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Affinity Purification of von Willebrand Factor Using Ligands Derived from Peptide Libraries

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Abstract—The chromatographic purification of vWF (von Willebrand Factor) from human plasma represents a challenge because it consists of multimers with molecular weights ranging from 0.5 to 10 million Daltons. Phage peptide library screening yielded a lead peptide (RLRSFY) that interacts with vWF. Conservative substitutions of terminal residues of the lead peptide led to a second peptide, RVRSFY, which was more efficient in the affinity chromatographic purification of vWF from protein mixtures. Adsorption isotherm measurements indicated multiple interactions between vWF and the immobilized peptide RVRSFY. Increases in peptide density on the chromatographic supports resulted in stronger association constants and higher maximum protein binding capacities. When the peptide density was lower than 32 mg/mL, there was no measurable interaction between vWF and immobilized peptide RVRSFY in HEPES buffer containing 0.5 M NaCl at pH 7. An increase in peptide density from 32 to 60 mg/mL increased the association constants from 0.9×10^6 to 2×10^6 (M^{-1}). Divalent salts (calcium and magnesium chloride) were used to elute the retained vWF with 82.5% of the activity recovered. The interactions between vWF and the immobilized peptide RVRSFY are dominated by ionic attractions and also involve hydrophobic interactions at close contact. Finally, the purification of vWF from crude material PEG filtrate of a cryoprecipitate of human plasma is demonstrated using affinity chromatography with immobilized *N*-acetyl-RVRSFYK.

Introduction

The glycoprotein von Willebrand Factor (vWF) stabilizes and transports Factor VIII (FVIII), which is directly involved in the blood coagulation cascade.[1] Deficiency of either vWF or FVIII results in diseases of haemostasis, e.g. hemophilia A and vWF disease in humans, which are treatable with the relevant protein or a complex of both.

The purification of vWF is a challenge because it is a multimeric protein with molecular weights ranging from 0.5 to 10 million Daltons.[1] Such a wide range of molecular weights poses difficulties for its purification using size exclusion or ion exchange chromatography. This has led to investigation on the use of immunoaffinity chromatography as a purification method.[2]

In immunoaffinity chromatography, however monoclonal antibodies are the usual affinity ligands and they must be purified extensively prior to use. Protein ligands, such as antibodies, make the procedures for obtaining regulatory approval of an industrial scale purification lengthy and the purity and activity of the antibodies may vary. In addition, antibodies are expensive and the immobilized antibodies are sensitive to the process operating conditions. They can lose activity or leach into products by the harsh elution and cleaning conditions commonly used in immunoaffinity separation processes. The leakage of the antibodies from a column can result in serious product contamination due to the immunogenicity of those proteins.

Small peptides consisting of a few amino acids may hold certain advantages[3] as ligands for industrial affinity separations since they are not likely to cause an immune response in case of leakage into the product. Peptide ligands are also much more stable in comparison to antibody ligands. They can be manufactured aseptically in large quantities under good manufacturing practices (GMP) conditions, and at significantly lower cost. Also, peptides may be easily modified by existing chemical methods to facilitate product elution under mild conditions.

Peptide ligands can be found by screening phage peptide libraries. Phage peptide libraries are created by the insertion of a random DNA sequence of a given length into bacterial phages which are cultivated for the expression of random peptides on the phage surfaces.[4–7] Millions of phage particles, each with a different peptide, are then incubated with target protein. Phage particles that are not bound specifically to the target protein are discarded. The specifically retained phages are used to infect *E. coli* cells for further gene selection. The amplified gene of individual phage isolate can be sequenced and the specific peptide sequence deduced.

Key words: peptide library, vWF, affinity chromatography, peptide ligands, blood products.

This paper describes the screening and optimization of peptides to be used as affinity ligands for vWF purification. Measurements of the association constant of vWF to immobilized peptides reveal that the attachment of vWF to the surface is multimeric. The effects of peptide density, solution pH, temperature, and ionic strength, on the adsorption of vWF to the affinity matrix were analyzed to understand the nature of the interactions. Finally, the purification of vWF from a crude PEG filtrate of human plasma by affinity chromatography is demonstrated.

Results

The selection of peptide from phage peptide libraries

The phage peptide library screening technique of Smith[6–8] was adapted for the screening of peptide ligands that bind to vWF. vWF (10 nM) was allowed to bind to 5×10^8 phage in 50 μL of solution. Complexes of vWF and phage were then captured onto rFVIII (recombinant FVIII) that had been adsorbed to wells of a microtiter plate. Interactions between vWF and FVIII involve two high affinity binding sites[9] ensuring efficient capture. This strategy was designed to avoid selection of peptides binding to vWF at FVIII contact sites.

The screening was performed with three rounds of phage capture, elution, and amplification. The summaries on the enrichment of phage for the first, second, and third rounds of capture by rFVIII (vs. control BSA) are listed in Table 1. Briefly, after the first round enrichment, the phage particles were amplified and were again contacted with vWF in solution and captured on microtiter wells coated with rFVIII (or BSA). There was a 65-fold enrichment in the phage binding of vWF in comparison to BSA.

Forty-five per cent of the second round enriched phages were contacted with vWF as above. There was a further 12-fold enrichment for phage with the ability to bind vWF. The DNA encoding the random peptides from approximately one-half of the 200 clones selected for vWF binding was sequenced and the peptide sequences were deduced according to standard coding.

The sequenced peptides were synthesized chemically, purified by HPLC, and coupled to an affinity support (Affi-gel BioRad, CA). The peptide–resins were contacted in batch format (20–50 μL each) with biotinylated-vWF for 18 h at 4–8 °C in a microfuge tube. Resins were washed and stained using streptavidin–alkaline phosphatase conjugate using the insoluble dye BCIP (5-bromo-4-chloro-3-indolyl phosphate)/NBT (nitro blue tetrazolium). Several potential peptides that showed interaction with biotinyl-vWF were identified and listed in Table 2. The peptide RLRSFY showed much stronger staining than the others. This peptide was selected for further studies in the following section.

