for purity and efficacy by the quality control department at Novo Nordisk. Bovine serum albumin (BSA) was purchased from Sigma (Dorset, UK). Pre-packed Sephadex G-25 (PD-10) desalting columns, HR 5/2 columns and Sepharose CL-6B were purchased from Pharmacia Biotech (Uppsala, Sweden). Amino-derivatized agarose 6XL (24 μmol amino groups/g moist weight gel; 110 g) was obtained from ProMetic BioSciences (Balasalla, Isle of Man, UK). Polypropylene columns (4 ml) and frits for screening purposes were purchased from Varian (Surrey, UK).

2.3. Analytical techniques

Protein concentrations were routinely measured using the Coomassie dye binding method according to standard protocols [16]. The protein (100 μl) or standard (BSA, 0.2–1.4 mg/ml; 100 μl) was incubated with Coomassie Brilliant Blue G-250 solution (1 ml) for 10 min and the absorbance measured at 595 nm.

Aliphatic amines were detected on TLC plates by dipping in a ninhydrin solution (0.2% (w/v) in ethanol) and drying with a heat gun until colour development was complete. The presence of an aliphatic amine was indicated by a brown to purple colouration. The presence of amines on beads was determined by the method of Kaiser [17]. A small volume of beads was pipetted into an Eppendorf tube (1.5 ml) and two drops each of 5% ninhydrin (w/v) in methanol, liquefied phenol (80 g phenol/20 ml ethanol) and potassium cyanide (1 m*M*; 2 ml) in pyridine (98 ml) added. The sample was heated to 120 °C on a hot plate for 4–6 min, following which a blue colouration indicated the presence of free amino groups. Analytical TLC was performed on commercial Macherey-Nagel plates coated with Polygram® SIL G/UV_{254} (0.25 mm thick).

2.4. Computer-aided molecular modelling

Molecular modelling was performed on an IRIX 6.3 Silicon Graphics workstation purchased from Silicon Graphics, Reading, UK at the Institute of Biotechnology, University of Cambridge. The software package Quanta was purchased from Molecular Simulations, Cambridge, UK. The X-ray crystal structure of factor VII was downloaded from the Protein Data Bank (1DAB) into the Quanta 97 modelling program. The Gla and EGF domains of

FVII were studied in detail to identify potential binding sites. Potential triazine based ligands were drawn in the Chemnote application in 2-dimensions, and imported in Quanta 97 in 3-dimensions. All the structures were minimised in vacuo, using Steepest gradient and then Conjugate gradient minimisation tools with CharmM forcefield. Docking of the ligands on factor VII was performed manually, and the putative ligands were brought into proximity of the chosen binding sites on the protein. After docking, the rotatable bonds of the ligands were adjusted, to have better contact with the target protein site. Within the defined computational parameters, the ligand–protein complex with least energy constraints was further minimised in vacuo, as described above. Hydrophobic interactions, involvement of metal ions and the formation hydrogen bonds were noted.

2.5. Instrumentation

The Biocad 700E Workstation was from Perseptive Biosystems (Boston, USA), while the UV/Visible Lambda 3 Spectrophotometer was purchased from Perkin-Elmer (Bucks, UK). Mass spectra were recorded on an AEI MS30 or MS50 Mass Spectrometers (in FAB positive mode) at the Chemical Laboratory, University of Cambridge.

2.6. Solid phase synthesis of FVII ligand library

Amino-derivatized agarose 6XL (24 μmol amino groups/g moist weight gel; 110 g) was suspended in a mixture of acetone–water (1:1, v/v; 200 ml, 0 °C) in an ice-salt bath and a solution of recrystallised 1,3,5-trichloro-triazine (5 mmol, 0.9 g) in acetone (30 ml) was added at 0 °C in small portions over a period of 2 h. When the primary amine groups on the agarose could no longer be detected with ninhydrin, the triazine-activated agarose was washed sequentially with increasing concentrations of acetone in water (1:3; 1:1, 1:0, by vol.; 4×500 ml) followed by decreasing concentrations of acetone in water (3:1, 1:1, 1:3, by vol.; 4×500 ml) and, finally, water (4×250 ml). The cyanuric chloride-activated agarose (100 g) was divided into four equal parts (25 g) and added to a solution of the first substituent (1.2 mmol, two equivalents) (Table 1) dissolved in 50% (v/v) acetone–water (25 ml) and agitated gently at 30 °C for 16 h. The substituted gels were separately recovered on a glass sinter (No. 2) and washed exhaustively and sequentially with 50% (v/v) acetone:water (1:3, 1:1, 1:0, 3:1, 1:1; by vol.; 200 ml), and finally with water (200 ml). Each of the four samples of filtered and washed monosubstituted agarose matrices was divided into four portions (5 g moist gel), and to each of the 16 gels was added a solution of 50% (v/v) DMF:water (5 ml) containing the second amine substituent (0.24 mmol, two equivalents) and the reactions allowed to proceed with rotational agitation at 80–90 °C for 72 h in an oven. In order to maintain the pH, a molar equivalent of NaOH was added to the reaction mixture where the amino ligand bore a carboxyl function. The bi-substituted gels were separately washed and filtered as described above and were stored in 20% (v/v) ethanol at +4 °C.

2.7. Solution phase synthesis of ligands 5/5 and 5/28

Cyanuric chloride was recrystallised by dissolving 1,3,5-trichloro-triazine (160 mmol; 30 g) in hot petroleum ether (b.p. 80–100 °C; 50 ml) by heating up to 90 °C in a water bath with constant stirring and allowing to cool overnight. The crystals (80% yield, m.p. 145 °C) were collected, dried under reduced pressure and stored in an air-tight container at room temperature. A fine slurry of recrystallised cyanuric chloride (1.1 g; 6 mmol) was prepared in acetone (24 ml) and dissolved in a well-stirred ice-water bath (36 ml) and a solution of the first amino substituent, 3-aminobenzoic acid (6 mmol; Table 1, 5) in acetone (10 ml) together with $NaHCO_3$ (504 mg; 6 mmol) in water (10 ml) was added and allowed to react at 0 °C for ~2 h. The progress of the reaction was followed by TLC. On completion, the white solid was filtered on a Büchner funnel containing ash-less filter paper (42 grade; 5.5 cm), washed with copious amounts of water and dried in vacuo over KOH pellets for ~12 h. The product, 2-(3-aminobenzoic acid)-4,6-dichloro-triazine, was subjected to TLC, ^{1}H-NMR and MS analysis.

