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One-step purification of vitronectin from human plasma by affinity chromatography on phage-displayed peptides

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A novel affinity purification method for rapid isolation of vitronectin (VN) from human plasma is described. Recently we have used phage display technology to obtain clones expressing peptides with high binding activity for VN. The isolated "strong VN binders" were covalently coupled to CNBr-activated Sepharose. Human plasma was applied to the column and bound VN was eluted using 0.5 M acetic acid, giving purity exceeding 90%. The developed method is a convenient alternative to conventional antibody-antigen affinity chromatography techniques for purification of VN, as it offers low ligand cost, is rapid and ensures good protein recovery from human plasma.

Keywords: affinity chromatography, phage display, vitronectin

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INTRODUCTION

Affinity chromatography methods are widely used for protein purification. There are many techniques based on antibody–antigen interactions but also other ligands are used such as non-antibody proteins (Fassina *et al.*, 2001), heparins (Utt *et al.*, 2002), metal ions (Roque *et al.*, 2007), and dyes (Jungblut *et al.*, 1989). Affinity purification resins offer powerful possibilities, however, due to their biological origin and methods of ligand preparation, are expensive and difficult to sterilize and clean. Proteolytic ligand degradation is a frequent problem as well (Tozzi *et al.*, 2003; Clonis, 2006).

To avoid these inconveniences, a lot of effort has been devoted to introducing synthetic affinity ligands which mimic the functional and structural properties of natural biomolecules. For example, combinatorial peptide synthesis is used for screening of affinity ligands. It enables selection of molecules from large random peptide libraries (Lam *et al.*, 1991; Houghten *et al.*, 1991; Liu *et al.*, 2003; Shin *et al.*, 2005).

Apart from chemical methods, peptide libraries of enormous diversity can be produced using biological systems, like the phage display technology (Smith, 1985; Smith & Petrenko, 1997). In this type of peptide libraries, about 10^8–10^9 different phage clones with random peptides on their surface are screened for affinity binding by incubating the library with the target of interest (Smith & Petrenko, 1997). The non-interacting or weakly binding phages are washed away, while the potentially interacting clones are eluted and recoverd and then amplified by infection of *Escherichia coli* cells to increase their copy number. This cycle is repeated until high-affinity binding phages expressing specific peptide(s) are isolated. Selection of phages expressing peptides specifically binding antibodies (Luo *et al.*, 2001), enzymes (Hyde-DeRuyscher *et al.*, 2000), cell surface receptors (Campa *et al.*, 2002), and even whole cells (Giordano *et al.*, 2001) has been reported.

In this report, a random 15-mer peptide library was used to find phages strongly binding human vitronectin that were next used to capture VN from blood plasma. For this purpose, the selected phages were immobilized on CNBr-activated Sepharose and then human plasma was run through the column. The large size range (45-165 μm) of beads allowed multi-point attachment of virus particles (1 μm long and 6 nm in diameter). Due to the high binding selectivity of the isolated clones for the target, highly purified preparations of human VN were obtained in a single isolation step.

MATERIALS AND METHODS

Materials and reagents. A linear pentadecamer peptide phage display library (GenBank Accession AF246445) was based on the fUSE5 filamentous phage vector expressing foreign peptides as N-terminal fusions to all five copies of minor coat protein pIII, and was a gift of G. P. Smith (University of Missouri, Columbia, USA). *Escherichia coli* TG-1 strain (*K12*, Δ[*lacpro*], *supE, thi, hsdD5,* F'*TraD36, proA+B+, LacI^q, LacZ*, Δ*M15*) was from Stratagene (La Jolla, CA, USA). Native human monomeric VN and rabbit anti-human VN antibodies were purchased from American Diagnostica GmbH (Stamford, CT, USA). Goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies (goat anti-rabbit/HRP) were from Chemicon International. Mouse polyclonal anti-M13 phage HRP-conjugated antibodies (anti-M13-HRP) were from GE Healthcare (Uppsala, Sweden). Acrylamide/Bis Solution 40 % (37.5 : 1) and protein kaleidoscope molecular weight standards used in Tris/glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis were obtained from Bio-Rad (Hercules, CA, USA). Protease inhibitor cocktail tablets were from Roche Diagnostics GmbH (Mannheim, Germany). Micro BCA Protein Assay was purchased from Pierce (Rockford,

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Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; CNBr, cyanogen bromide; ELISA, enzyme-linked immunosorbent assay; K-EDTA, potassium EDTA; LB, Luria-Bertani medium; PBS, phosphate-buffered saline; PEG, polyethylene glycol; SDS/PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ssDNA, single stranded DNA; TPBS, PBS containing 0.1 % Tween 20; VN, vitronectin.