Table 1. Screening of phage peptide libraries for vWF binding peptides

	Phage applied	Phage captured by vWF	Phage captured by BSA
Initial	5×10^8	Not counted	Not counted
Second round	5×10^8	2.4×10^4	370
Third round	1×10^4	200	16

Table 2. Selection of peptide from phage peptide libraries

Peptide selected	Blue staining for vWF[a]
R-L-R-S-F-Y	+ + + (strong staining)
H-L-N-S-L-S	+ (weakly staining)
R-V-L-S-A-H	+
K-G-T-T-Y-Y	+
E-I-N-V-P-T	+
S-F-A-K-L-S	+
L-P-P-P-K-L	+
S-H-L-P-W-H	+
Control with no peptide	– (no staining)

[a]Staining was observed from peptide immobilized columns.

Affinity chromatography for the purification of vWF using immobilized peptide RLRSFY

Peptide RLRSFY was directly synthesized[10] on a modified Toyopearl resin (-(β-Ala)$_2$-Toyopearl) and the resin was packed in a 1 mL column (0.7 × 2.5 cm). 0.1 mL of Koate®-HP (human plasma-derived factor VIII concentrate containing vWF, FVIII, and human albumin as the main components) was applied to the column at a flow rate of 2 mL/min in binding buffer (10 mM HEPES, 5 mM calcium chloride and 0.1 M NaCl, pH 7). After 1 min of washing in binding buffer, a linear gradient over 5 min from 0.1 to 1 M NaCl in HEPES buffer was applied to the column. The elution with high salt concentration was maintained for another 1 min, before 2% acetic acid was used to remove any retained protein. The resulting chromatogram is shown in Figure 1, line (a). About 60% of the protein did not bind to the peptide resin or was loosely

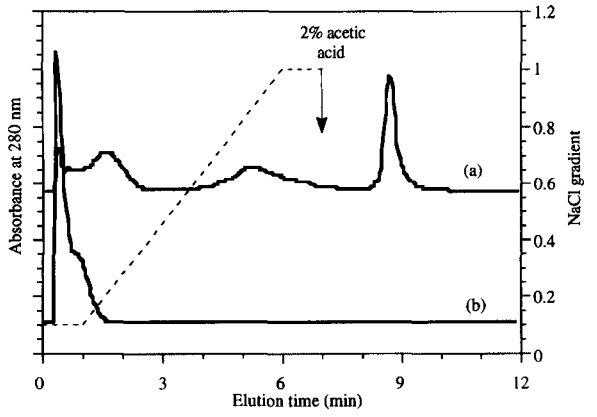

Figure 1. Elution profiles of Koate®-HP from columns with (a) and without (b) peptide RLRSFY. Flow-through peak is at 0.5 min, low and high salt peaks in line (a) are at 1.8 and 5.5 min, and acid peak is at 9 min.

Table 3. Mass balances based on A_{280} and ELISA measurements

Samples	RLRSFY-Toyopearl		(β-Ala)$_2$-Toyopearl	
	% area of acid peak	% vWF by ELISA	% area of acid peak	% vWF by ELISA
Flow-through	8	1.6	94	83.3
Salt	48	0.09	3	0.9
Acid	39	49.8	1	0.06
% recovery	95	51.5	98	84.3

bound and eluted by sodium chloride. The remainder was eluted by the 2% acetic acid wash. In a control experiment in which no peptide was attached to the resin [only -(β-Ala)2-Toyopearl], almost all the protein from a 0.1 mL injection of Koate®-HP flowed through the column under the same conditions used for the peptide column. This chromatogram is also shown in Figure 1, line (b).

The peaks in Figure 1 were collected and analyzed for vWF by ELISA and for purity by SDS–PAGE. Most of the vWF was retained by the RLRSFY-Toyopearl column and could not be eluted by 1 M sodium chloride (Table 3). Approximately one-half of the vWF was recovered in the acid peak. In comparison, 83% of the total vWF was found in the flow through peak from the (β-Ala)$_2$-Toyopearl control column.

Table 3 also shows that the total protein (represented as absorbance at 280 nm, A_{280}) recovered from the two columns is similar. However, as discussed above, only one half of the applied vWF was recovered from the peptide–Toyopearl column. This suggests that a portion of the vWF was denatured by elution with 2% acetic acid. This was confirmed by ELISA measurements on vWF standards that had been treated with acid and then neutralized (data not shown).

Analyses of the column fractions by SDS–PAGE in a reducing buffer revealed that the major protein in the acid eluate from the peptide–Toyopearl column had a subunit molecular weight of 225 kD. In the acid elution fraction, the gel also showed a small amount of albumin at 66 kD (data not shown). We concluded that the peptide-Toyopearl resin could retain vWF from the Koate®-HP mixture, but some modification on the peptide sequence might increase selectivity. That led us to the following studies to optimize the amino sequence of the peptide ligand for vWF purification.

Sequence optimization

Single conservative amino acid substitutions at the first three positions of RLRSFY were synthesized directly on (β-Ala)$_2$-Toyopearl. Amino acid substitutions used for both positions of arginine (R) were histidine (H), lysine (K), glutamine (Q), and asparagine (N). Substitutions for leucine (L) included isoleucine (I), valine (V), phenylalanine (F), and tyrosine (Y). These individual point mutations created 12 unique peptide sequences.