The product (1 mmol) recovered from the first substitution, 2-(3-aminobenzoic acid)-4,6-dichloro-triazine, was dissolved in acetone (4 ml) and added to water (6 ml) at 40 °C. To the resulting suspension,

Table 1
Amines used for the construction of the Factor VII ligand library

Ligand	Amines
1	1-Amino-6-naphthalene sulphonic acid
2	1-Amino-5-naphthol
3	Benzylamine
4	3,5-Diamino-benzoic acid
5	3-Aminobenzoic acid
6	4-Aminobenzoic acid
7	Tyramine
8	Naphthylamine
9	4-Nitro-1-naphthylamine
10	2,7-Diaminofluorene
11	3,6-Diaminoacridine
12	3-Amino-1,2-propanediol
13	3-Amino-1-propanol
14	5-Amino-1-propanol
15	3-Aminophenol
16	6-Aminocaprioc acid
17	3-Amino-phenylboronic acid
18	3-Amino-2-naphthoic acid
19	4-Amino-1-naphthbol
20	1-Amino-2-naphthol
21	3-Amino-2-naphthol
22	1-Amino-6-naphthol
23	1-Amino-7-naphthol
24	2-Amino-1-naphthol
25	6-Amino-1-naphthol
26	3-Amino-benzamidine
27	4-Amino-benzamidine
28	2-Amino-benzimidazole
30	2-Amino-benzothiazole
32	2-Amino-4-chlorobenzothiazole
34	2-Amino-5-chlorobenzoxazole
36	2-Amino-5,6-dimethylbenzimidazole
37	2-Amino-5,6-dimethylbenzothiazole
38	7-Amino-2,4-dimethyl-1,8-naphthyridine
40	6-Aminoindazole
41	2-Amino-6,8-dihydroxypurine
42	5-Aminoindan
43	5-Amino-3-methyl-1-phenylpyrazole
44	4-Aminophenol
45	2-Aminophenol
46	2-Aminobiphenyl
47	1-Aminoindan
48	2-Aminoindan
49	Glucosamine
50	1-Aminobenzoic acid

the second substituent (Table 1, 3-aminobenzoic acid (5); 2-aminobenzimidazole (28); 1 mmol) in acetone (2 ml) together with Na_2CO_3 (1 mmol) in water (2 ml) was added in separate flasks and the mixture allowed to react at 40 °C for 12 h. Completion of the reaction was assessed by TLC until only bis-substituted triazine could be detected, after which the product was precipitated from the reaction mixture by the addition of 1 *M* HCl. The solid product was filtered on a Büchner funnel containing ash-less filter

paper (42 grade; 5.5 cm), washed with copious amounts of water and dried in vacuo over KOH pellets for ~12 h. The products, 2,4-bis-(3-aminobenzoic acid)-6-chloro-triazine (5/5) and 2-(3-aminobenzoic acid)-4-(2-aminobenzimidazole)-6-chloro-triazine (5/28) were subjected to TLC, ^{1}H-NMR and MS analysis. Overall yields for ligands 5/5 and 5/28 were 96 and 90%, respectively, with purities >95% by TLC on Macherey-Nagel plates coated with Polygram® SIL G/UV$_{254}$ (0.25 mm thick) with 2% (v/v) aqueous acetic acid as eluent.

2.8. Ligand immobilisation

Aminated agarose (1 g moist weight; 17.5 μmol amino groups/g moist weight gel) was washed with water followed by increasing ratios of DMF:water (1:3, 1:1, 1:0, by vol.) until the gel was suspended in DMF (2 ml). The bis-substituted ligand (10-fold molar excess over amino group concentration) in DMF (1 ml) together with $NaHCO_3$ (10-fold molar excess over amine concentration) in water (600 μl) was added and allowed to react for 72 h at 90 °C. The completion of the reaction was confirmed by removing small aliquots of the gel and detecting the presence of residual amines [17]. Upon completion of the reaction, the coupled gel was washed exhaustively with DMF:water (1:0, 3:1, 2:1, 1:1, 1:2, 1:3, 0:1 (v/v)) and packed (1 ml total gel volume) into disposable polypropylene columns for evaluation of their chromatographic properties and performance.

2.9. Stock solution of rFVIIa

Purified rFVIIa (Novo Nordisk; lot no. RENS7; 1.52 mg/ml) was exchanged on G-25 (PD-10) columns previously equilibrated with 30 column volumes of 20 m*M* Tris–HCl, 50 m*M* NaCl, 2.5 m*M* $CaCl_2$, pH 8.0 (binding buffer), by loading purified protein (2.5 ml; 3.8 mg) onto the column and eluting with the above buffer (3.5 ml). The concentration of rFVIIa was determined by Coomassie Blue binding (Bio-Rad, CA, USA) according to the manufacturer's protocol, using BSA as a standard. The stock solution (~1 mg/ml; 20 μ*M*) was apportioned into 5-ml aliquots and frozen at −20 °C until required. The extinction coefficient (1-cm light path) for a 1-mg/ml solution of rFVIIa at 280 nm was 1.37.