IL, USA). Chemiluminescence kit (ECL Western Immunoblotting detection system) was from GE Healthcare. ECL film was from Kodak (New York, USA). *o*-Phenylenediamine was from Sigma-Aldrich (St. Louis, MI, USA). Maxisorp ELISA plates and high-binding polystyrene immunotubes were obtained from Nunc (Roskilde, Denmark). K-EDTA tubes were from Medlab-Products (Raszyn, Poland). CNBr-activated Sepharose 4 Fast Flow was from GE Healthcare. Econo-Chromatography columns (200 mm × 5 mm) were purchased from Bio-Rad.

Isolation of VN-binding phages, tube biopanning procedure. Selection of phages was performed as described earlier (Noppe *et al.,* 2009). Briefly, a high-binding polystyrene tube was coated overnight with human VN (20 μg/ml in 3 ml of PBS). After blocking the tube with 5% skimmed milk in PBS (3 h), 2×10^{12} phages (in 0.5% skimmed milk in PBS) from an amplified pentadecamer library were added and incubated overnight on a tube rotator at 4 °C. Next, the input phages were removed and the tube was washed 10 times with TPBS to remove the nonspecific binders. The bound phages were eluted with 0.1 M glycine buffer (pH 2.2) and the eluate was immediately neutralized with 1 M Tris/HCl (pH 8). Phages were amplified by infection of *E. coli* TG-1 and partially purified from supernatant by PGE precipitations; two additional biopanning rounds were performed. After the last panning round, cells were infected with eluted phage pool (different dilutions) and plated on LB agar plates containing tetracycline (40 μg/ml). Single colonies were picked from these plates and were grown overnight in 2 × TY medium containing tetracycline (40 μg/ml) in a 96-well plate. The plate was centrifuged and the phage-containing supernatant was tested for the presence of VN-binding phages in ELISA tests. Phage ssDNA was prepared by phenol/chloroform extraction and sequencing reactions were performed. For the pentadecamer peptides, a 5' AGCATTCCACAGACAGCCCTCATAGTT 3' primer was used.

Amplification of selected phages. Amplification of selected clones was performed as described earlier (Noppe *et al.*, 2007). For this purpose, a single cell colony corresponding to individual phage clone was transferred to a sterile Erlenmayer flask containing 40 ml of 2 × TY medium with tetracycline (40 μg/ml) and incubated overnight at 37 °C on a shaker (220 rpm). The obtained cell suspension was centrifuged and the clear supernatant containing phages was precipitated twice with 20% PEG-6000/2.5 M NaCl solution (1/5 volume). Finally, the recovered phage particles were resuspended in sterile PBS containing 5% glycerol and stored at –20 °C until further use. Phage concentration was measured as described earlier (Noppe *et al.,* 2009).

Measurment of phage binding to VN, phage-ELISA. A 96-well plate was coated overnight with VN (5 μg/ml in PBS). After 2 h of plate blocking with 5% skimmed milk in PBS, a dilution series of either a phage suspension obtained after different rounds of biopanning or of individual phage clones selected after the last round were added to the wells (all samples in PBS with 0.5% skimmed milk), and then the plates were incubated at room temperature for 90 min. Bound virions were detected after 1 h incubation with polyclonal anti-M13-HRP antibodies. Visualization was obtained with *o*-phenylenediamine and the reaction was stopped with 4 M H_2SO_4. The absorbance was determined at 490 nm. After each incubation step the plates were washed 3–6 times with TPBS. To detect nonspecific binding, all assays were done in parallel on plates coated with 5% skimmed milk in PBS alone. The background binding was subtracted from all readings before data analysis.

Plasma preparation. Blood was collected in K-EDTA anti-coagulation tubes, plasma was obtained by centrifugation of non-coagulated blood and stored at –20 °C until further use. For VN capturing, frozen plasma was melted at 37 °C and the precipitated proteins were discarded by centrifugation followed by filtering through a 0.22 μm filter.