Table 4. Comparison of vWF capture from different peptide-Toyopearl resins

Peptide resins	% area of acid peak	% vWF by ELISA		
		Flow-through	Salt	Acid
HLRSFY	26.7	0.75	0.28	32.7
KLRSFY	27.1	1.03	9.83	26.94
NLRSFY	14.8	19.96	11.88	6.98
QLRSFY	9.2	29.45	11.69	2.2
RLHSFY	24.9	1.77	2.89	50.72
RLKSFY	25.1	0.6	3.59	45.58
RLNSFY	24.5	0.26	1.5	47.68
RLQSFY	20.1	0.46	0.54	50.68
RFRSFY	27.8	0.2	0.07	47.96
RIRSFY	28.0	1.92	4.09	44.78
RVRSFY	30.2	0.09	0.08	55.78
RYRSFY	27.7	1.65	0.14	51.72

After the resins were packed into individual 1 mL glass columns, 0.1 mL of Koate®-HP was applied to each column and eluted with a sodium chloride gradient and 2% acetic acid, the same elution protocol used in the experiment described in Figure 1. Fractions were collected and analyzed for total protein by A280 absorbance and for vWF by ELISA. The results are listed in Table 4. When the R at the N-terminus was replaced by N or Q, the percentage of vWF captured by the column was significantly reduced. The substitution of the R at the third position from the N-terminus had no effect on the capture of vWF from Koate®-HP. This seemed to suggest that the R at the N-terminal played a more important role than the R at the third position. When the second position (L) was substituted with F, I, V, or Y, the percentage of the total protein in the acid peak (calculated from absorbance at 280 nm) was reduced from 39% in Table 1 to around 30% in Table 4.

These fractions from the 12 different peptide columns were further concentrated by filtration in an Amicon Centricon-10 (molecular weight cut off 10,000), then analyzed by SDS–PAGE (reducing). A comparison of all the gels showed that the fractions eluted from the column of RVRSFY-Toyopearl gave the best resolution, as shown in Figure 2. In Figure 2, the lanes for the flow through and salt peaks showed mostly albumin, which was consistent with the ELISA results listed in Table 4. The acid fraction from the RVRSFY column was essentially pure vWF. The selectivity of the RVRSFY column for vWF was much better than that of the original RLRSFY column.

PEG filtrate was tested in the above columns for the purification of vWF and results similar to those using Koate®-HP were obtained (data not shown). In particular, no detectable amount of albumin was retained by the RVRSFY column after a 1 M NaCl wash. Approximately 76% of the vWF activity (from the FVIII binding assay) was recovered after elution from the column with 2% acetic acid. Therefore, the peptide RVRSFY was chosen for further studies.

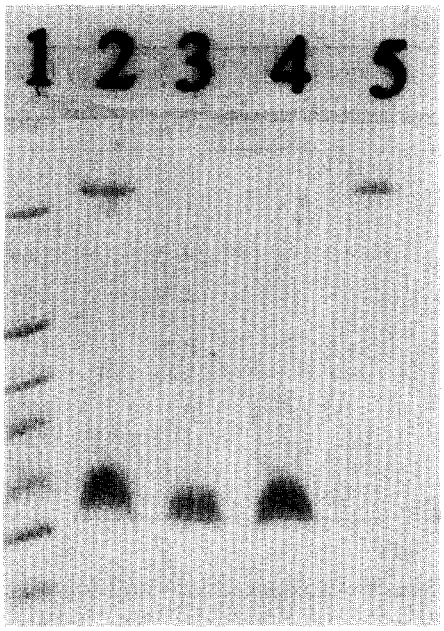

Figure 2. SDS–PAGE of fractions collected from the chromatography using column RVRSFY–Toyopearl. lane 1: molecular weight standard; lane 2: starting material, Koate®-HP; lane 3: flow-through peak; lane 4: salt peak; lane 5: acid peak.

Adsorption isotherm of vWF on RVRSFY–Toyopearl

Interactions between RVRSFY directly synthesized on Toyopearl and vWF in solution were studied through adsorption isotherm measurements. Because of the wide range of molecular weights of vWF, complex theoretical analyses are required to obtain binding constants from the adsorption isotherms since different multimers of vWF may have different binding constants. To simplify the analyses, a vWF fraction with a molecular weight around 1000 kD was used for the isotherm measurement. This size fraction was obtained from Koate®-HP by size exclusion chromatography using Bio-Gel A-5M (BioRad) and confirmed by nonreducing Agarose gel electrophoresis (not shown).

The adsorption isotherm for vWF on directly synthesized RVRSFY on Toyopearl at 25 °C is shown in Figure 3. The adsorption isotherm was fitted using the Langmuir equation, resulting in an association constant of 1.31×10^6 M^{-1}. The curve fit also showed that the maximum capacity of the resin was approximately 15 mg vWF/mL resin, corresponding to 60 mg vWF/g resin.

The surface density of a typical monolayer of protein on a surface is about 2 mg/m^2.[11] The surface area of RVRSFY–Toyopearl is 31 m^2/g, as determined by the BET surface area measurement method.[12] A monolayer of vWF is therefore expected to correspond to a vWF binding capacity of 62 mg/g of resin. This theoretical capacity is about the same as the maximum capacity obtained from the adsorption isotherm measurement, indicating that the adsorbed vWF is able

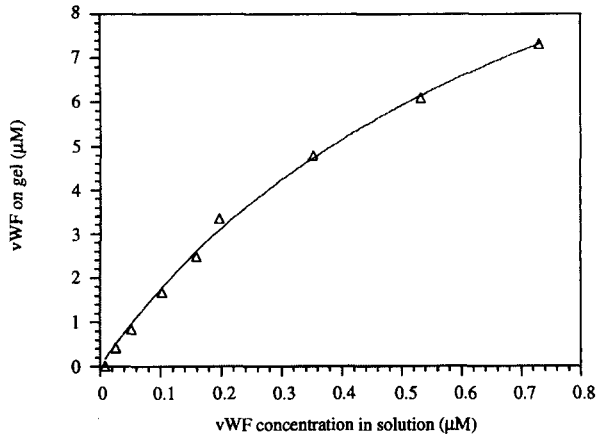

Figure 3. Adsorption isotherm of vWF on RVRSFY–Toyopearl. Experimental data (△) were measured in batch experiment in a buffer containing 10 mM HEPES, 5 mM $CaCl_2$, and 0.5 M NaCl at pH 7. The solid line is the fit of the Langmuir equation.

to cover almost the entire surface of the pores of the resin. Therefore, the vWF fraction used in these experiments apparently had no difficulty in entering the 1000 Å pores in this support material.