2.10. Screening of the ligand library

Each affinity gel was packed to a 1-ml volume in 4-ml polypropylene columns and retained between 20-μm frits. The columns were washed with regeneration buffer (30% (v/v) iso-propanol–0.2 *M* NaOH, 70 ml) until no ligand leakage was detected at 254 nm and then with water (20 ml). The columns were equilibrated (30 column volumes) with starting buffer (20 m*M* Tris–HCl, 50 m*M* NaCl, 2.5 m*M* $CaCl_2$, pH 8.0; 30 ml total; 1-ml aliquots) at room temperature, after which a sample of rFVIIa (1 mg/ml in starting buffer; 1 ml) was applied. The columns were washed with the starting buffer (10 ml; 1-ml aliquots) followed by elution with elution buffer (20 m*M* Tris–HCl, 50 m*M* NaCl, 5 m*M* EDTA, pH 7.5; 3 ml total in 1-ml aliquots). The sample, flow through and elution fractions collected for each column were analysed for the presence of rFVIIa by measuring the absorbance at 280 nm. Columns were rejuvenated by washing with regeneration buffer (30 ml) and eventually stored in 20% (v/v) ethanol–water.

2.11. Determination of the association equilibrium constants (K_{AX})

Each of the immobilized affinity ligands 5/5 and 5/28 were sucked to dryness on a No. 3 sinter funnel, an aliquot (0.2 g) was weighed, rehydrated (≈0.325 ml wet volume gel) in deionised water and packed in a Pharmacia HR 5/2 column. The columns were equilibrated with the starting buffer (30 column volumes) on a BioCad 700E Workstation prior to evaluation. Purified rFVIIa (0–1 mg/ml; 15 ml) was applied through a 15-ml sample loop at a flow rate of 0.3 ml/min, after which the regeneration buffer (30% (v/v) iso-propanol–0.2 *M* NaOH; 30 ml) was applied at 0.3 ml/min and the column re-equilibrated with the binding buffer. The frontal chromatograms were monitored with a UV detector set at 280 nm. From the breakthrough curves generated at each rFVIIa concentration in triplicate, 50% of the optical density at steady state was used to determine the elution volume of rFVIIa ($\bar{V}_A$). The column bed

volume (V'_A) was determined by using the same gel volume of unmodified matrix and determining the breakthrough curve of rFVIIa at 1 mg/ml. The association equilibrium constant (K_{AX}) was determined from the resulting Scatchard plot according to Winzor [18–21].

2.12. Capacity of the adsorbents

The capacity of the immobilized 5/5 adsorbent for pure rFVIIa was measured by frontal analysis at 23 °C in 20 m*M* Tris–HCl, pH 8.0/5 m*M* $CaCl_2$/50 m*M* NaCl using immobilized ligand concentrations

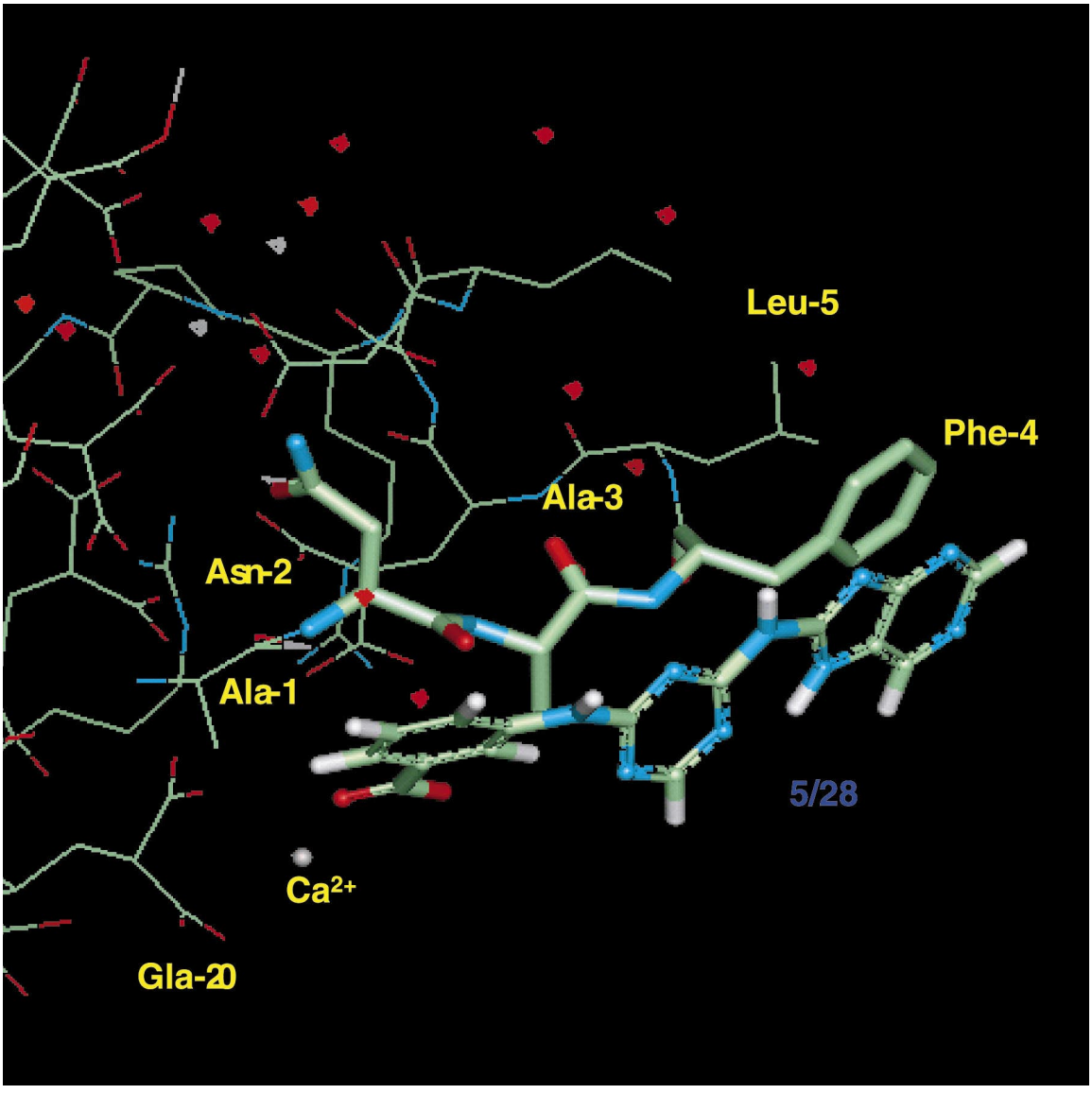


Fig. 2. Structure of part of the Gla-domain of human coagulation factor VII showing a putative ligand (Table 1, 5/28) interacting with key aromatic (Phe-4) and γ-carboxyglutamate (Gla-20) residues with the possible formation of stacking interactions between substituent 28 (Table 1) and the side chain of Phe-4 and the formation of a coordination complex between Gla-20, a Ca^{2+} ion and substituent 5 (Table 1).

in the range 0–33 μmol/g moist weight gel and a flow rate of 18 column volumes/h.

