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Isolation of a peptide ligand for affinity purification of factor VIII using phage display

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Abstract

Polypeptides for use in affinity chromatography of factor VIII were identified using phage display technology. Phage libraries were designed to express polypeptide fusions containing five to seven residues flanked by two cysteines that form a disulfide bond. Individual bacteriophage were selected for the ability of these polypeptides to bind factor VIII, and then release the protein under mild elution conditions. Strong consensus sequences were observed that appear to be necessary for this reversible interaction. Chemically synthesized ligands identified by this screening were immobilized onto a chromatographic support and used for affinity purification of factor VIII from a complex feedstream. A chromatographic step was developed that provided a 10 000-fold reduction in host cell proteins and DNA, while providing exceptional product recovery.

Keywords: Affinity chromatography; Phage display; Peptides; Factor VIII

1. Introduction

The B-domain deleted form of human factor VIII (BDDrFVIII) is produced in recombinant Chinese hamster ovary (CHO) cells and is approved in both the North American and European markets for the treatment of hemophilia A. The current BDDrFVIII manufacturing process, which uses five orthogonal chromatography steps to provide a highly pure product, includes affinity chromatography employing a monoclonal antibody (mAb) that is specific for factor VIII [1]. The use of an immunoaffinity chromatography resin is common to the manufacturing processes for all recombinant factor VIII products [2], and many plasma-derived factor VIII products.

The motivation to replace the monoclonal antibody column used in the purification of BDDrFVIII arises from several limitations or concerns arising from the use of an immobilized monoclonal antibody in a current good manufacturing practice (cGMP) purification process. The immunoaffinity resins are used for a limited number of cycles of use before they are retired, which is the result of a slow reduction in factor VIII yield through the lifetime of the resin, a property that is not uncommon for immunoaffinity resins. In addition, the lability of the antibody precludes sanitization by caustic or denaturing buffers. Finally, the antibody immobilized to the chromatographic resin is produced by a murine hybridoma culture. Because many murine hybridomas harbor viable retroviruses [3,4], their use as a production host for the mAb presents a low, but non-zero, risk that viruses could be introduced into the factor VIII manufacturing process stream, and potentially contaminate the product. (Of course, there is an excellent assurance of the viral safety of BDDrFVIII produced by the current process [5].) The substitution of a chemically-synthesized peptide ligand for the antibody in the affinity chromatography step has the potential to eliminate all of these shortcomings. Further, an improved manufacturing process has been developed for production of BDDrFVIII which employs no animal- or human-derived proteins, to decrease the potential for introduction of infectious agents such as viruses into the manufacturing process.

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The elimination of the immunoaffinity chromatography resin is necessary to support the claim that no animal-derived raw materials are used in the improved process.

Phage display is a method for obtaining specific binding molecules [6–8]. Novel DNA is inserted into the genome of a phage (or phagemid) so that the DNA is an in-frame insertion into a gene encoding one of the phage coat proteins. As a result, the phage particle may display on its surface one or more copies of an altered coat protein containing a novel amino acid sequence. In a phage display library, the inserted DNA is diverse in sequence and generates a large and diverse population of phage. Each phage in the library could carry a different insert. Each phage in the library has a single DNA insert and the translation of that DNA is presented on its surface. To isolate specific binding proteins or peptides, the population is allowed to contact the target material, non-binding phage are washed away, and the remaining population, enriched in binding phage, is amplified. Because the selection process is conducted in vitro, the experimentalist can specify the conditions under which binding and elution will occur.

Through the use of phage display technology described further, a suitable peptide ligand was identified which substitutes for the antibody in the affinity chromatography step, and provides BDDrFVIII of equivalent purity and with an equivalent product isoform spectrum.

2. Experimental

2.1. Phage library construction

Phage display libraries were constructed in the previously described M13mp18 phage derivative, MANP [9,10]. DNA encoding the libraries was synthesized by MorphoSys (Munich, Germany) using TRIM technology [11]. The use of TRIM allows addition of preformed trinucleotides to the growing synthetic DNA to express the desired specification of amino acid residue distributions at each varied position (see Table 1). The library DNA was purified, and ligated between the *Nco*I and *Pst*I sites in the prepared MANP vector. The ligated DNA products were used to transform cells of the *Escherichia coli* strain XL1-Blue (Stratagene). Library size estimates, based on counts of plaque forming units (pfu) from the initial transformations, are included in Table 1.

2.2. Selection using BDDrFVIII

Recombinant B-domain deleted factor VIII (described in [12]) was obtained from Wyeth BioPharma (Andover, MA, USA). For use in phage selection and screens, this material was lightly biotinylated, using NHS-LC-biotin (Pierce No. 21335), with no loss of activity.

For each round of selections, 10 μg of biotinylated BDDrFVIII was added to one 350 μL aliquot of washed streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, Dynal Biotech No. 112.05) and the mixture was incubated on a rotating mixer for 2 h at room temperature (RT) (or overnight at 4 °C). The BDDrFVIII-treated beads were washed one time with 0.1% Tween 20 in phosphate-buffered saline (PBST) and then blocked with 350 μL of PBS containing 0.5% bovine serum albumin (BSA) and 114 μM biotin for 3 h at room temperature.

For each separate library, 10^{11} pfu were added to a 100 μL aliquot of prepared beads in a final volume of 250 μL of binding buffer (0.1 M ammonium acetate, 0.8 M NaCl, 5 mM $CaCl_2$, 0.02% Triton X-100, pH 6.3) and the suspension was mixed by tumbling for 30 min at RT on a rotating mixer. The supernatant solution, containing unbound library phage, was removed and the beads were washed seven times with PBST. Phage that remained bound to the beads were removed using either an ethylene glycol solution (50% ethylene glycol, 20 mM histidine, 0.25 M NaCl, 20 mM $CaCl_2$, 0.01% Tween 20, pH 7.0), which is a relatively gentle elution condition or extremely low pH (150 mM NaCl, 50 mM sodium citrate, 0.1% BSA, pH 2.0), which will strip off almost all of the bound phage. The neutralized phage eluates were used to infect aliquots of XL1-Blue MRF′ *E. coli* cells which were then plated onto NZCYM agar containing 50 μg/mL of ampicillin. After overnight incubation at 37 °C, phage were harvested. The amplified phage preparations from the ethylene glycol and the low pH elutions were titered and used separately to provide input to the next rounds of selection.