Total amino acid analysis showed that the peptide density was approximately 200 μmol of peptide per gram of resin. This is equivalent to 25.8 μmol/m^2 (based on the 31 m^2/g surface area), or about 4 peptide molecules/nm^2. Apparently, the peptide was densely packed on the surface of pores in the resin. Since the maximum capacity of vWF is only 15 mg/mL (or 0.015 μmol/mL), the molar ratio between peptide and protein is 333 to 1. The following section describes the adsorption behavior of vWF on resins with varying peptide densities.

The effect of peptide density on the adsorption isotherms

The peptide Ac-RVRSFYK-amide was chemically synthesized on Rink resin, cleaved from the solid and purified by HPLC. The peptide was then immobilized to Toyopearl-Epoxy-650M resin through the ε-amino group of the lysine. This resin consists of the same matrix composition as the resin used in the direct peptide synthesis. The acetyl group at the N-terminus prevented the peptide from coupling at the N-terminal amine so that the immobilized peptide had an orientation that was a similar to the orientation during screening. The acetyl group at the N-terminus had no effect on binding to vWF when the peptide was directly synthesized on the resin (data not shown). The peptide coupling efficiency and peptide density on the resin were calculated from reversed-phase HPLC measurements of peptide concentrations before and after coupling. Various resin batches were made using different peptide concentrations during the coupling step. The coupling efficiencies were in the range of 95 to 75% and the final peptide densities were 5–60 mg peptide/mL of resin.

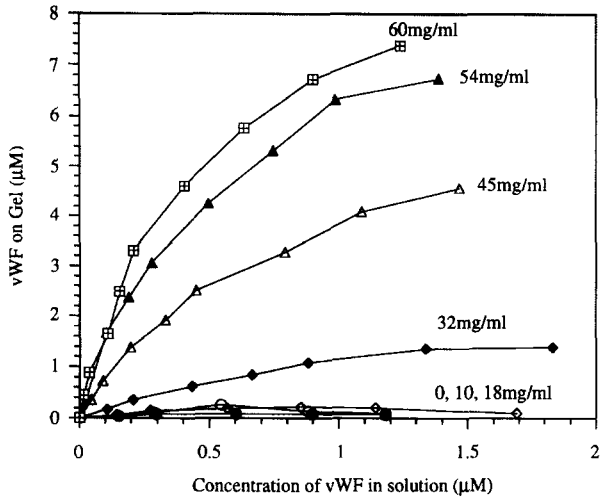

Figure 4. Effect of peptide density on the adsorption of vWF to Ac-RVRSFYK–Toyopearl. Experimental data (△) were measured in batch experiment in a buffer containing 10 mM HEPES, 5 mM $CaCl_2$, and 0.5 M NaCl at pH 7.

The effect of peptide density on the adsorption isotherms is shown in Figure 4. As expected, an increase of peptide density resulted in increased adsorption of vWF. There is a minimum peptide density, about 32 mg peptide Ac-RVRSFYK/mL resin (32 μmol/mL), required for binding vWF to the solid particles under these conditions (0.5 M NaCl in 10 mM HEPES and 5 mM $CaCl_2$ buffer, pH 7).

The Langmuir equation was used to fit the isotherms in Figure 4 and the association constants and maximum capacities obtained from the curve fits are listed in Table 5. They show an increase in the association constant and the maximum protein binding capacity with an increase in the peptide density immobilized on the Toyopearl resin. When the peptide density is less than 32 mg/mL, no reliable fitting parameter could be obtained.

The resins with peptide densities of 4.7, 10, 18, 32, 45, and 54 mg/mL were packed in columns for the separation of vWF from Koate®-HP. When 0.1 mL solution of Koate®-HP was injected into columns equilibrated with the same buffer used in the adsorption isotherm measurements, the percentage of vWF in Koate®-HP captured by the columns was also dependent on the peptide density. Figure 5 shows the effect of peptide density on the capture of vWF from Koate®-HP in the chromatography format. When the peptide density was

Table 5. Effect of peptide density on the association constant and maximum capacity

Peptide density (mg/mL)	Association constant (M^{-1})	Maximum capacity (mg/mL)
32	8.82×10^5	2.32
45	1.12×10^6	7.3
54	1.68×10^6	9.7
60	2.06×10^6	10.33

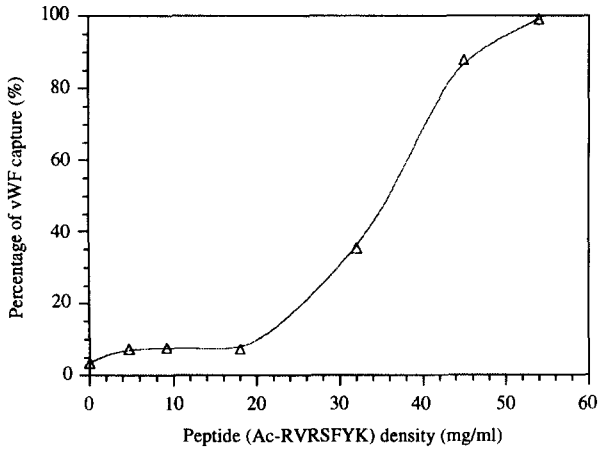

Figure 5. Effect of peptide density on the capture of vWF from Koate®-HP. The percentage of vWF capture was calculated from the amount of vWF retained by a peptide column divided by total vWF injected.

less than 32 mg/mL, almost no capture of vWF was obtained. As the peptide density increased, the capture of vWF went through a transition from no capture to almost complete capture at 54 mg/mL density. This trend was consistent with the results obtained from the adsorption isotherm measurements.

The effects of salts and temperature on the elution of captured vWF from Ac-RVRSFYK–Toyopearl column

Generally, elution with high salt concentrations may result in proteins with higher biological activities than acid elution. With this motivation, the NaCl concentration during the elution step was increased to 2 M, however, the vWF from Koate®-HP captured on the Ac-RVRSFYK–Toyopearl resin was not released. When divalent salts, such as $CaCl_2$ and $MgCl_2$, were used for elution, most of the vWF captured was recovered. Figure 6 shows the dependence of the recovery of vWF (from A_{280} measurement) on calcium and magnesium chloride concentrations. About 80% of captured vWF could be eluted by $CaCl_2$ and $MgCl_2$ at concentration larger than 0.3 M. Divalent salts were more efficient in disrupting the interactions between the immobilized peptide and vWF than sodium chloride at similar ionic strength.