2.13. Assessment of the purity of eluted fractions

The purity of eluted recombinant FVIIa was assessed by an RP-HPLC procedure. A column (4× 250 mm) of C4-substituted silica (300-Å pore size) was equilibrated in 0.1% (v/v) TFA in water/acetonitrile at a flow rate of 1 ml/min. Elution of bound fractions was effected with a 37–52% (v/v) gradient of acetonitrile in water over 45 min at 70 °C; eluted proteins were detected by absorbance at 214 nm. Under these conditions, minor impurities appear after approximately 10, 16 and 24 min, whilst rFVIIa and rFVII appear at 25 and 26 min, respectively. Peaks were characterised by SDS–PAGE and N-terminal sequence analysis. Samples for SDS gel electrophoresis were heated at 70 °C for 10 min in SDS NuPAGE™ non-reducing sample buffer and an aliquot (1 μg) applied to an SDS NuPAGE 4–12% precast gel. Electrophoresis was effected in an Xcell II mini cell system at 200 V for 35 min.The gels were stained with Coomassie Brilliant Blue R-250.

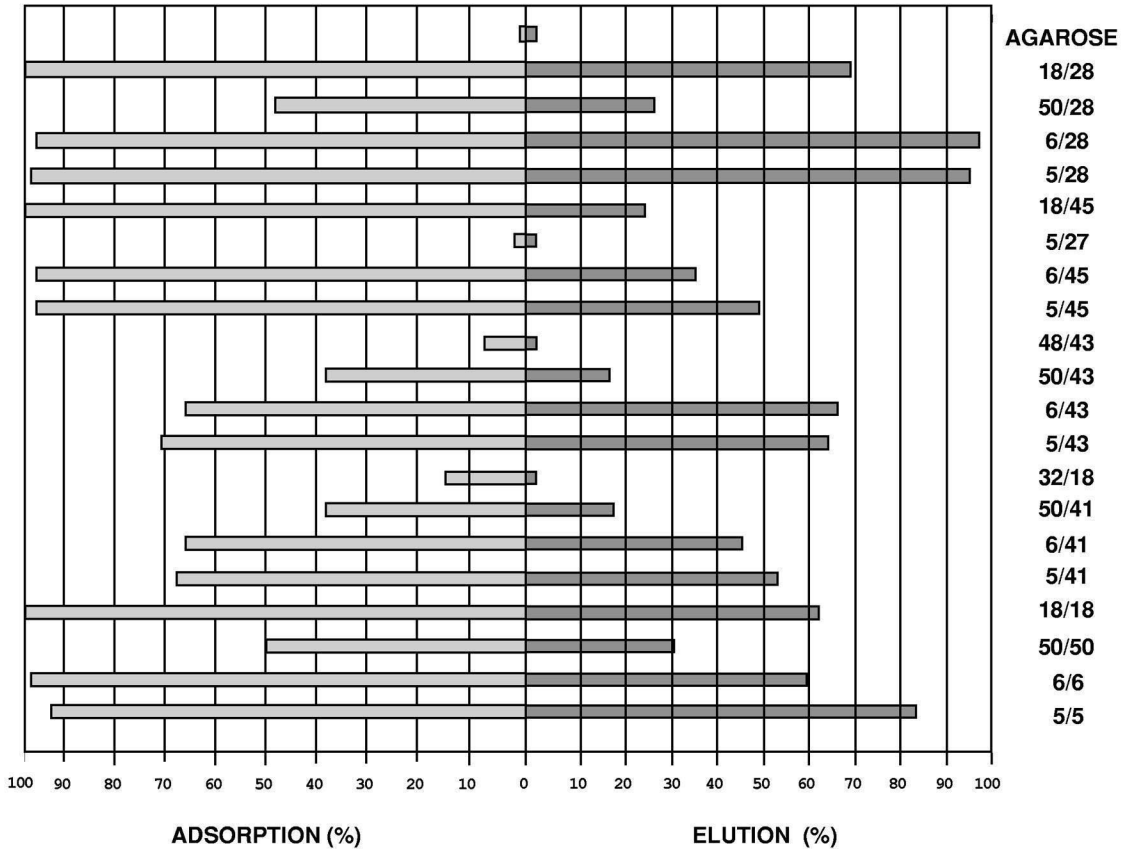


Fig. 3. Initial chromatographic screening of representative examples of the rFVIIa binding combinatorial library. Pure rFVIIa (1 mg) was applied to a column (1 ml) of the appropriate adsorbent equilibrated in 20 m*M* Tris–HCl, pH 8.0/50 m*M* NaCl/5 m*M* $CaCl_2$ at 23 °C, washed in the equilibration buffer and FVIIa eluted with 20 m*M* trisodium citrate, pH 8.0. % Adsorption relates to the applied sample. % Elution is the % of FVIIa eluted of that adsorbed. Library members were synthesised on the solid phase by sequential reaction with the two amines (Table 1).