2.3. Indirect phage enzyme-linked immunosorbent assay (ELISA) protocol

Analysis of binding by individual phage isolates to immobilized BDDrFVIII was performed in an ELISA format similar to that described [13]. Wells of Immulon 2HB plates were coated with streptavidin (100 μL per well at 1 μg/mL),

Table 1
Flanking sequences and variegation patterns in TN libraries

Library	Left flank	Insert	Right flank	N ($\times 10^8$ pfu)
TN7/1	AEGTGD	X1X2$\overline{\text{CX3X3X3X3X3C}}$X2X1 (X1 = ADFHLPR; X2 = $\overline{\text{ADFGHLPRS}}$; $\overline{\text{X3}}$ = ADEFGHILNPQRSTVWY)	DPGPTDN	1.1
TN8/6	AEGTGD	X4X2$\overline{\text{CX5X5X5X5X5X5C}}$X2X4 (X4 = ADHR; X2 = $\overline{\text{ADFGHLPRS}}$; $\overline{\text{X5}}$ = ADFGHLNPQRSVW)	DPGPTDN	1.7
TN9/1	AEGTGD	X2$\overline{\text{CX5X5X5X5X5X5X5C}}$X2 (X2 = ADFGHLPRS; $\overline{\text{X5}}$ = $\overline{\text{ADFGHLNPQRSVW}}$)	DPGPTDN	1.2

then washed and coated with 100 μL of biotinylated BDDrFVIII at 10 μg/mL. Blank control wells were blocked with biotin (instead of BDDrFVIII) and biotinylated BSA was immobilized in BSA control wells. Equal volumes of each phage isolate preparation to be tested were added to immobilized BDDrFVIII, BSA, and blank wells and plates were incubated for 1 h at room temperature. After the phage binding step, the plates were washed 5 times with PBST and a 100 μL aliquot of a 1:10 000 dilution of horseradish peroxidase-labeled anti-M13 antibody conjugate was added to each well. Plates were incubated for 1 h at RT and then washed five times with PBST. The plates were developed with tetramethylbenzidine solutions and read at 630 nm using an ELISA plate reader.

2.4. DNA sequence analysis

Phage DNA for sequence analysis was prepared from individual phage isolate stocks by polymerase chain reaction (PCR) amplification using PCR primers that bracket the peptide-encoding DNA insert. PCR products were sequenced using standard dye-terminator methods and sequencing products were analyzed with an Applied Biosystems 310 DNA sequencer.

2.5. Peptide synthesis

Peptides corresponding to the deduced amino acid sequences of peptides displayed by positive phage isolates were synthesized on solid phase using standard 9-fluorenylmethoxycarbonyl (FMOC) or *tert*-butyloxycarbonyl (tBOC) protocols. Peptides were purified by reverse phase chromatography. Cyclic peptide masses were confirmed by electrospray mass spectrophotometry and peptides were quantified by absorbance at 280 nm.

2.6. Isothermal titration calorimetry measurements

Samples of TN8.2 peptide and BDDrFVIII were dialyzed against the column load buffer at pH 6.4. The BDDrFVIII solution was placed into the sample cell of a MicroCal (Northampton, MA, USA) isothermal titration calorimeter (ITC), and measured volumes of the TN8.2 peptide solution were injected into the sample cell. The heat released upon ligand-protein binding was measured (after correction for the heat of mixing and dilution), and the change in heat released per injection was plotted against the ligand concentration. The data were fit to an equilibrium binding equation [14] providing estimates of the equilibrium dissociation constant and stoichiometry of binding.

2.7. BDDrFVIII assays

Factor VIII activity was determined using either the Coatest VIII:C assay kit (Chromogenix, Milan, Italy) that was adapted to run on a Cobas FARA (Roche Diagnostics Systems), or a two-stage chromogenic substrate assay [15]. Both assays measure activated factor Xa activity generated via the intrinsic pathway where factor VIII:C acts as a co-factor in the presence of factor IXa, phospholipid and calcium ions. Factor Xa concentrations are estimated by measuring the colored product resulting from hydrolysis of the chromogenic substrate.

2.8. TN8.2 Sepharose resin coupling

NHS Sepharose 4 Fast Flow was obtained from Amersham Bioscience. Peptide coupling reactions were performed in jacketed stirred reactors controlled to 25 °C. The isopropanol solution used for resin storage was removed by sequential washing with 1 mM HCl on sintered funnels. One half-resin volume of 1 mM HCl was added, yielding a resin slurry of 67% (v/v). The peptide was dissolved in 300 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at a concentration of 2.8 mg/mL. Coupling reactions were initiated by the addition of one resin volume of peptide solution to the resin suspension. Samples taken for the analysis of immobilization kinetics used Millipore Ultrafree-MC 0.45 μm centrifuge filter units to separate resin beads from the reaction liquid. Reactions proceeded for 1 h. The coupled resin was treated with alternating washes of pH 4 and 8 as per the vendor's recommendations. Immobilization efficiency was determined by quantifying the peptide present in the reaction supernatant using RP-HPLC.

2.9. TN8.2 Sepharose chromatography

Chromatographic columns of approximately 10 cm bed height were packed and equilibrated with six column volumes of equilibration buffer (100 mM ammonium acetate, 400 mM sodium chloride, 5 mM calcium chloride, 1.02% Triton X-100, 0.3% tri-*n*-butyl phosphate, pH 6.8). The load containing BDDrFVIII was applied to each column at a challenge of up to 25 000 IU/mL resin. Following the load, the column was washed with six column volumes of wash 1 (100 mM ammonium acetate, 400 mM sodium chloride, 5 mM calcium chloride, 0.02% Triton X-100, pH 6.8) and six column volumes of wash 2 (100 mM ammonium acetate, 1.0 M sodium chloride, 5 mM calcium chloride, 0.02% Triton X-100, pH 6.8). Three column volumes of wash 3 (50 mM histidine, 50 mM calcium chloride, 0.02% Triton X-100, pH 6.6) preceded elution with elution buffer (50 mM histidine, 50 mM calcium chloride, 0.02% Triton X-100, 50% ethylene glycol, pH 6.6) flowing in the reverse direction. Residual protein was removed with the strip buffer (6.0 M guanidine hydrochloride, 50 mM acetic acid, pH 3.0).