The capture of vWF from Koate®-HP was not very sensitive to temperature. However, at higher temperatures, calcium was more efficient in eluting the captured vWF from a column packed with Ac-RVRSFYK-Toyopearl. For example, when the temperature was increased from 22 to 26 and 30 °C, the percentage of vWF eluted by 0.5 M calcium chloride increased from 80% to 85% and to 89%, respectively. The efficient elution of the captured vWF by divalent salts at higher temperatures indicates that ionic interactions between immobilized peptide and vWF play an important role in the adsorption process.

To examine vWF recovery by calcium chloride elution, vWF was separated from 0.1 mL of Koate®-HP using a

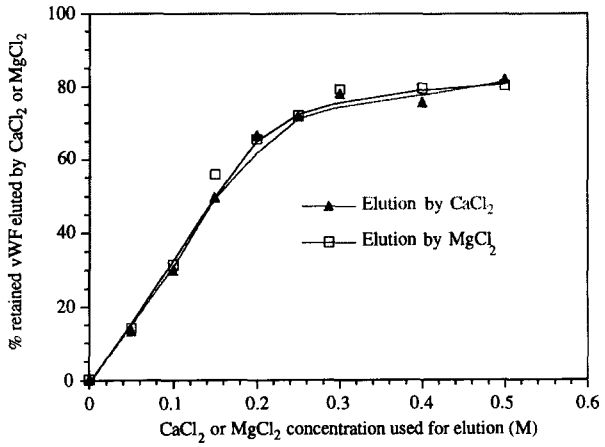

Figure 6. The effect of calcium concentration on the elution of captured vWF from a column packed with 54 mg/mL Ac-RVRSFYK–Toyopearl.

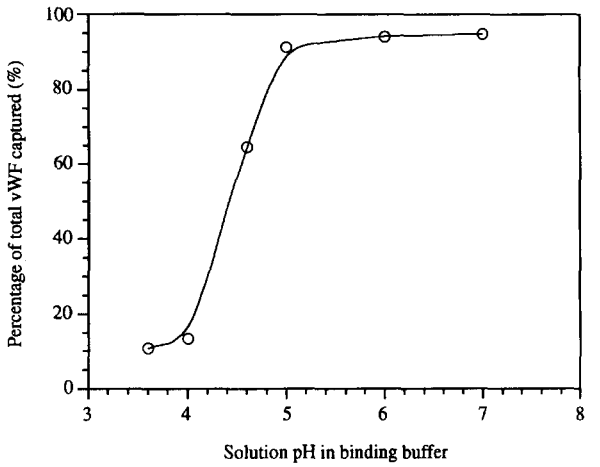

Figure 7. The effect of solution pH on the capture of vWF from Koate®-HP. The percentage of total vWF captured was from the amount of vWF retained by the peptide column (peptide density 54 mg/mL Ac-RVRSFYK) divided by total vWF injected.

0.4 mL column containing 54 mg/mL of the peptide Ac-RVRSFYK. The fractions of flow-through, elutions by salt (1 M NaCl), 0.5 M $CaCl_2$, and by 2% acetic acid were collected and vWF in each fraction was measured by ELISA: 82.5% of total vWF was recovered from the $CaCl_2$ fraction and the vWF recovered in all fractions was 87.8%. In comparison, when only 2% acetic acid was used for elution, only 55.8% of the total vWF applied was recovered (ELISA results in Table 4).

The effect of solution pH on the capture of vWF from Koate®-HP

The immobilized Ac-RVRSFYK on Toyopearl is positively charged at any pH lower than 12. At a solution pH higher than 5.8, the isoelectric point for vWF,[13] vWF is negatively charged so that the interaction between vWF and peptide is favorable. As shown in Figure 7, between pH 6 and 7, about 95% of vWF was captured by the peptide column. However, when the solution pH was between 5 and 4.6, significant percentages (91 and 65% respectively) of vWF were still captured by the immobilized peptide, as shown in Figure 7. Since the local pH at a positively charged surface is higher than the solution pH,[18] it is possible that vWF near the resin surface still has a sufficiently high negative charge, even at a bulk solution pH below its isoelectric point, to bind to the resin. In addition, it also shows that it is possible to capture vWF over a wide range of solution pH.

The effect of peptide alteration on the capture of vWF from Koate®-HP

To further understand the interaction between the immobilized peptide and vWF, the peptide RVRSFY was truncated or scrambled. Three new peptides, Ac-RVRK, Ac-SFYK, and Ac-SRYVRFK, were synthesized and immobilized to Toyopearl at a density of 54 μmol/mL, equivalent to 54 mg/mL Ac-RVRSFYK. The elution profiles of Koate®-HP from these new columns are shown in Figure 8. The fractions in each chromatogram were collected and the vWF recovery analyzed by ELISA. The chromatogram (a) in Figure 8 was obtained from a column of Ac-RVRK–Toyopearl. It shows similar behavior to an ion exchange column. vWF was eluted by NaCl at 8 min and albumin flowed through at 0.8 min or was eluted by low NaCl concentration at 3.6 min. In the column of Ac-SFYK–Toyopearl, vWF was released by very low concentration of NaCl (at 2 min) and all the albumin flowed through the column at 0.8 min, as shown in chromatogram (b). In chromatogram (c), 95% of the total vWF was retained by the immobilized scrambled peptide Ac-SRYVRFK and was released only by 2% acetic acid. The results from the scrambled peptide indicate that the sequence dependency is not absolute for RVRSFY. Also, although the immobilized peptides Ac-RVRK and Ac-SFYK interact separately with vWF, the combination of these two regions in RVRSFYK and SRYVRFK enhances the interaction with the protein.