3. Results and discussion

3.1. The rational design and combinatorial selection strategy

Since an important feature of the ligand selection focused on imparting calcium ion dependence to the interaction between the putative affinity ligand and the target protein, the prominent Ca^{2+}-binding Gla-domain was inspected in detail. Numerous proteins containing a Gla-domain [15] have been successfully purified by elution from adsorbents with buffers containing EDTA. In order to achieve this singular specificity from the affinity ligand, the known crystallographic structure of the protein was inspected. The 2.0-Å X-ray crystal structure of the tissue factor (TF)–FVIIa complex shows that the FVIIa molecule adopts an extended conformation with the structures of all four domains, including the seven Ca^{2+}-binding sites on the Gla-domain, well resolved (Fig. 1B) [22]. Our attention initially focused on the inter-domain region of the protein where the Gla-domain (residues 1–45) joins EGF-1 (residues 46–86) and binds hydrophobically to the tissue factor C-terminal domain (TF-2; residues 107–210). This ''hydrophobic stack'' region involves residues of the two C-terminal helices of the Gla-domain, runs vertically from about Phe-24 to Gly-47, and includes the residues Phe-24, Phe-31, Phe-40, Trp-41 and Tyr-44 (Fig. 1B). Some of these residues are retained in the des-Gla-FVIIa (Fig. 1C). The close proximity of this region to the side chain functionalities of residues Gla-35 and Lys-38 suggested that bifunctional ligand structures comprising aromatic acids or benzamidine/benzimidazole substituted on a triazine scaffold might prove effective as they combined aromatic stacking, electrostatic and hydrogen bonding capabilities, which were complementary to the target residues. A putative ligand was designed to overlap Phe-24 at one end of the hydrophobic stack and present an amidine function towards Gla-35 and Lys-38. The ligand was synthesised by substitution of 2-amino-3-naphthoic acid and 2-aminoben-

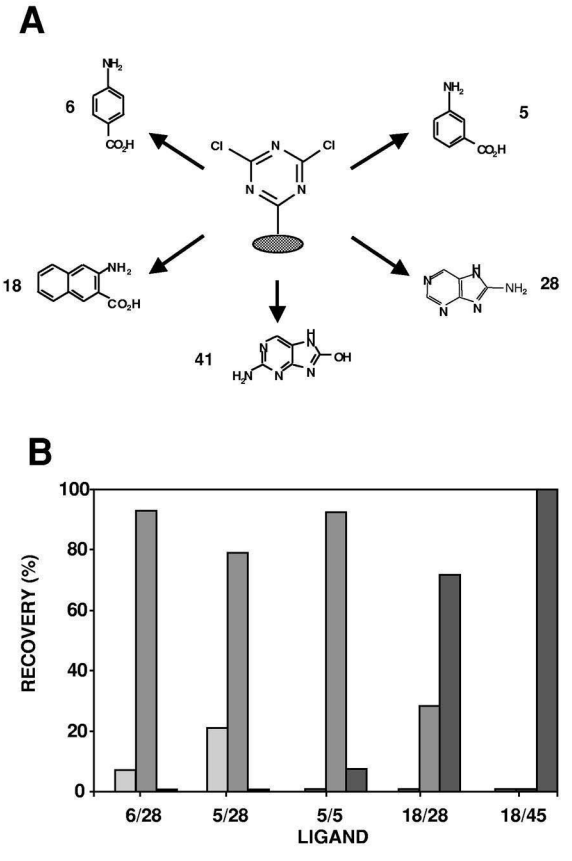


Fig. 4. Chromatographic evaluation of the key members of the FVIIa library. (A) Structures of the lead aromatic amines substituted on the triazine scaffold. (B) Analysis of the flowthrough (▫), citrate elution (▪) and regeneration (■) fractions for six lead affinity adsorbents showing how the adsorbents bind FVII weakly (6/28) to very tightly and requiring harsh regeneration conditions to effect elution (18/45).

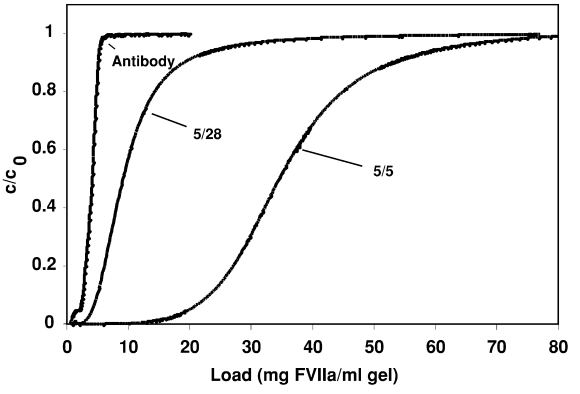


Fig. 5. Frontal analysis showing the breakthrough curves (c/c_0) of 1-ml columns containing the immunoadsorbent, 5/28 and 5/5 equilibrated in 20 m*M* Tris–HCl, pH 8.0/50 m*M* NaCl/5 m*M* $CaCl_2$ at 23 °C.

zimidazole on a triazine scaffold (Table 1, 18/28). Computer-aided molecular modelling showed that ligands bearing such features were able to interact with the target residues on the protein, with the carboxyl group on the naphthalene ring bringing additional interactions with the side chains of Gla-25 and Arg-28.

The three hydrophobic residues Leu-8, Leu-5 and Phe-4 (Fig. 1B) which protrude from the Gla-domain when calcium is bound may provide an alternative binding site for an affinity ligand, since these residues are retracted on removal of Ca^{2+} from FVII [22]. Ligands comprising of the triazine moiety substituted in the first position with either 2-aminobenzimidazole (Table 1, 28) or with 3- or 4-aminobenzoic acid (Table 1, 5/6), and with the latter in the second position would provide aromatic stacking to Phe-4 and the possibility of the formation of hemicalcium salts with the Ca^{2+} ions associated with the γ-carboxyglutamate (Gla) residues, particularly Gla-20, located nearby (Fig. 2). This model was generated with least geometric constraints and offers the most accessible binding on the Gla-domain to the putative ligand 5/28. However, despite the value of computer modelling in allowing the visualisation of putative protein–ligand interactions in solution, it is clear that in order to generate effective affinity adsorbents many other factors must be taken into consideration. Thus, the complexity of the three dimensional matrix environment, with largely unknown interactions between the ligand and the matrix, coupling-, activation- and spacer chemistries, suggests that the design of affinity ligands can never be truly rational and computer based and that directed combinatorial chemistry should be evoked as a useful adjunct to develop effective affinity ligands for target proteins. Furthermore, conformational changes may occur in the target protein to accommodate the ligand at a biding site that may seem inaccessible in the X-ray crystal structure.