Coupling of phages to CNBr-activated Sepharose fast flow. One gram of dry CNBr pre-activated resin was transferred into a sterile 50 ml conical tube and suspended in cold 1 mM HCl for 30 min in order to swell and remove sugar additives present in the gel beads. After incubation, the beads were washed with 10 gel volumes of coupling buffer (0.1 M carbonate buffer pH 9). After washing, 7×10^{11} VN-binding phages in 30 ml of coupling buffer were added and incubated for 16 h on a tube rotator (10 rpm) at 4 °C. After the incubation, the beads were washed with 10 gel volumes of coupling buffer to remove all non-bound phages, followed by incubation for at least 2 h with 15 gel volumes of 0.2 M glycine in coupling buffer on a tube rotator (10 rpm) at room temperature (to block the remaining active amino groups). Next, the gel was washed 4 times using alternating coupling buffer and low-pH washing buffer (0.1 M acetic acid, 0.5 M NaCl). Finally, Sepharose with coupled phages was transferred to a glass econo-chromatography column and equilibrated with PBS. To prevent microbial growth, the column was stored in PBS containing 0.1% sodium azide until further use. After each incubation-washing step, the suspended gel was decanted by centrifugation and supernatants were removed. All buffers used were filtered through a 0.22 μm filter.

Capturing of VN on Sepharose-phage column. Human plasma (3 ml) was diluted five-fold and applied on a previously prepared column, followed by washing with 20 ml of PBS. Elution was performed with 10 ml of 0.5 M acetic acid, 0.5 ml fractions were collected and immediately neutralized by addition of 0.5 ml of 0.5 M Tris. All buffers used were filtered through a 0.22 μm filter with the addition of protease inhibitor cocktail, according to manufacturer's instruction. Finally, the column was washed with sterile water and PBS with sodium azide (0.1%) and stored at 4 °C for further use.

Micro BCA protein assay. The amount of protein recovered was measured with the Micro BCA Protein Assay test according to manufacturer's instruction. The values for blank standard were subtracted from of all readings.

SDS/PAGE and Western immunoblotting analysis. The purity and homogeneity of eluted human VN from the phage columns was tested by SDS/PAGE and Western immunoblotting. Reduced samples were run on a 12% gel (Laemmli, 1970), followed by blotting onto nitrocellulose membrane. After blotting, the membrane was coated with 4% skimmed milk in PBS for at least 2 h at room temperature. After washing with TPBS, the membrane was incubated overnight with rabbit anti-human VN antibodies (1/5000) in PBS containing 0.4 % skimmed milk at 4 °C. After extensive washing with TPBS, goat anti-rabbit/HRP antibodies (1/10000 in PBS with 0.4% skimmed milk) were added to the membrane and incubated for 2 h at room temperature. Finally, after extensive washing with TPBS the reaction was developed by membrane incubation for 1 min in ECL reagents, followed by incubating with ECL film for visualization.

RESULTS AND DISCUSSION

In earlier studies (Noppe *et al.*, 2006; 2007) an interesting and novel method was proposed for rapid isolation of proteins from crude feeds, using cryogels with immobilized phages from phage display library as affinity ligands. Their macroporous monolithic gel columns allowed capturing recombinant human lactoferrin or von Willebrand factor from defatted milk and whole blood, respectively.

In this study, a similar approach was developed to isolate VN from blood plasma using phage as affinity ligand. Instead of cryogels, CNBr-activated Sepharose was used as a matrix for phage immobilization. First, phages potentially interacting with human VN were screened as described in Methods. After the third cycle of selection a mixed population of different phage clones which can bind to VN was obtained (Fig. 1). The use of individual selected clones in ELISA experiments revealed eight phage clones that bound to VN in a dose-dependent manner with high affinity (Fig. 2). Further sequencing analysis showed that those clones presented four different peptides on their surface, referred to as: VnD, VnN, VnNT, and VnY (Table 1). Those peptides differed either by a single amino acid (aspartic acid, asparagine or tyrosine were present in position number 4 of VnD, VnN and VnY clones, respectively) or had a double amino acid substitution, like in the VnNT clone, in which aspartic acid and alanine residues were replaced by asparagine and threonine in positions 4 and 11 respectively (Table 1, underlined). These small differences in primary structure of the identified clones/peptides did not affect their binding affinity to VN (Fig. 2) and all these phage clones expressed the same biological activity in terms of interactions with the target protein. In the following experiments, the ability of the selected phage clones expressing the described peptides to capture VN from human plasma was tested. Each of the identified clones was amplified and then immobilized on CNBr-activated Sepharose. In preliminary experiments, VN binding to the affinity resin was evaluated. For this purpose, 200 μg aliquots of purified VN were loaded onto phage