2.10. Impurity assays

Residual levels of host cell proteins in the elution pool were determined using a polyclonal sandwich ELISA. DNA

concentrations were determined by an immunoassay using antibodies against single-stranded DNA [16].

3. Results

3.1. Library screening results: sequences and analysis

Phage display technology was used to identify cyclic peptides that could be used for the affinity purification of BDDrFVIII. This was undertaken using three phage peptide display libraries differing in the number of amino acid residues comprising a constrained disulfide loop: TN7/1, TN8/6, and TN9/1 (Table 1).

DNA was inserted into gene *iii* of a derivative of M13 filamentous phage which results in expression of the novel peptide in all displayed III protein (Fig. 1). The DNA inserts encode peptides that contain two cysteine residues at fixed positions giving rise to a disulfide-constrained cyclic peptide, of defined size for each library, at the amino terminus of each phage gene III protein. The disulfide bond constrains the conformation of the other residues in the displayed peptide so that they act more like residues in surface exposed loops of proteins rather than residues in linear peptides. As shown in Table 1, the TN libraries used here allow sequence diversity both within the central disulfide-constrained structure and in flanking residues.

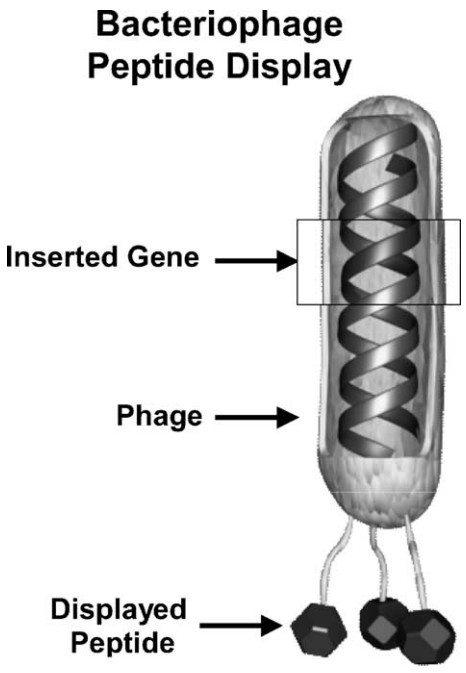

Fig. 1. Schematic illustration of the peptide phage display process. The M13 filamentous phage is drawn showing the circular single-stranded DNA phage genome enclosed within a sheath consisting of phage coat proteins. In the phage display process used in this report, a synthetic DNA construct encoding a disulfide-constrained cyclic peptide fused to the amino terminus of the phage gene III protein ("inserted gene") is incorporated into the phage genome. The expressed gene product comprising the gene III protein with the cyclic peptide fused to the amino-terminus ("displayed peptide") is incorporated into the coat structure of the phage particle containing the fusion gene, providing a direct physical link between gene and gene product.

The repertoire of allowed amino acids at the varied (or variegated) positions is also shown in Table 1. In contrast to other peptide display libraries used at Dyax Corp, the sequence diversity allowed in these three libraries was deliberately restricted. The rationale for restricting library diversity was two-fold. First, to ensure greater coverage of the encoded sequence space in the relatively small libraries (ca. 10^8 transformants, Table 1), the diversity of the regions flanking the central disulfide-constrained structures was limited to sets of amino acids providing representative ranges of amino acid chemistries and sizes. Greater sequence diversity was allowed within the critical disulfide-constrained region of each library. Second, three residues were excluded at all positions of sequence variation. Cysteine was excluded to assure the formation of the proper cyclic structure in the displayed peptide. Methionine was excluded because the residue may oxidize under conditions of use in chromatography, thus limiting the effective lifetime of the affinity resin. Lysine was excluded to facilitate the directed immobilization of synthetic peptide ligands onto chromatographic supports through amine-based coupling chemistry (see further). As reported here, these restrictions did not preclude identification of suitable binding peptides from these libraries.

Biotinylated BDDrFVIII protein that was confirmed to be active after derivatization was immobilized onto streptavidin-coated magnetic beads and used as a target in six separate selection campaigns. Each campaign incorporated three rounds of selection from a single library, using one elution condition for phage bound to the immobilized BDDrFVIII. A round of selection consisted of a binding step in which the phage library was first incubated in suspension with the target, followed by a plate wash step to remove unbound or weakly-bound members of the phage library, and an elution step to recover a pool of phage. This results in a population of phage isolates enriched in members that bind the target under binding and wash conditions and release the target under elution conditions. Finally, elution pools from each round of selection were separately amplified by infection and growth in *E. coli* cultures. The purified phage preparations from this amplification step served as the input for subsequent rounds of selection.

The binding, wash, and elution solutions used in the phage selection protocols were chosen to closely approximate the operating conditions of the immunoaffinity column. This approach would allow the process conditions for the peptide affinity chromatography step to be essentially unchanged as a consequence of the resin substitution. Those conditions preserve the integrity of the BDDrFVIII molecule by avoiding extremes of pH or solvent composition during elution. The binding buffer for the phage selection process simulated the immunoaffinity column load conditions, which are derived from the eluate pool from the ion-exchange column

step upstream of the current immunoaffinity column. Two elution conditions were tested for release of phage bound to BDDrFVIII. The first elution condition (50% ethylene glycol, 20 mM histidine, 0.25 M NaCl, 20 mM $CaCl_2$, 0.01% Tween 20, pH 7.0) is very similar to the elution buffer used for the immunoaffinity column. A second elution employed a low pH buffer [citrate-buffered saline (CBS), pH 2.0] to essentially denature the target and recover all phage isolates bound to the target. The ethylene glycol and pH 2 elution selections were performed separately for each library.

Upon completion of the second and third rounds of selection, 96 randomly-chosen individual phage isolates from pools generated by both elution conditions were screened for binding to BDDrFVIII by indirect phage ELISA. Biotinylated BDDrFVIII was immobilized in streptavidin-coated wells and bound phage were detected with HRP-conjugated anti-M13 polyclonal antibody and TMB peroxidase substrate. Background binding levels were established using wells coated with streptavidin alone or with BSA. Phage isolates exhibiting ELISA signals significantly above background ($A_{630} > 0.25$) were scored as positive for binding to BDDrFVIII. The amino acid sequences of the peptides displayed by these isolates were deduced from DNA sequence analysis. Further analytical tests allowed identification of a set of high scoring isolates, characterized by high ELISA signals and good discrimination of target over background.