The purification of vWF from PEG filtrate of human plasma

Crude PEG filtrate from the cryoprecipitate of human plasma was used to demonstrate a practical separation. The PEG filtrate was treated with virus inactivation reagents, 1% TNBP (tri-n-butylphosphate) and 0.5% Tween 20 at 30 °C for 3 h. Treated PEG filtrate was injected directly into the column. As shown in Figure 9, vWF in the PEG filtrate was captured and resolved using a calcium gradient from 5 to 500 mM using a column of 54 mg/mL of Ac-RVRSFYK–Toyopearl. The fraction in the small peak at 10 min contained 74% of the applied vWF. In all other fractions, which contained 95% of the total applied protein, only 5% of vWF was detected. Other applications using this peptide column are under investigation.

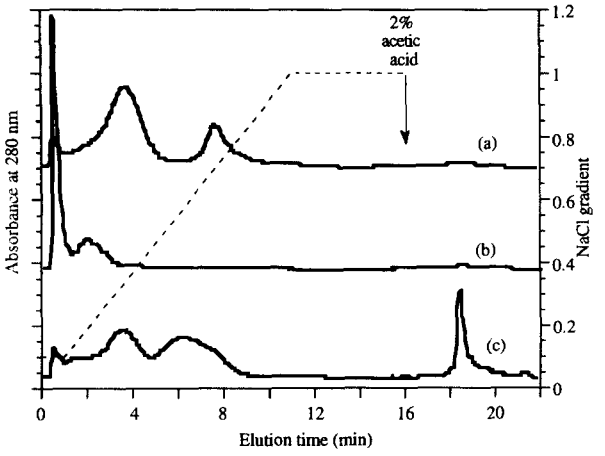

Figure 8. Elution profiles of Koate®-HP from peptide columns packed with (a) Ac-RVRK, (b) Ac-SFYK, and (c) Ac-SRYVRFK immobilized on Toyopearl. Peptide densities are 54 μmol/mL, equivalent to 54 mg/mL Ac-RVRSFYK. Flow-through peaks are at 0.5 min. Low salt peaks for lines (a), (b), and (c) are at 3.6, 2 and 3.7 min. respectively. High salt peaks for line (a) and (c) are at 8 and 6.2 min. respectively. Acid peak for line (c) is at 18.5 min.

Discussion

The phage vector fd-TET displays the peptide as a fusion product to the pIII protein.[6] Four or five copies of pIII protein are presented at the trailing end of each phage particle. Multiple copies of the peptide ligand should allow for multi-point attachment to vWF, itself a large multimeric protein, and amplify weak affinity interactions as the apparent arithmetic product of the dissociation constants. It was with this strategy in mind that a ligand suitable for affinity chromatography was discovered.

The peptide RLRSFY was obtained from phage library screening against vWF, from nearly 100 unique clones sequenced and from which deduced peptides were synthesized, purified and attached to resin. Only this peptide interacted with vWF. It is amphipathic with respect to amino acid side groups, namely hydrophilic guanidiniums (R), hydroxyl (S), and possibly a tyrosinate (Y), and hydrophobic aliphatic (L) and aromatics (F, Y). The N-terminal guanidinium (R) is apparently an important contact point, although α-NH_3 is not critical for binding. Other anions can substitute for guanidinium, namely the ε-NH_3 (K) and imidazole (H). BET analyses suggest that the peptide at effective densities is closely packed and may be displayed as a surface.

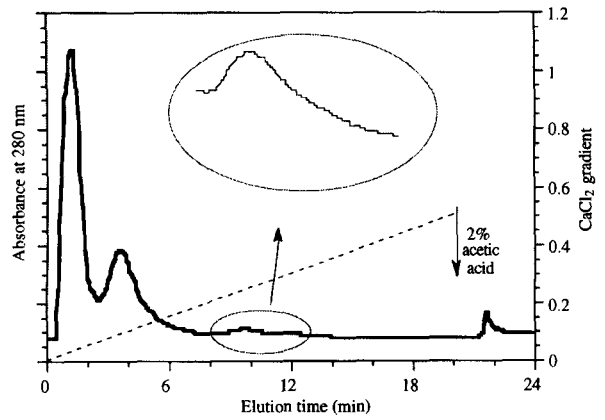

Figure 9. The purification of vWF from PEG filtrate from a cryoprecipitate of human plasma. Flow-through peak is at 0.8 min, salt peak is at 3.8 min, vWF peak is at 10 min and acid peak is at 21.8 min.

After the L in the peptide RLRSFY is substituted with V, the interaction of immobilized peptide with albumin is weakened while the captured vWF remains undisturbed. Although the hydropathies of L and V are similar,[14] the shorter side chain of valine compared to leucine may have contributed to the difference. For the purpose of affinity chromatography, the peptide RVRSFY is a better ligand for the separation of vWF from protein mixtures.

The interaction between vWF and immobilized RVRSFY is dependent on the peptide density. A minimum density of 32 mg/mL (32 μmol/mL) is required for a detectable interaction as determined from adsorption isotherm measurements (Figure 4). In addition, an increase of peptide density increases the apparent binding constant and maximum capacity. These are characteristic of a multi-point interaction system, since an increase of ligand density will increase the possibility of interaction between immobilized ligand and protein.[15] In contrast, when a peptide binds to the cleft of a protein, the binding affinity decreases with increases in peptide density and the protein–peptide interaction is measurable even when the peptide density is as low as 0.05 mg/mL (0.05 μmol/mL resin).[16]

The mechanistic studies reveal that the multi-point interaction between vWF and immobilized peptide Ac-RVRSFYK is dominated by ionic forces, but probably involves other interactions. Classical ion exchange chromatography has been used to purify vWF in a three step process using DEAE–Fractogel.[17] In each step, vWF was eluted easily with less than 0.2 M NaCl. Even with a strong anion exchanger, TMAE–Fractogel (trimethylaminoethyl), vWF could be eluted with sodium chloride concentrations as low as 0.32 M in our laboratory. In comparison, the interaction of vWF with immobilized RVRSFY at high density is resistant to 2 M NaCl and susceptible to divalent cations, consistent with the relatively strong binding constant of around 10^6 M^{-1}. In addition, the two trios of terminal residues (RVR and SFY) alone could not interact strongly with vWF, even though both are amphipathic. Strong interaction involves the combination of both components, although not necessary in a unique sequence.