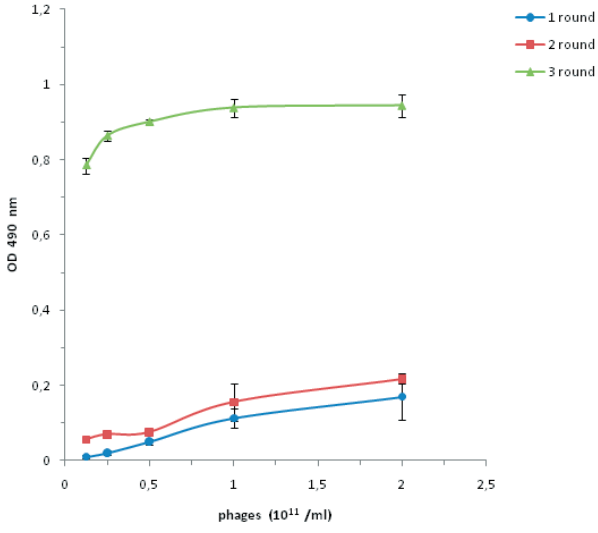

Figure 1. Binding of phages to VN observed during subsequent biopanning rounds
After each biopanning round, phages were pooled and tested for binding to VN. After the final, third biopanning round, a strong positive signal for binding of pooled phages to VN was observed.

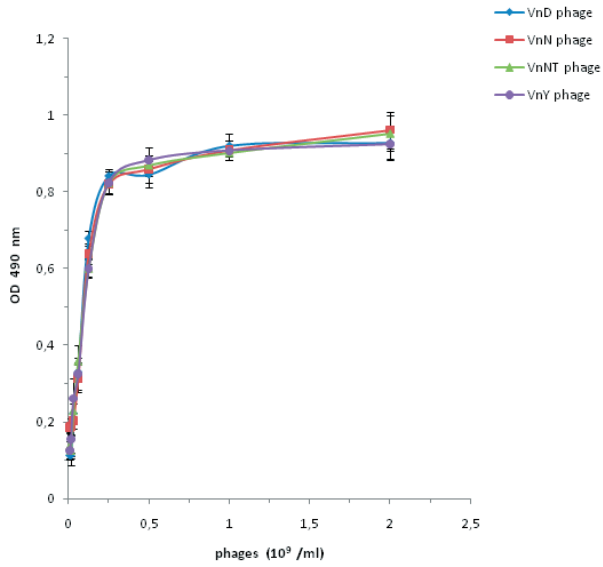

Figure 2. Dose dependent binding of individual selected phage clones to VN
A dilution series of phage clones: VnD, VnN, VnNT and VnY were prepared, and then added to wells previously coated with VN. Bound virions were detected with polyclonal anti-M13 HRP-conjugated antibodies.

columns containing VnD, VnN, VnNT or VnY clones. The flow through and washing step fractions were collected and next the bound protein was eluted with 0.5 M acetic acid and immediately neutralized by addition of 0.5 M Tris. The amount of VN in the eluted fractions was estimated by micro BCA assay. The capacity of the phage columns was found to be 34 ± 2 μg (SEM, n = 8), regardless of which selected phage clone was coupled to Sepharose. Next, diluted plasma was loaded onto the same columns and after the washing step with PBS, the bound VN was eluted as described above. SDS/PAGE of the eluted samples showed the presence of VN of high purity and homogeneity. Under reducing conditions, there two bands were visible corresponding to a single chain of 75 kDa (VN75) and a clipped form composed of two chains 65 kDa (VN65) and 10 kDa (not visible due to low molecular mass) held together by a disulfide bond (Fig. 3), respectively. Few impurities could be seen in these preparations. The amount of the 75 kDa VN form (Fig. 3, lane 2 and 3) differed when blood plasma samples were taken from various individuals reflecting the VN gene polymorphism since three distinct VN types differing in the ratio of the 75 kDa and 65 kDa VN forms have been reported: type I (75 kDa-rich), II (75/65 kDa-even), III (65 kDa-rich) (Conlan *et al.*, 1988; Kubota *et al.*, 1988; 1990; Tollefsen *et al.*, 1990).