Table 2 summarizes sequence and ELISA data for high scoring (target/background >5) isolates identified from post-selection screening analyses. High scoring isolates were obtained from all three libraries. ELISA signals were, on average, higher for peptides with larger loop sizes so that, in terms of average ELISA signal magnitudes, TN9 > TN8 > TN7. As a result, peptides with the greatest discriminations between target and background in the ELISA assays were recovered from the TN9 library.

Two related sequence families, which segregate among library types, are apparent among the sequences listed in Table 2. The first sequence motif is found only in isolates from the TN7 and TN8 libraries and is located precisely relative to the second of the two cysteine residues that comprise the disulfide bond in the peptides: . . . SWϕxPC. . . The symbol "ϕ" is used to indicate presence of V, I, L, or F and "x" indicates no strong residue preference. In the TN8 peptides, which are one residue larger than the TN7 peptides, the motif is expanded to include additional residues: . . . hpCGSWϕrPCxa/h. . . where upper case indicates a strong residue preference at the position. [Although histidine was the only residue recovered at the first variegated position, the sequence diversity allowed at this position (Table 1) was limited.]

The second sequence motif is found only among isolates from the TN9 library, but bears some relationship to the first motif. This common sequence encompasses the entire nine-residue disulfide-constrained peptide macrocycle: . . . fCWVFpFxHCx. . . Both sequence motifs include the sequence WϕxPxxH, regardless of the difference in loop size, suggesting that these sequences interact with the target in a similar fashion at a single site, with some accommodation of differing loop sizes and geometries.

3.2. Peptide synthesis and immobilization

Following the sequencing of the phage display selectants, analysis of the superfamily of sequences in Table 2 provided a rationale for choosing nine unique peptides for synthesis and an initial round of resin screening to identify the best peptide sequence for the affinity chromatography step. The peptide sequences were selected to include all three loop sizes, a range of hydrophobicities as determined by their Kyte–Doolittle scores, a range of isoelectric points, and examples of the minor sequence motif variations identified within the major superfamily consensus sequence (Table 3).

Peptides were designed with constant amino- and carboxy-terminal sequences flanking the unique cyclic core regions identified from the phage library selection experiments. The amino-terminal sequence, AEGTGD, was conserved from the displayed phage sequence to provide the original context. The constant carboxy-terminal linker sequence, EPGEGGGGK, was chosen to promote solubil-

Table 2
High scoring isolates from rounds 2 and 3

Library	AA sequence	N_{occ}[a]	R[b]	Mode[c]
TN7/1	RLC**SW**VS**P**CSA	1	10	E
	FGC**SW**LF**P**CPF	2	8	E
	RLC**SW**IS**P**CSA	4	6	E
TN8/6	**H**PC**G**A**W**LR**P**CYN	1	20	E
	HPC**GSW**LR**P**CLH	10	16	E/2
	HPC**GSW**FR**P**CFH	3	16	E
	HSC**GSW**LF**P**CFA	7	10	E
	HPC**GSW**FN**P**CAH	4	8	E/2
	HLCFA**W**FR**P**CDA	1	8	2
	HPC**GSW**LH**P**CAA	1	6	2
	HAC**GSW**FR**PC**HA	3	6	E/2
	HLCGA**W**FR**P**CDA	6	6	E/2
	HRC**GSW**LH**P**CLA	1	6	E
TN9/1	**FCWVF**A**F**D**H**CH	14	24	2
	FCWVFP**F**Q**H**CA	2	24	E
	FCWVFN**F**S**H**CS	3	24	2
	FCWVFP**F**N**H**CS	6	18	E
	FCWVFNWV**H**CD	1	14	E
	FCWVFP**F**N**H**CD	6	8	2
	FCWVFQ**F**R**H**CH	1	8	2
	FCH**VF**N**F**V**H**CS	3	8	2
	FCWVFP**F**H**H**CF	1	6	E

Consensus residues are shown in bold (uppercase letters stronger than lowercase). TN7: rl**CSW**ϕs**PC**sa; TN8: **H**p**CGSW**ϕ**rPC**xa/h; TN9: **FCWVF**p**F**x**HC**x (ϕ = **V**, **I**, **L**, **F**).

[a] N_{occ} is the number of isolates that had the given sequence.

[b] R is the ratio of the ELISA signal with target to the ELISA signal without target.

[c] Mode is the mode of elution used to obtain the given isolate. "E" means elution with 50% ethylene glycol, "2" means pH 2 elution, and E/2 means the peptide sequence was found in isolates obtained with both elution protocols.

Table 3
Performance of lead clones immobilized on chromatographic resins

Library	Clone ID	Amino acid sequences in variegated region	Percentage of factor VIII activity	
			Elution peak	Load eluate
TN7	04 G10	**RLCSWVSPCSA**	0	64
	04 A08	**FGCSWLFPCPF**	43	5
	01 A09	**FHCIGVWFCLH**	1	61
TN8	06 C10	**HPCGSWLRPCLH**	26	0
	06 E02	**HLCGAWFRPCDA**	42	2
	08 A11[a]	**HRCGSWLHPCLA**	20	34
TN9	11 A01	**FCWVFAFDHCH**	56	1
	12 E09	**FCWVFPFQHCA**	31	6
	12 D06	**FCWVFPFHHCF**	0	28

[a] 08 A11 (later called TN8.2) was the sequence eventually selected for the process step; its relatively poor performance in this screen was the result of this resin having significantly lower ligand density than the others and hence resulting in excessive losses in the load eluate.

ity and proteolytic stability while separating the attachment site (the ε amino group of lysine residue) from the rest of the peptide. Specifically, since DP sequences are prone to cleavage under acid conditions, the first two residues of the linker sequence (EP) preserve the acidic residue after the last selected core residue for context while avoiding the specific DP dipeptide sequence present from the phage presentation of the peptides (Table 1).