A unique peptide sequence is normally required for the interaction to a specific cleft of a protein.[16] The independence of specific peptide sequence in the interaction of RVRSFY and vWF suggests that the peptide

RVRSFY may not interact to a specific cleft on the surface of vWF.

The concept of pseudo-affinity most closely describes the interaction of immobilized Ac-RVRSFYK with vWF. Typical pseudo-affinity chromatography uses ligands of a more general nature such as dyes,[15] heparin, and histidine.[19] Immobilized dyes interact with proteins in multipoint attachment and an increase of dye density increases adsorption capacity.[15] The binding constant increases to a maximum then drops when more dyes are immobilized. Such interactions are observed at low ionic strength (less than 0.1 M). Vijayalakshmi[19] used histidine as ligand for the purification of several proteins, including vWF from plasma. Like dyes, immobilized histidine interacted with vWF near its isoelectric point and only at a critical ionic strength. The solution pH was so critical for the interaction that even a deviation of 0.3 from the optimized pH would result in a failure for the separation. In contrast, the immobilized peptide Ac-RVRSFYK could capture vWF in a wide range of pH and ionic strengths.

In conclusion, we have demonstrated that peptides derived from peptide libraries can be used for the affinity purification of vWF after being directly synthesized or immobilized on an affinity matrix. Conservative substitutions on the peptide sequence is a useful technique for modifying selectivity. Finally, peptide density, solution pH, and $CaCl_2$ concentration can be controlled to optimize the separation of vWF from crude PEG filtrate from a cryoprecipitate of human plasma.

Experimental

Biopanning for peptide screening

Microtiter plate wells were coated overnight at 4 °C with 10 μg/mL rFVIII in a final volume of 50 μL/well in 0.1 M $NaHCO_3$, pH 8.6. Following blocking with 3% BSA (bovine serum albumin), the wells were washed three times with Buffer A (10 mM HEPES, 100 mM NaCl, 2.5 mM $CaCl_2$, 20 mg/mL mannitol, pH 6.8). 5×10^8 phage from the 6-mer bacteriophage display library[6] were added to vWF (final concentration of 20 nM) in a final volume of 50 μL Buffer A. Phage were contacted with vWF at 20–26 °C to allow binding for 40 min in a siliconized microcentrifuge tube. The solution was then transferred into the rFVIII-coated microtiter wells (BSA-coated wells served as a control) for 20 min at room temperature to effect capture of vWF and vWF:phage complexes by rFVIII. The solution was aspirated from the wells in order to remove unbound phage. Weakly bound phage were removed from the captured vWF by six consecutive washes with Buffer A containing 0.05% (v/v) Tween 20, followed by six consecutive washes with Buffer A. Bound phage were eluted with 50 μL/well of 0.025 N HCl, 0.037 M glycine, 0.15 M NaCl (pH 2.0) for 10 min. The phage eluates were neutralized to pH 7.0–7.5 with 1 M Tris pH 9.1, and then were amplified in *E. coli*.

The amplified phage particles were then subjected to second and third rounds of screening using the same procedures as in the first round. Upon finishing the third round screening, the neutralized eluates were used to infect *E. coli* and plated on agar plates in order to quantify numbers of phage captured on either rFVIII, or on the BSA control. The clones selected for vWF binding were subjected to DNA sequencing and the peptide sequence in each clone was deduced according to standard coding.

Amination of resin and solid phase peptide synthesis

Toyopearl AF Chelate 650M resin from TosoHaas (Montgomeryville, PA) was chosen for the amination and direct synthesis of peptides.[10] The resin was rinsed in a 25 g reaction vessel with H_2O, MeOH and dimethylformamide (DMF, Burdick & Jackson). A five-fold molar excess of ethylenediamine (Aldrich, Milwaukee, WI) over resin carboxylate was coupled onto the carboxylate moiety with a slightly molar excess of benzotriazole-1-yl-oxy-tris-pyrrolilidinophosphonium (PyBOP, NovaBiochem, La Jolla, CA) and *N*-methylmorpholine (NMM, three-fold molar excess over PyBOP, Aldrich) in DMF for 60 min. This aminated resin was washed with DMF, then methanol, then dried in vacuo. To generate non-cleavable peptide-resins, two β-alanine (Nova Biochem) spacer residues were coupled by standard solid phase peptide synthesis couplings.

Peptides were synthesized by the solid phase method on a Gilson AMS422 Multiple Peptide Synthesizer (Middleton, WI) utilizing FMOC (9-fluorenylmethoxycarbonyl) as the α-amino protection. Briefly, each amino acid (five-fold molar excess; 1 mL of 0.5 M in DMF) was activated in situ with PyBOP (0.5 mL of 0.3 M in DMF) and NMM (0.25 mL of 1.19 M in DMF) with our modified TosoHaas resin (0.3 g, 120 μmol) or Rink amide resin (Nova Biochem, 0.5 g, 200 μmol). Coupling was allowed to proceed with argon-bubbling agitation for 45 min. All peptides were cleaved and/or deblocked with Reagent R (5 mL of 90% trifluoroacetic acid (TFA), 5% thioanisole, 3% ethanedithiol, 2% anisole, all from Aldrich) for 3.5 h. The TosoHaas resin–peptides were deprotected in the synthesis vessel, extensively washed with MeOH and dried in vacuo. The peptide mixtures from Rink resin were filtered away from resin directly into 40 mL cold anhydrous Et_2O (Aldrich) through a medium porosity sintered glass funnel. The filter cakes were dissolved in 50% MeCN–H_2O and lyophilized in a scintillation vial. These precipitated, unpurified peptides were dissolved at 25–50 mg/mL in 50% MeCN–H_2O and 1 mL was purified by preparative HPLC (Gilson, Inc., Middleton, WI) with a 22×250 mm (C18 15μ 300 Å, Vydac, Hesperia, CA) reversed phase column. The analytical HPLC system, Ultrafast Microprotein Analyzer, was purchased from Michrom BioResources, Inc. (Sacra-

mento, CA). Molecular weights and sequences of peptides were verified by MS/MS determinations using fast atom bombardment mass spectrometry on a Jeol HX110HF instrument.

