Bacteriophages and Cryogels: A New Efficient Tool in Bioseparation

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ABSTRACT

An affinity purification procedure for the direct purification of a protein from a complex sample has been developed. The procedure is based on using selected phage clones expressing a peptide with high binding affinity for a selected protein which were covalently coupled to a macroporous monolithic column. Large pore size (10-100 μm) makes it possible to couple phage particles (~ 1μm long) as ligands without any risk of blocking the monolithic column. The bound protein was eluted with a purity of >95%. The technique presents a good alternative to conventional immuno-affinity chromatography for purification of a protein of interest from complex samples due to (i) the robustness of the system in terms of recovery and ligand leakage and (ii) economical aspect in terms of low ligand cost. Two case studies are presented: the capture of lactoferrin from defatted milk and of von Willebrand Factor from whole blood or plasma.

Keywords: macroporous monolithic cryogel, phage, affinity purification

METHODS

Macroporous Cryogel

6% and 4.4% Dry epoxy-activated monolithic cryogel columns (50mm x 8 mm) were kindly provided by Protista Biotechnology AB (Lund,Sweden) and prepared as described earlier [1]. Briefly, a mixture of acrylamide as monomer, *N*,N'-methylene-bis-acrylamide as cross-linker and allyl glycidyl ether as co-monomer to introduce reactive epoxy groups were cryo-polymerized by free-radical polymerization at subzero temperature (-12°C).

Coupling of phages to macroporous monolithic cryogel

6% dry epoxy-activated cryogels were inserted in a chromatographic column and ré-swollen in water. The columns were equilibrated with 0.1M carbonate buffer pH 9.2. Phage solutions (HuA, HuK, HuN and Hu14 phages against lactoferrin [2]) in carbonate buffer were subsequently re-circulated over separate cryogel columns overnight at 4°C. After coupling, non-bound phages were washed out with carbonate buffer. Remaining free epoxy groups were neutralized by re-circulating 0.1M ethanolamine in carbonate buffer for 3 hours at room temperature. Finally, the column was washed with water and equilibrated in PBS buffer. Also a 4.4% column was prepared with C7-VWF phages as described above.

Capturing of protein

Human lactoferrin

The different columns were washed with PBS buffer and a sample of defatted human milk or transgenic cow milk containing recombinant human lactoferrin was loaded on the column. Non bound proteins were washed out and elution of bound lactoferrin was performed with 1M NaCl. After a wash step with 2M NaCl the columns were re-equilibrated with PBS buffer. Elution fractions were collected and assayed by measuring at 214nm and by Phage ELISA [3] for detection of human (rec) lactoferrin. Purity was checked by SDS-PAGE or RP-HPLC.

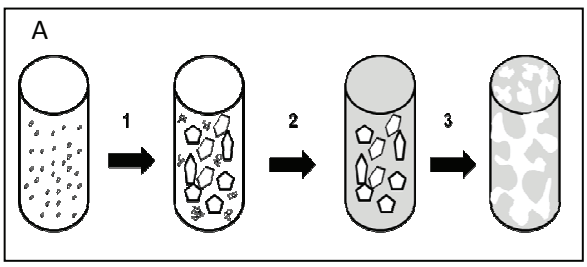
Von Willebrand factor

VWF is a large multimeric blood protein with a molecular mass weight up to 10^4 kDa. The concentration in blood is 5-10 μg/ml. Due to its high MW we used a 4.4% cryogel column with some larger pore size. Due to the relatively high content of aromatic amino acids in the peptide on the C7 phage, a more specific buffer system was needed. The column was equilibrated in a MES buffer at pH 6.0. Elution was performed with MES buffer containing 250mM $CaCl_2$. The column was also thermostated at 4°C. Elution fractions were collected and assayed by Phage ELISA. Purity was checked by SDS-PAGE and Western Blotting.

RESULTS and DISCUSSION

Macroprous cryogel.

The dimensions of the filamentous bacteriophage M13 are approximately 65nm x 1μm. Due to this property, conventional beads are not suitable as a chromatographic matrix. As we also want to purify proteins starting from a crude matrix, such as milk or whole blood, a new matrix should be selected. The macroporous cryogel with pores up to 100 μm is in this case an excellent choice. By changing e.g. the monomer concentration or freezing temperature different pore sizes within the cryogel can be obtained. Figure 1 shows the scheme of cryogel polymerization. The monomer mixture containing TEMED and ammoniumpersulphate is poured in a column (1) and directly immersed in a cryostate at -12°C. Ice crystals are quickly formed and grow until they come in contact with each other, creating a "non-frozen liquid microphase" (2) wherein the polymerization reaction proceeds in time (3). After overnight freezing, the formed monolith is thawed and the merged ice crystals form the interconnected pores (4). In this way pores are created of 5-100 μm. After washing, the gels are used or dried. The wet cryogel (Fig.1) has a sponge-like structure, while the dried cryogels are more brittle keeping their interconnected pore size. Re-swelling of the dry cryogel in water occurs in seconds and yields an elastic and spongy-like material as the initial cryogel after polymerization.