Peptides were synthesized using either standard FMOC or tBOC solid-phase synthesis, and purified to greater than 95% purity by RP-HPLC. All peptides were designed to contain only one primary amine provided by a lysine residue at the carboxyl terminus. This strategy required acetylation of the amino terminus, which was shown by calorimetry studies to have no effect on the peptide–BDDrFVIII dissociation constant. The single primary amine enabled a directed immobilization to the chromatographic resin, which in turn presents the peptide in the same orientation presented by the phage during the initial library screening, i.e., C-terminal linkage with a free amino terminus. An N-hydroxysuccinimide activated agarose matrix was chosen as the base chromatography medium due to its rapid and well-understood reaction chemistry with primary amines [17], and its similarity to the base matrix of the immunoaffinity step (Sepharose 4 Fast Flow). The immobilization reaction was completed within 30 min under typical conditions, and the reaction was found to be robust to minor changes in operating pH, ligand density, and *N*-hydroxysuccinimide (NHS) ester concentration. The immobilization efficiency was high, with peptide incorporation levels typically exceeding 90% [18].

The solution phase dissociation constants of BDDrFVIII with several of the ligands identified from the screen were determined to be approximately 1×10^{-6} M by isothermal titration calorimetry (ITC). This determination was made under solution conditions similar to those used during the loading of the affinity column. While this is at least four to five orders of magnitude weaker binding than the monoclonal antibody currently used for BDDrFVIII purification, a significantly higher molar concentration of ligand on the resin forces the binding equilibrium to favor adsorption and compensates for the weaker affinity for BDDrFVIII, and provides excellent capture and retention of BDDrFVIII during the chromatographic cycle. Although phage display technology has been shown to be capable of identifying peptides with much tighter binding to their target, having dissociation constants in the nanomolar range [19], the elution of the target protein from immobilized peptide ligands is more easily achieved with ligands having only micromolar binding affinity.

3.3. Screening of candidates on chromatographic resins

A panel of nine resins (Table 3) was generated, with ligand concentrations in the range of 1–3 mg of peptide/mL of resin (about 0.3–1 μmol peptide/mL of resin). These resins were then evaluated using a complete chromatographic cycle of operation, based on the solutions and conditions for the immunoaffinity resin operation. The columns were first equilibrated to neutral pH, challenged with a representative BDDrFVIII-containing load, briefly washed to remove unbound BDDrFVIII, and then eluted with a buffer containing 50% ethylene glycol. Since the phage library screen was designed to select for peptides which had high affinity for BDDrFVIII under these load conditions and had reduced affinity in the presence of glycol, there was a reasonable probability that this chromatographic cycle would demonstrate both adequate product capture and efficient elution.

The factor VIII activity in the load eluate (LE) and elution fractions for the nine resins is shown in Table 3. Several candidate resins failed to capture the product (for example, 04 G10 and 01 A09), or released the BDDrFVIII that had been bound during the wash regime (12 D06); subsequent studies suggest that these limitations may be overcome by increasing the ligand density of the resins. Other candidate resins completely captured the product, but did not provide a high recovery in the elution peak (12 E09). Likewise, a reduction in ligand density may alleviate this problem. (Low pH elution solutions similar to those used during the phage

Table 4
Chromatographic column performance of top three sequence candidates

Clonal designation and trivial name	06 E02 (TN8.1)	08 A11 (TN8.2)	11 A01 (TN9.2)
CHO protein removal (log_{10} reduction)	3.8	4.2	n.d.
DNA removal (log_{10} reduction)	3.7	4.4	n.d.
BDDrFVIII recovery (%)	46	48	<1
Elution peak volume (column volumes)	<1.0	<1.5	n.d.
Light chain/heavy chain ratio	1.3	3.1	n.d.

screening could not be used in the chromatography step due to the loss of factor VIII activity under those extreme conditions.) The sequence ultimately selected for the cGMP purification step, the TN8.2 sequence, did not distinguish itself from the other sequences in this first resin screen. A control experiment using resin produced by hydrolyzing the NHS Sepharose without peptide present in the coupling buffer failed to capture appreciable BDDrFVIII activity. Also, resin which presented a reduced, linear peptide lacking the disulfide bond failed to bind BDDrFVIII, confirming the need for the constrained loop in order to function effectively.

The three most promising candidates based on product recovery and purity assessments were taken into a more stringent round of resin screening, which included further chromatographic runs, and analysis of product purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), host cell protein ELISA, and DNA residuals. The data in Table 4 provide a summary of the performance of a panel of runs conducted with the same lot of load material. The TN9.1 resin failed to capture the product with sufficient capacity to be a viable candidate; a higher ligand density would likely improve the capture efficiency and allow a true comparison to the two other resins. Due to the superior performance of the other two resins, the TN9.1 resin was dropped from consideration. Additional criteria were used to assess the remaining two peptide resins, including ease of resin reuse and cleaning, evaluation of the extent of elution and factor VIII activity balances across the step (since irreversible adsorption could lead to resin cleaning problems after repeated use), robust performance within the normal variations in process conditions, and a more rigorous evaluation of product yield. In the final determination of the best ligand to be used for the process step, optimized process steps using TN8.1 and TN8.2 resins were evaluated in parallel. Superior product yield and smaller elution peak volumes were observed for the TN8.2 resin. The TN8.2 sequence was eventually selected for the cGMP process step upon consideration of these combined performance factors.

The BDDrFVIII cell culture process expresses a small quantity of BDDrFVIII light chain that is not associated with the complementary heavy chain. Given the binding specificity of the TN8.2 ligand for the C2 domain of the BDDrFVIII free light chain (data not shown), it would be expected that the TN8.2 Sepharose would also capture this free light chain. The TN8.2 Sepharose elution pool does contain a small amount of captured light chain, but during development of the process step it was observed that the levels of free light chain could be reduced by the incorporation of a high salt wash prior to column elution. This suggests that there is reduced binding affinity between BDDrFVIII and free light chain presumably arising from structural differences imparted by the presence or absence of the heavy chain partner. Additional removal of free light chain is provided by two subsequent chromatography steps in the BDDrFVIII purification process, yielding bulk drug substance with no excess free light chain.

Following the selection of the TN8.2 ligand sequence, the column step operating conditions were subjected to additional development efforts to optimize the column performance. Minor modifications were made to the column wash buffers to increase product purity and to the elution buffer to ensure complete elution. The peptide sequence identified by the screen, NHS immobilization chemistry and resin backbone were unchanged during optimization.