Peptide immobilization and affinity column packing

Purified peptides were dissolved in 0.2 M sodium bicarbonate and 0.5 M NaCl at pH 10.3. Resin powder, Toyopearl-Epoxy-650M (TosoHaas, Montgomeryville, PA), was directly mixed with peptide solution at a ratio of 0.5 g resin to 3.2 mL peptide solution. The slurry was rotated at 40 °C for 24 h, then the solution was separated from the resin. Peptide concentrations in the solution before and after immobilization were determined by analytical C18 reversed-phase chromatography. Since there was negligible dilution when the Toyopearl powder was mixed with the peptide solution, the decrease of peptide peak area after mixing was due to immobilization and was used to calculate the peptide coupling efficiency. The final peptide density on the resin could then be deduced.

After peptide coupling, the reactive sites on the gel were blocked by reacting with 1 M ethanol amine at pH 11 for 24 h at 40 °C. The gel was washed with degassed PBS (phosphate buffered saline) buffer and 1 M NaCl at pH 7.4.

The gel slurry (1 mL wet gel mixed with 2 mL degassed PBS and 1 M sodium chloride buffer) was transferred into a packing device (from PerSeptive Biosystems, Framingham, MA) and packed into a PEEK column (0.75 × 5 cm from Alltech, Deerfield, IL) at a flow rate of 8 mL/min. The pressure drop was 60 psi, which was within the maximum pressure drop of 120 psi suggested by the manufacturer. The column was washed with at least 4 bed vols of binding buffer (10 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$ at pH 7) and elution buffer (2% HAC), then equilibrated with binding buffer.

vWF ELISA

vWF concentration was measured by ELISA. Briefly, rabbit anti-vWF antibody (catalog number BYA-3058–1 from Accurate Inc. NY) was coated on each well of a microtiter plate (96 wells from Corning, NY) overnight using 100 μL solution of antibody diluted 200 times in 0.1 M sodium bicarbonate buffer, pH 9.6. Each well was then blocked with 300 μL solution of 1% BSA in PBS for 1 h at room temperature. The plate was washed five times with PBS. Pure vWF and the collected samples were diluted to a concn range between 0.02 and 0.2 μg/mL using 1 mg/mL BSA in PBS. Each sample (100 μL) was incubated with the anti-vWF coated well for 1 h at room temperature and the plate was washed five times with PBS plus 0.1% Tween 20. A second anti-vWF antibody with horseradish peroxidase (HRP) conjugate (catalog number p226 from DAKO, Glostrup, Denmark) at a concn of 1 to 500 dilution was introduced to each well and incubated for 1 h at room temperature. The plate was washed again with PBS plus 0.1% Tween 20 five times. Substrates ABTS (2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)) and H_2O_2 were used for the kinetic reading at 410 nm using a Bio Kinetics Reader from BioTek (Winooski, VT).

vWF activity assay (factor VIII binding assay)

Briefly, 0.01 μg rFVIII was adsorbed onto a microtiter plate (96-wells from Corning, NY) overnight using 100 μL soln in 0.1 M sodium bicarbonate buffer, pH 9.6. After washing and blockage with BSA, pure vWF and the collected samples were added to each well at a concentration range between 0.02 and 0.2 μg/mL. The detection of bound vWF was the same as in the ELISA measurement above.

Gel electrophoresis

The molecular weights and purity of the collected samples were determined by SDS–PAGE under reducing condition using Phastsystem from Pharmacia (Piscataway, NJ). To determine the multimers of vWF, 1.5% agarose gel electrophoresis was used. The agarose gel electrophoresis was performed on the BioRad mini gel system.

Adsorption isotherm measurements

Adsorption isotherms were measured in a batch mode. In a siliconized microcentrifuge tube, 0.1 mL wet gel was mixed with a vWF soln of 10 mM HEPES, 5 mM $CaCl_2$ and 0.5 M NaCl at pH 7 to a total volume of 0.3 mL. The microcentrifuge tube was incubated at 25 °C for 20 min, then the gel was sepd from the soln by microcentrifugation. The concs of vWF in solutions were measured both by absorbance at 280 nm with an extinction coefficient of 1.2 for 1 mg/mL vWF and by ELISA. By a total material balance, the concn of vWF adsorbed on the gel was calculated. The total amount of vWF in the system was increased by adding concentrated vWF solution to the mixture while maintaining the same total volume.

Highly purified vWF (plasma derived) and recombinant FVIII were from Bayer Corp. (Berkeley, CA). Koate®-HP containing vWF, FVIII, and albumin was a product of Bayer Corp (Clayton, NC). Other chemicals used in our experiment were from Sigma in analytical grade or purer. All aq solns were prepared using deionized water purified by Barnstead nanopure water purification system (Dubuque, IA).

Treatment with solvent and detergent

The crude starting material, PEG (polyethylene glycol) filtrate, is an process intermediate derived from human plasma cryoprecipitate for making Koate®-HP (Bayer Corp, Clayton, NC). The PEG filtrate was treated with 1% TNBP and 0.5% Tween 20 (from Aldrich) at 30 °C

for 3 h. After the treatment, the mixture was directly injected onto peptide affinity columns.

BET measurement of surface areas on Toyopearl gels and peptide surface density calculation

BET (Brunauer–Emmett–Teller) surface area measurements[12] were performed with a 30% N_2 in He gas mixture using a FlowSorb II 2300 instrument (Micromeritics, Norcross, GA). The samples were weighed in a sample holder and degassed at room temperature (20–23 °C) for 1 h. The sample holder was immersed for 3–4 min in liquid N_2 to allow surface condensation at a constant gas flow rate until no changes in N_2 pressure could be measured.

The sample holder was then brought quickly to room temperature and the desorbed N_2 volume was measured with a calibrated conductivity cell. The calibration was performed with 1 and 6 mL of N_2 sets at room temperature.

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