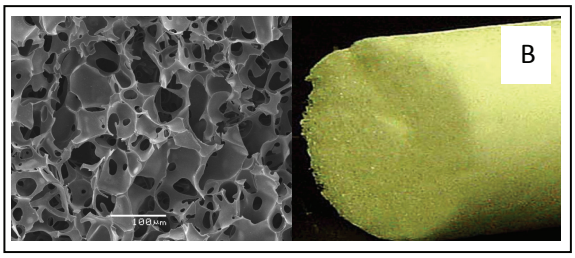


Figure 1. (A) Polymerization scheme of cryogel. (B) Pictures of cryogel: (left) TEM of a cross section of cryogel; (right) a dried cryogel [2].

Column robustness.

Four different phage columns have been prepared, each with a different peptide expressed on the pIII protein of the phage. The peptides have a different pI, net charge and number of hydrophobic amino acid residues. A sample of pure human lactoferrin was loaded on the column in order to determine the binding capacity and reproducibility, recovery and robustness of the phage columns [3]. Capacity was lower as compared to a column packed with conventional beads due to the lower surface area of the monolith column and the structural properties (~1μm long) of the phage as affinity ligand. Steric hindrance, possible multiple coupling, non-attainability of the epoxy groups inside the dense pore walls could in addition account for lower binding capacity. No substantial decrease in binding capacity was observed after intensive re-use of the column. Recovery is directly related to the strength of the elution solvent and should be optimized for each target protein separately. In case of lactoferrin 1M NaCl was a very effective elution solvent and recovery was quantitative. Ligand leakage is also an important issue in affinity chromatography. The phages are covalently bound via the reactive epoxy group and are above all very stable. After intensive use of the column, a additional run was performed and the elution fractions were collected. A sample of each fraction was used to infect *E.coli* cells to amplify phages if present in the elution fraction. No cells were detected on the agar plates in contrast to a positive sample. No viable phages were present in the elution peak, as even the presence of a few phages in the elution peak would result in the growth of cell clones.

Capture of human lactoferrin from milk.

Milk is a complex biological mixture of proteins, lipids, sugars, minerals and other small molecules. The

concentration of lactoferrin in human milk varies from 1-7 g/l depending on the lactation period, whereas in bovine milk less than 0.1 g/l is present. A sample of defatted human milk was loaded on the Hu14 phage-cryogel. After washing out the non-binding proteins, lactoferrin was eluted with 1M NaCl (fig. 2). The fractions were analyzed for purity on SDS-PAGE (fig.2). A similar sample of transgenic bovine milk containing recombinant human lactoferrin was also run on the column and analyzed as described above. Similar results were obtained with the other phage columns (data not shown).

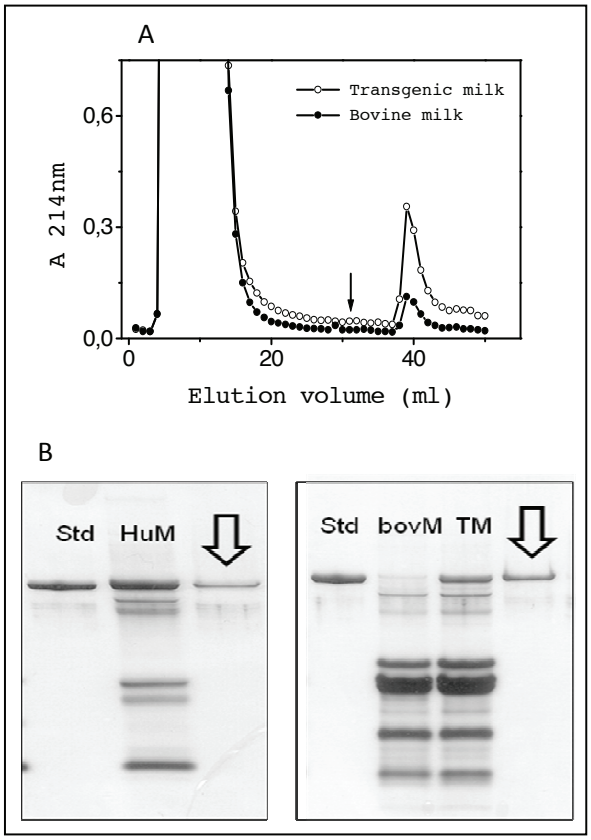


Figure 2. (A) Elution pattern of human milk and transgenic milk on Hu14 Cryogel. (B) SDS-PAGE of 1M NaCl fractions after cryogel. Std: pure HuLF; HuM: human milk; bovM: bovine milk; TM: transgenic cow milk; arrow: Lactoferrin eluted from the cryogel column (1M NaCl fraction).