3.2. Construction of the combinatorial library

Since it is known that the affinity of an immobilized ligand is determined not only by the characteristics of the ligand per se but also by the matrix, activation and coupling chemistry, the putative lead structures were optimised by a directed combinatorial approach [5–10]. A catalogue of 44 amino derivatives of bi-, tri- and heterocyclic aromatics, aliphatic alcohols, benzamidines, benzothiazoles, benzoxazoles, benzamidines, fluorenes and acridines substituted with various carboxyl, sulphonate, nitro

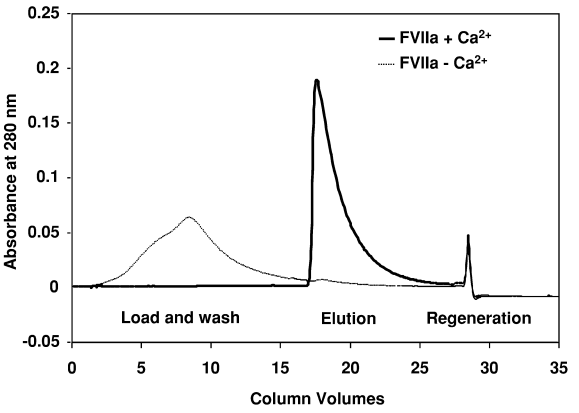


Fig. 6. Comparison of the chromatography of rFVIIa on an immobilized 5/5 adsorbent (~20 μmol 5/5/g moist weight gel) in 20 m*M* Tris–HCl/50 m*M* NaCl±5 m*M* $CaCl_2$.

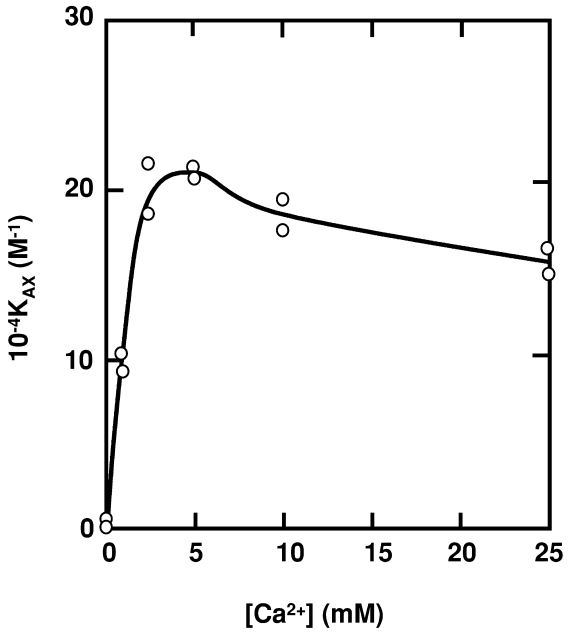
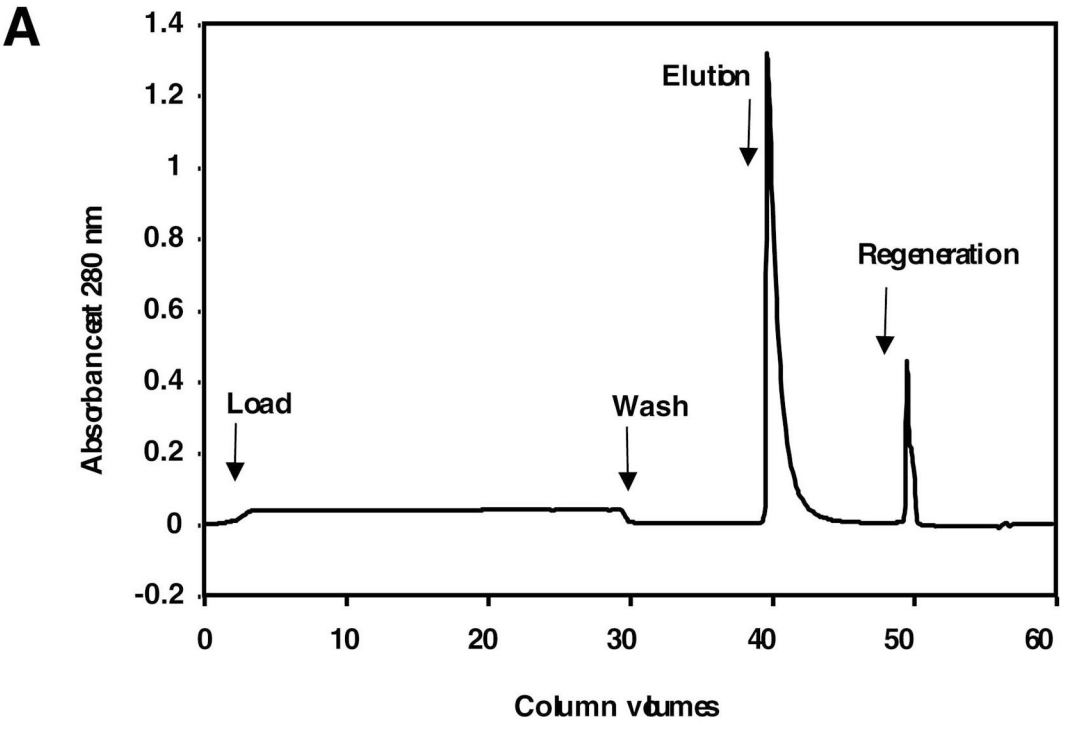
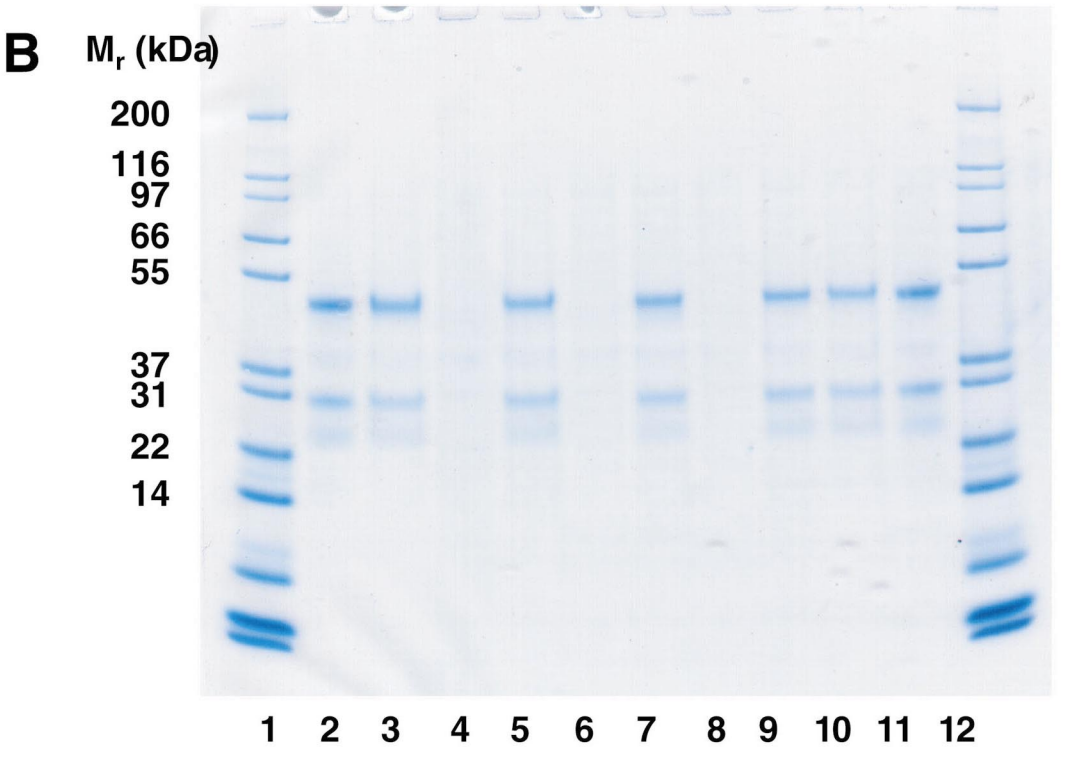