One critical variable identified during the optimization phase was the ligand concentration on the resin. Table 5 summarizes the observations made from chromatographic evaluation of TN8.2 Sepharose resins with ligand densities in the range of 1.0–5.5 mg/mL resin. Insufficient product retention was observed with ligand densities of 2.0 mg/mL and below. Poor recovery was also observed at 5.5 mg/mL ligand density due to incomplete elution, even when ethylene glycol concentrations were increased to 60%. Ligand densities in the range of 2.25–3.5 mg/mL resin showed good product recovery, but the resin at 3.5 mg/mL ligand density gave a higher retention of free light chain. These observations indicate that a narrow four-fold difference in ligand density markedly modulates product recovery from the extreme of failure to capture product at low ligand density, to a reduc-

Table 5
Effect of the TN8.2 ligand density on chromatographic performance

Ligand density (mg/mL)	Chromatographic performance
5.5	Incomplete elution (even with 60% ethylene glycol), poor overall recovery
3.5	Good recovery, but higher light chain levels
3.0	Good recovery (no significant losses in load eluate and washes)
2.5	Good recovery (no significant losses in load eluate and washes)
2.25	Good recovery (<3% loss in load eluate and washes)
2.0	Insufficient product retention (5% loss in load eluate and washes)
1.5	Significant losses (15% loss in load eluate and washes)
1.0	Almost no product capture (90% in load eluate)

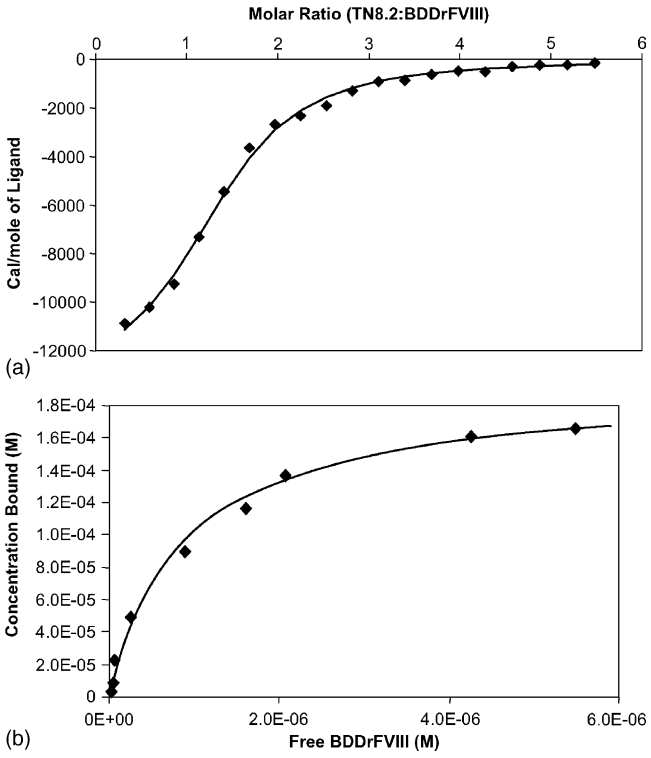

Fig. 2. Equilibrium binding measurements. The TN8.2 and BDDrFVIII interaction was characterized by: (a) isothermal titration calorimetry, which was conducted in free solution. (b) measurement of the adsorption isotherm directly on TN8.2 Sepharose resin. Both methods show excellent fits of the mathematical models that assume a single site of interaction between ligand and protein, and give very similar estimates of the dissociation constant (0.72×10^{-6} M for the calorimetry study, and 0.92×10^{-6} M for the adsorption isotherm).

tion in product recovery arising from incomplete elution at high ligand density. Considering this chromatographic performance and the cost considerations of producing the resin, the target ligand density was set at 2.5 mg/mL resin. The cGMP resin production is capable of controlling ligand density to tight tolerances (within 10% of the target ligand density), and a formal robustness study of the process step at the high and low limits of ligand density showed consistent product recovery and purity [18].

3.4. Characterization and performance of the TN8.2 Sepharose resin

The TN8.2 resin was characterized for chromatographic cycle performance and modeled by a software programs simulating affinity chromatography which confirmed the sensitivity to ligand density described above. The equilibrium adsorption isotherm followed the expected Langmuir form, with a dissociation constant of 0.92×10^{-6} M, in excellent agreement with the solution ITC measurement of 0.72×10^{-6} M (Fig. 2). The equilibrium binding capacity of the resin is very high, in excess of 33 mg of BDDrFVIII/mL of resin. This is a large increase in capacity over the immunoaffinity resin, which has an immobilization density of 1 mg antibody/mL of resin, and an equilibrium binding capacity of less than 2 mg BDDrFVIII/mL of resin. The TN8.2 Sepharose column was subjected to breakthrough analysis to determine the dynamic binding capacity under typical column load conditions; greater than 13 mg/mL of BDDrFVIII bound at the point of 10% column breakthrough. This allows for a significantly higher process control limit to be set on the maximum loading capacity of the TN8.2 Sepharose resin in comparison to the immunoaffinity step [18].

A typical chromatogram for the optimized process step is shown in Fig. 3. The load eluate contains a large amount of protein impurities as well as high concentrations of solvent and detergent, and as a result the UV signal is well off-scale. The UV rapidly drops during the wash steps, with little concomitant loss of BDDrFVIII activity in the load eluate and wash fractions. The column is eluted in the reverse direction to the load and wash, which results in a very sharp elution peak of less than two column volumes. Product recovery in the elution pools averages 85%, significantly higher than the immunoaffinity step recovery average of 63%. Sanitization and cleaning of the resin after use is achieved with a solution of 6 M guanidine hydrochloride at low pH, which reduces product carry-over between cycles of use to less than 0.001% (data not shown). The chemical stability of the TN8.2 ligand allows for a much more rigorous sanitization regime than can be used for monoclonal antibody resins.