Capture of VWF from whole blood / plasma.

Whole blood is an even more complex sample containing also a large number of cells (2-20 μm). An initial experiment was performed with whole blood on a naked cryogel to check the behavior of the blood sample on the column [4]. The plug of blood travels through the column without substantial tailing. Fractions were also collected and negligible lysis of the red blood cells was observed, meaning that all cells flow freely through the

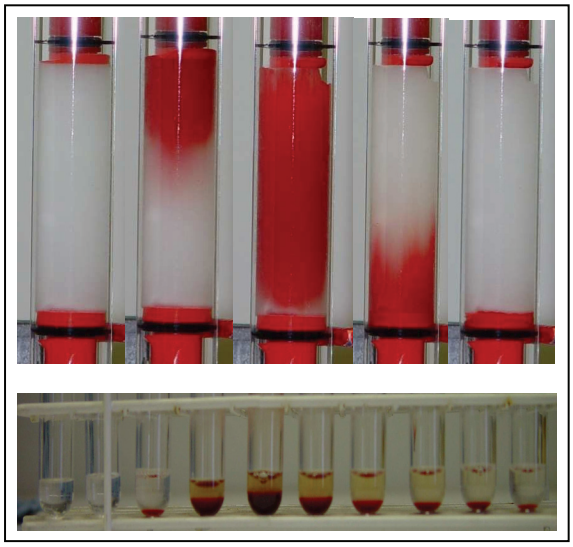


Figure 3. Flow pattern of whole blood through a naked cryogel.

column pores without clogging the column (fig. 3). A sample of whole blood or plasma was then applied to a C7-VWF-phage cryogel. Column fractions were collected and analyzed with Phage ELISA and SDS-PAGE/Western Blotting (fig.4).

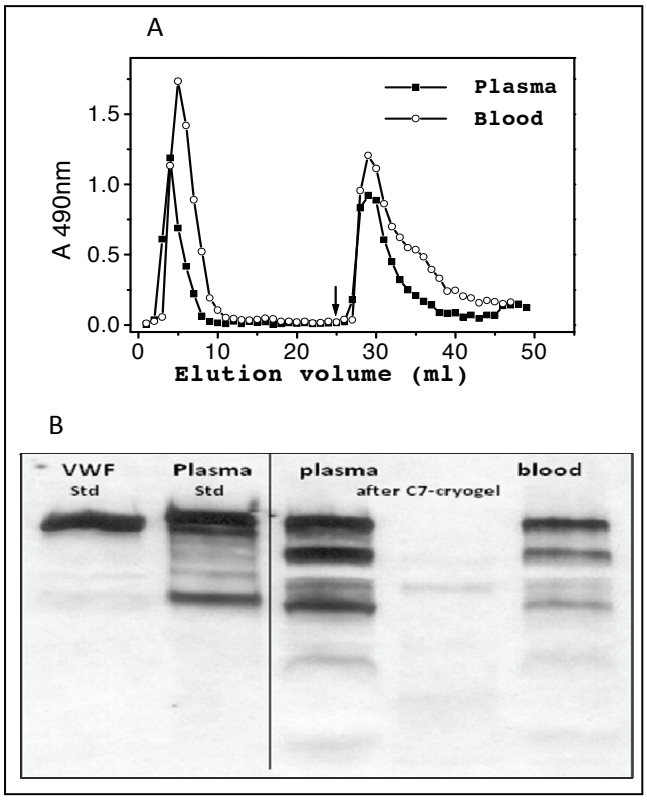


Figure 4. (A) Elution pattern of plasma/blood on C7-VWF-cryogel. (B) SDS-PAGE/Western blot of vWF fraction eluted from C7-cryogel

CONCLUSIONS

The data presented above provide "proof of principle" that bacteriophages, expressing peptides on one of their coat proteins, can be used as affinity ligands in combination with macroporous monolith columns. When Compared to conventional antibodies, the bacteriophages, once they are selected against a specific target, are very cheap to produce. Also the production of the monolith can be done at low cost. Due to the high specificity of the expressed peptides towards the target, the number of chromatographic steps can be reduced resulting in an overall time and cost saving procedure. Further research is on-going to improve coupling procedures and to increase column capacity.

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