Table 1. Peptides identified on the surface of selected phage clones
The sequence of peptides present on the surface of individual phage clones was deduced from the nucleotide sequence the coding region of isolated ssDNA.

Phage clone	Peptide sequence
VnD	C R G D P F C D F V A F L M A
VnN	C R G N P F C D F V A F L M A
VnNT	C R G N P F C D F V T F L M A
VnY	C R G Y P F C D F V A F L M A

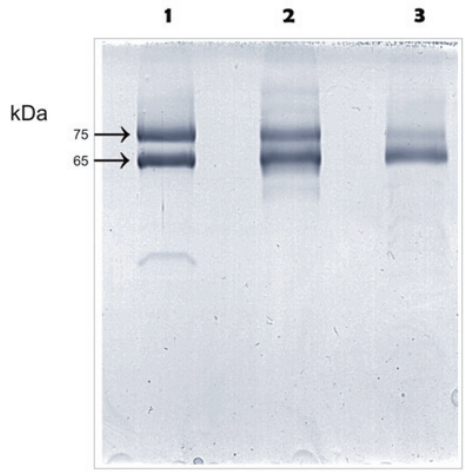

Figure 3. SDS/PAGE of VN present in fractions eluted from phages immobilized on CNBr-activated Sepharose
VN standard (lane 1) and VN fractions (lanes 2 and 3) eluted with 0.5 M acetic acid from phage columns were separated in 12 % polyacrylamide gel. About 2 μg of protein was applied to each well.

Next, the robustness of the tested columns in terms of binding capacity and phage leakage after multiple use was checked. Several cycles of loading, washing and re-equilibration were performed. Equal amounts of human plasma were applied to phage columns, followed by washing and elution. The fractions collected during different steps of the purification procedure (such as washing and elution) were next tested for the presence of virions. Precipitation of fractions with a PEG/NaCl mixture followed by measuring the absorbance at 260 nm did not reveal any leakage of phage to the tested samples. No appreciable decrease in the binding capacity was observed after intensive column re-use. Each time the binding capacity of the columns was about 30 μg. The lack of phage leakage and the unchanged yield for VN binding make these Sepharose columns suitable for rapid one-step purification of the target protein.

The CNBr-activated Sepharose column with immobilized phages is a convenient platform for VN purification from human plasma. Sepharose beads (particle size range 45–165 μm) have been shown to be a suitable matrix for phage coupling to their surface. On large structures such as the phages used here (about 1 μm in length) there are five copies of the same peptide, which means that only a small part of the virion is involved in the direct binding to the target protein (Noppe *et al.*, 2006; 2007). In this context, the proposed approach to purify VN may only be an analytical rather than preparative application. However, in comparison with other affinity chromatography techniques using monoclonal antibodies, their fragments or synthetic peptides, phages have some advantages. The phage selection procedure called biopanning (Koivunen *et al.*, 1999) against the target molecule is simple, fast and inexpensive. Once the phages are selected, they can be easily amplified and purified in large quantities. In contrast, the use of synthetic peptides may cause some problems during experimental procedures. Peptide synthesis is a sophisticated multiple-step process (Tozzi *et al.*, 2003; Noppe *et al.*, 2007). Attempts at direct immobilization of synthetic peptides on a chromatographic matrix may cause the loss of their binding properties (Noppe *et al.*, 2007). Moreover, the selected peptide often functions as an affinity ligand when it is an integral part of phage coat protein or when all its copies are closely located on the phage surface (Cwirla *et al.*, 1990; Noppe *et al.*, 2004).

To sum up, in view of the presented data, the developed method has been shown to be an inexpensive, efficient, reliable, and, most of all, rapid approach for isolation of high purity VN from human plasma.

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