The excellent selectivity provided by the TN8.2 peptide is reflected by the high degree of removal of extraneous host cell proteins and DNA (Table 6). SDS-PAGE analysis indicates that the load to the TN8.2 Sepharose step is enriched in BDDrFVIII, but total protein measurements indicated that the load is less than 25% pure (see Fig. 4). (The initial step in the purification process is a cation-exchange column that provides over a 100-fold concentration of BDDrFVIII, but does not contribute greatly to product purity [1].) The resultant BDDrFVIII in the TN8.2 elution peak pool is greater than 99.9% pure. The extent of host cell protein removal was determined using a polyclonal ELISA that recognizes a broad spectrum of host cell protein impurities. Typically, the TN8.2 column step provides a 10 000-fold reduction in host cell protein levels. Similar reduction values are observed for removal of host cell-derived DNA. In total, these impurity removal values are consistent with a highly selective affinity chromatographic step, comparable to the performance provided by immunoaffinity steps.

The BDDrFVIII product is a heterogeneous mixture of isoforms, as is expected for production of complex glycoproteins in recombinant CHO cells [9]; one minor isoform identified in the SDS-PAGE gel shown in Fig. 4 is the M_r 170 000 fusion of the heavy and light chains, which is the translated product of the BDDrFVIII expression construct that is later cleaved to form the BDDrFVIII heterodimer. The substitution of one affinity chromatography ligand for another could result in an altered spectrum of product isoforms due to differential affinities for various

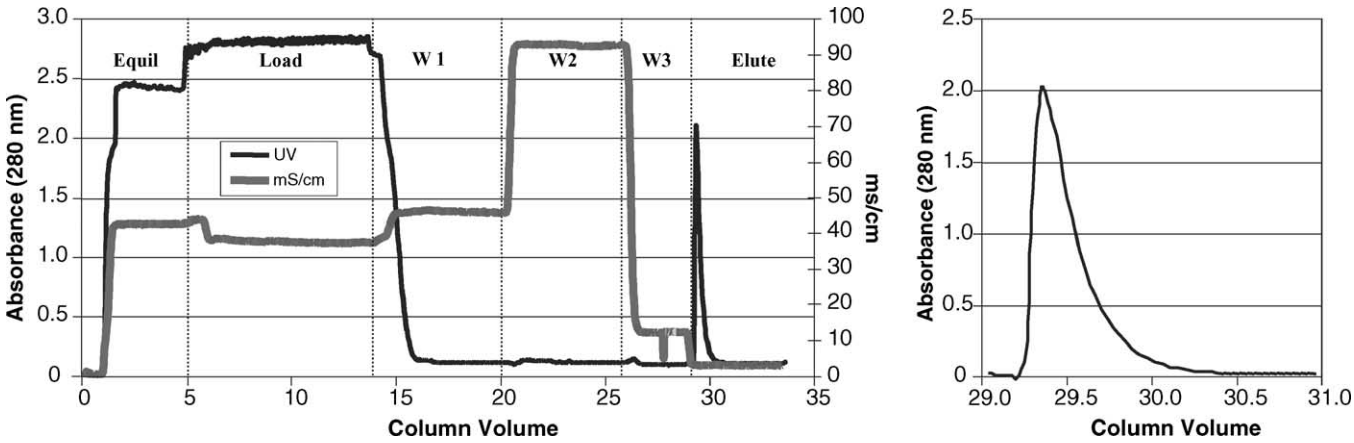

Fig. 3. Typical TN8.2 Sepharose chromatogram. The UV and conductivity profiles indicate the transitions between the equilibration, load, wash, elution and regeneration stages. The elution peak is expanded, and has a very steep ascending side and a rapid return to baseline without evidence of post-peak trailing or resolution of subspecies during elution.

Table 6
Comparison of TN8.2 and immunoaffinity purification step performance

	Immunoaffinity	TN8.2
BDDrFVIII recovery (average of >10 cGMP runs)	63	85
CHO protein removal ($\log_{10}$ reduction)	3.7	4.0
DNA removal ($\log_{10}$ reduction)	4.1	4.1
BDDrFVIII isoforms captured	M_r 170 000 fusion protein + multiple heterodimer forms	M_r 170 000 fusion protein + multiple heterodimer forms
Elution peak volume (column volumes)	<1.0	<1.5
Dynamic capacity (IU/mL)	20 000 (estimate)	180 000
Process control loading limit (IU/mL)	5000	25 000
Sanitization solution	0.1 M acetic acid, 20% ethanol, pH 4.0	6 M GuHCl, 50 mM acetic acid, pH 3.0

isoforms or a biased selection based on the distinct binding sites. The TN8.2 Sepharose step, however, provides a spectrum of BDDrFVIII product isoforms that is indistinguishable from immunoaffinity-purified BDDrFVIII as determined by high resolution techniques including peptide mapping, mass spectrometry, and oligosaccharide fingerprinting (data not shown).

4. Discussion

Phage display technology was used to identify peptides that appropriately substitute for a monoclonal antibody in the development of an affinity chromatography step for purification of BDDrFVIII. The peptides bind BDDrFVIII under conditions compatible with the load solution, and release BDDrFVIII under non-denaturing conditions. At the start of this investigation, it was unclear if a relatively small peptide could be identified that would have sufficient selectivity and affinity to be competitive with immunoaffinity chromatography. Several peptides were isolated under the allowed process conditions for both the product binding and releasing steps. The isolates from TN7 and TN8 are closely related; the consensus sequences (TN7: CSWϕsPC; TN8: HpCGSWϕrPCxh/a) differ mainly by the insertion of a glycine after the first cysteine. The TN9 isolates have a slightly different consensus (TN9: fCWVFpFxHCx) and are likely to bind to the same site in a different orientation.

While peptides with much tighter binding (dissociation constants in the nanomolar range) to their target may be identified from phage display screening [19], it may generally prove that the elution of the target protein from immobilized peptide ligands is more easily achieved for ligands with low micromolar binding affinity.

A simple resin screening based on product recovery was used to pare the nine peptide candidates to the three most effective candidates, which included the TN8.2 peptide. However, subsequent optimization studies with TN8.2 Sepharose identified ligand density on the chromatographic resin as a critical variable. It is likely that the ligand density on the resin in combination with the ligand dissociation constant modulates product capture efficiency (poor product capture at low ligand densities) and elution efficiency (incomplete product elution at elevated ligand densities). Subsequent resin screens should be designed to account for this sensitivity, as highly selective ligands could have been eliminated from the first resin screening because the ligand density was not optimized.