Fig. 7. Effect of $[Ca^{2+}]$ on the apparent equilibrium association constant (K_{AX}, M^{-1}) for the binding of rFVIIa to immobilised 5/5 in 20 m*M* Tris–HCl/50 m*M* NaCl, pH 8.0.


A
Absorbance at 280 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
10
20
30
40
50
60
Column volumes
Load
Wash
Elution
Regeneration


B
Mr (kDa)
200
116
97
66
55
37
31
22
14
1
2
3
4
5
6
7
8
9
10
11
12

and hydroxyl functionalities (Table 1) formed the basis for the solid-phase synthesis of the directed combinatorial library on a Sepharose CL-6B support matrix [5–10].

3.3. Screening of the combinatorial library for Factor VII binding and elution

The adsorbents were screened for FVII binding under the same conditions used for the immunoadsorbent; adsorption in the presence of Ca^{2+} and elution by chelating Ca^{2+} with citrate [15]. Analysis of the chromatographic behaviour of a number of individual members of the library (Fig. 3) showed that ligands comprising 3-aminobenzoic acid (Fig. 4A; 5), 4-aminobenzoic acid (6), 2-amino-3-naphthoic acid (18), 2-amino-benzimidazole (28) and 6-hydroxy-2-aminopurine (41) were particularly effective for the Ca^{2+}-mediated interaction with FVII. The binding of rFVIIa to the immobilized ligands was dependent on ionic strength within the range 50–300 m*M* NaCl but almost independent of pH within the range 6.0–9.0.

The bi-symmetric ligand 5/5 and the asymmetric ligand 5/28 showed high adsorptive capacity for FVII and recovery of >80% on removal of Ca^{2+} (Fig. 4B). Frontal analysis of these adsorbents showed that under the conditions tested, 10% breakthrough occurred at 23.1, 4.9 and 2.6 mg FVII/ml gel, respectively, for immobilized 5/5, 5/28 and the immunoadsorbent (Fig. 5). A maximum capacity of ~25 mg pure FVII/ml gel was observed when the immobilized ligand (5/5) concentration was >20 μmol ligand 5/5/g moist weight gel [18–21]; this capacity corresponds to a ligand usage of 2.5%, i.e. 2.5 μmol FVIIa/100 μmol immobilized ligand 5/5.

3.4. The effect of Ca^{2+} ions

The synthetic affinity ligands 5/5 and 5/28 were designed and selected to bind to a region of FVII known to interact with tissue factor and which should exhibit Ca^{2+}-sensitivity by virtue of proximity to the Gla-domain of the zymogen. Immobilized ligand 5/5 tightly adsorbs rFVII only in the presence of 5 m*M* Ca^{2+} and subsequently releases the protein in quantitative yield on chelation of the Ca^{2+} with citrate (Fig. 6). In contrast, loading purified rFVII in the absence of Ca^{2+} results in unretarded passage of the rFVII through the 5/5 adsorbent [23]. Quantitative affinity chromatography was used to characterize the binding of FVII to two of the immobilized ligands, 5/5 and 5/28, in order to assess their apparent equilibrium association constants and the sensitivity of the interaction to the Ca^{2+} ion concentration [24]. Frontal chromatographic analysis of the interaction of FVII with immobilised 5/5 and 5/28 in the presence of 50 m*M* NaCl and in the absence of Ca^{2+} yields apparent association constants (K_{AX}; M^{-1}) of $1.8\pm0.5\times10^3$ and $2.3\pm0.5\times10^3$, respectively. The dependence of the apparent equilibrium constant K_{AX} on the concentration of Ca^{2+} for FVII in Tris–HCl buffer containing 50 m*M* NaCl for immobilized ligands 5/5 and 5/28 shows a pronounced maximum at ~2–5 m*M* Ca^{2+} (Fig. 7). This maximum is also discernible at higher ionic strength (150 m*M*) for the interaction of FVII with immobilized 5/5, although, as anticipated for a largely electrostatic interaction, the overall affinity between FVII and 5/5 is reduced almost 10-fold. At the optimum concentration of Ca^{2+}, and in low ionic strength media, the apparent equilibrium association constant increases to approximately 7.5×10^4 and 22×10^4 M^{-1} for the interaction between FVII and 5/28 and 5/5, respectively [24]. Factor VII devoid of its Gla-domain binds relatively weakly (K_{AX} $6–8\times10^3$ M^{-1}) to immobilized 5/5 and 5/28 in a manner which is independent of the Ca^{2+} concentration. A value of 500 M^{-1} was assigned to the equilibrium association constant for the interaction of Ca^{2+} with the Gla-domain of FVII [23].

Fig. 8. Chromatography of rFVIIa on immobilized 5/5. (A) Elution profile obtained when semi-purified rFVIIa (84% pure; ex-ion-exchange chromatography [13,14] was applied to a 1-ml column of immobilized 5/5 (~20 μmol 5/5/g moist weight gel) equilibrated with 20 m*M* Tris–HCl, pH 8.0/50 m*M* NaCl/5 m*M* $CaCl_2$ at 23 °C, washed with 10 column volumes (CV) of the same buffer and eluted with 20 m*M* trisodium citrate, pH 8.0, at a flow rate of 10 CV/h. Column regeneration was effected with 0.2 *M* NaOH in 30% (v/v) iso-propanol. (B) SDS–PAGE of fractions eluted from the immobilized 5/5 column. Lanes 1, 12: molecular weight markers; lanes 2, 11: pure rFVIIa; lanes 3, 10: eluted fraction from immobilized 5/5 column; lanes 5, 7: eluted fraction from immunoadsorbent column; lanes 4, 6, 8: fraction eluted with regeneration buffer.

3.5. Optimization of the affinity adsorbent and operational parameters

The Ca^{2+}-dependent adsorption process was scaled up in order to assess the purity of the eluted FVII. The column was loaded with partially purified FVII (84% pure; ex-ion-exchange chromatography [13,14], the column washed, the FVII eluted with trisodium citrate and the adsorbent regenerated with 0.2 *M* NaOH in 30% (v/v) iso-propanol; Fig. 8A). Factor VII was eluted from the immobilized 5/5 adsorbent by chelation of Ca^{2+} with citrate in an overall yield of 99%. Analysis of the purity of rFVIIa by RP-HPLC and reducing SDS–PAGE from the immobilized 5/5 adsorbent (Fig. 8B) revealed that the eluted fractions displayed a purity >99%. Inspection of the SDS–PAGE gels reveals that in addition to the principal band at ~50 kDa, the eluted FVII is subject to degradation during the course of the purification protocol. It is known that FVII can lose its N-terminal Gla-domain, become hydrolysed at different sites in the heavy chain and have different extents of post-translational modification, such as *N*-glycosylation and aspartate β-hydroxylation [13,14]. The bands detectable at molecular weights corresponding to 38 and 15–17 kDa represent heavy chain degradation products generated during the activation of rFVII and co-purifying with the intact rFVIIa [13,14]. The des-Gla-FVIIa degradation product is removed in the polishing ion-exchange step. However, chromatography on the immobilized 5/5 adsorbent and subsequent elution with citrate appears to convert a significant proportion (~50–60%) of rFVII into rFVIIa. Conversion of the single chain rFVII into the active two-chain form (rFVIIa) occurs by autoactivation when rFVII is bound in sufficient density to charged surfaces [23].

4. Implications of the work

To our knowledge this is the first time that a synthetic affinity ligand has been designed to mimic and replace a metal ion-dependent immunoadsorption step in the purification of a human biopharmaceutical. The current protocol exploits an immobilized monoclonal antibody, which, in the presence of Ca^{2+}, increases the affinity for the Gla-EGF-1 hinge region of FVII some 75-fold. The performance of the immobilized ligand 5/5 described in this report compares more than favourably with the immunoadsorbent. For example, the interaction between 5/5 and FVII is Ca^{2+}-dependent with a 122-fold increase in affinity in the presence of ~2.5 m*M* Ca^{2+}. Comparison by RP-HPLC of the fractions eluted from the immobilized 5/5 and antibody adsorbent by citrate chelation shows that the FVII has almost identical purity (>99%), yield (98–99%), degradation profile (rFVIIa/rFVII ratio) and impurity content (~1000 ppm) both before, and after, the final ion-exchange polishing step. However, two important and significant differences are not only that the capacity for FVII of the immobilized 5/5 adsorbent is almost 10-fold higher than the immunoadsorbent, but also that the synthetic ligand is substantially more durable and able to withstand the rigours of CIP/SIP in the harsh manufacturing environment. The adsorbent is known to tolerate CIP with 1 *M* NaOH, 1 *M* acetic acid and 60% (v/v) ethanol and SIP with 1 *M* NaOH and steam at 121 °C without degradation of performance. Furthermore, desorption of the protein from the adsorbent under mild conditions is an asset for manufacturing a highly purified and fully functional FVIIa for therapeutic use.

Acknowledgements

The authors would like to thank James C. Pearson, Tad Podgorski and Steven J. Burton of ProMetic BioSciences Inc. for helpful discussions.

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