After optimization of the TN8.2 resin step, the purification process for BDDrFVIII was scaled up from the laboratory to

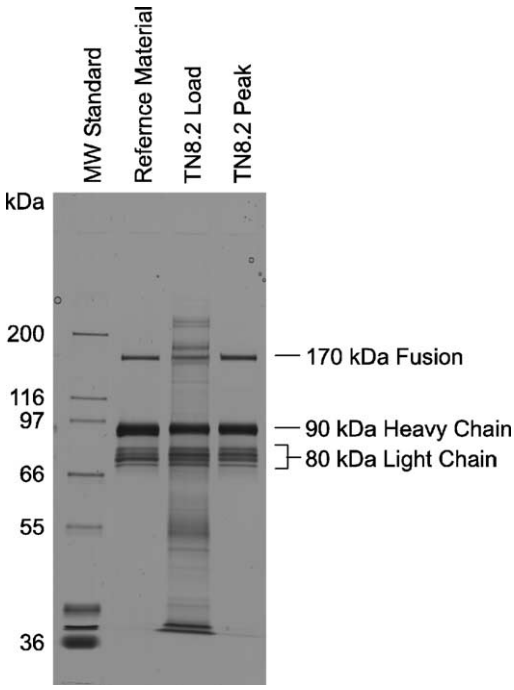

Fig. 4. SDS-PAGE analysis of TN8.2 Sepharose load and purified BDDrFVIII peak. Silver stained SDS-PAGE analysis of the TN8.2 Sepharose load and peak fractions indicate that protein impurities are greatly reduced by this affinity chromatography step. The major product isoforms and subunits are present in the prominent bands, including the M_r 170 000 product of the translational fusion expression vector, and the M_r 90 000 heavy chain and the M_r 80 000 light chain of the fully processed BDDrFVIII heterodimer (kDa = kilodalton).

full-scale cGMP production, with over a 1000-fold increase in the TN8.2 Sepharose column volume. No scale-related differences were observed, and the process step performance was in accord with developmental data. Validation studies have been completed, and demonstrate excellent column reuse and cleaning, robust operation over a range of operating parameters, and low and consistent levels of TN8.2 ligand leaching into the elution peak [18]. Significant removal of leached TN8.2 ligand is provided in subsequent purification steps, such that TN8.2 ligand levels in the BDDrFVIII product are below the level of detection of a sensitive polyclonal ELISA test method.

In this first example of phage display selection used for identification of affinity ligands for purification of a cGMP therapeutic protein, a process step was developed using a peptide ligand resin which was equivalent to an immunoaffinity resin in terms of the resulting product quality and ligand selectivity, but provided improved product recovery, and superior reuse and sanitization profiles. The step has been incorporated into the BDDrFVIII manufacturing process, and clinical trials are underway using BDDrFVIII purified with this ligand.

References

[1] R.K. Eriksson, C. Fenge, E. Lindner-Olsson, C. Ljungqvist, J. Rosenquist, A.L. Smeds, A. Ostlin, T.C. Charlebois, M. Leonard, B.D. Kelley, A. Ljungqvist, Semin. Hematol. 38 (Suppl. 4) (2001) 24.
[2] C. Lee, Thromb. Haemost. 82 (1999) 516.
[3] S.R. Adamson, Dev. Biol. Stand. 93 (1998) 89.
[4] A.H. Bartal, C. Feit, R.A. Erlandson, Y. Hirshaut, Med. Microbiol. Immunol. (Berl) 174 (1986) 325.
[5] T.S. Charlebois, B.D. O'Connell, S.R. Adamson, H. Brink-Nilsson, M. Jernberg, B. Eriksson, B.D. Kelley, Semin. Hematol. 38 (Suppl. 4) (2001) 32.
[6] H.M. Azzazy, W.E. Highsmith Jr., Clin. Biochem. 35 (2002) 425.
[7] W.G.T. Willats, Plant Mol. Biol. 50 (2002) 837.
[8] A.E. Nixon, Curr. Pharm. Biotechnol. 3 (2002) 1.
[9] H. Sandberg, A. Almstedt, J. Brandt, V.M. Castro, E. Gray, L. Holmquist, M. Lewin, U. Oswaldsson, M. Mikaelson, M.A. Jankowski, M. Bond, H.A. Scoble, Semin. Hematol. 38 (Suppl. 4) (2001) 4.
[10] A.K. Sato, D.J. Sexton, L.A. Morganelli, E.H. Cohen, Q.-L. Wu, G.P. Conley, Z. Streltsova, S.W. Lee, M. Devlin, D.B. DeOliveira, J. Enright, R.B. Kent, C.R. Wescott, T.C. Ransohoff, A.C. Ley, R.C. Ladner, Biotechnol. Prog. 18 (2002) 182.
[11] B. Virnekas, A. Pluckthun, K.C. Schneider, G. Wellnhofer, S.E. Moroney, Nucleic Acids Res. 22 (1994) 5600.
[12] US Patent 5 661 008, 1997.
[13] G.A. Kingsbury, R.P. Junghaus, Nucleic Acids Res. 23 (1995) 2563.
[14] D.R. Bundle, B.W. Siguskjold, Methods Enzymol. 247 (1994) 288.
[15] S. Rosen, Scand. J. Haematol. 33 (Suppl. 40) (1984) 139.
[16] J. Briggs, V.T. Kung, G. Pontis, P. Nagainis, P. Panfili, C. Chow, T. Smith, E. Sheldon, D. Ross, R. Smethers, B. Gomez, M. Lacy, V. Drbal, R. King, R. Zuk, Am. Biotechnol. Lab. 7 (1989) 34.
[17] A.P.G. van Sommeren, P.A.G.M. Machielsen, T.C.J. Gribnau, J. Chromatogr. 639 (1993) 23.
[18] B.D. Kelley, M. Tannatt, R. Magnusson, S. Hagelberg, J. Booth, Biotechnol. Bioeng. (2004), in press.
[19] L. Huang, D.J. Sexton, K. Skogerson, M. Devlin, R. Smith, I. Sanya, T. Parry, R. Kent, J. Enright, Q.-L. Wu, G. Conley, D. DeOliveira, L. Morganelli, M. Ducar, C.R. Wescott, R.C. Ladner, J. Biol. Chem. 278 (2003) 15532.