Concentration of a Multimeric Membrane protein complex

The use of pH elution of proteins from Vivapure spin columns as desalting tools

R. McRae and M. D. Brownleader
Vivascience, Sartorius Group, Stonedale Road, Stonehouse, Gloucester GL10 3RQ.

Introduction

It is a requirement of many analytical methods, that samples be prepared in the absence of salt. For this reason the use of standard, high ionic strength buffers to elute protein samples purified by ion exchange chromatography, is undesirable. In this report, it is shown that proteins loaded on to Vivapure spin columns can be eluted completely by altering the pH conditions of the buffer.

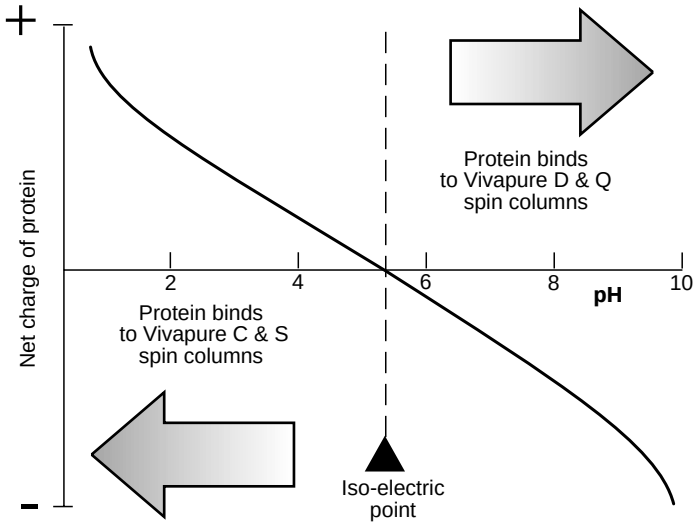
Background

Mass spectrometric techniques such as Matrix Assisted Laser Desorption/Ionisation-Time of Flight mass spectrometry (MALDI-TOF MS) and Electrospray Ionisation mass spectrometry (ESI MS) are routinely being used for the identification and characterization of macromolecules such as proteins with molecular weights less than 200 kDa. The accuracy of the method, which is often better than 0.1 %, exceeds that obtainable by any other physical measurements of mass. In addition, mass spectrometry provides rapid, highly accurate measurements of masses of all but the very largest molecules using only a very small amount of material. MALDI-TOF MS, for example, is particularly sensitive to salt. Many purified proteins are recalcitrant to mass spectrometry and even a small amount of salt is very difficult to remove by conventional methods such as dialysis. In this report, we demonstrate the effectiveness of the Vivapure spin columns to desalt proteins for downstream analyses such as mass spectrometry. Ion exchange chromatography is based on the reversible electrostatic interaction between charged protein molecules and the oppositely charged chromatographic membrane. Anion exchangers, Vivapure Q and D, are positively charged and will bind negatively charged proteins, cation exchangers, S and C, are negatively charged and will bind positively charged proteins.

Concentration of a Multimeric Membrane protein complex

The net charge on a protein is established through the interplay between the acidic and basic properties of the amino acids making up the protein, and the pH of the buffer solution in which the protein is dissolved. The pH at which there is no net charge on the protein is termed the iso-electric point, (pI). At a pH above the pI, the protein will be negatively charged. Below the pI, the protein will be positively charged (Figure 1).

Fig 1. Dependence of net charge of a protein as a function of pH



Bound proteins are usually eluted by increasing the ionic strength or salt content of the buffer. The excess of counter ions compete for the charged groups on the membrane, displacing the bound protein. The eluted protein and the salt both pass into the collection tube. Since this high concentration of salt would interfere with any of the analysis techniques outlined above, elution by this method would necessitate a further desalting step before proceeding with the analysis. Advantage can be taken of the variable charge properties of amino acids when the time comes to elute the bound protein. By changing the pH conditions of the buffer, the net charge on the protein can be altered. An acidic protein with a pI of 5.0 will bind to a Vivapure Q using a loading buffer at pH 8.0. If buffer at pH 3.0 is introduced, the net charge on the protein changes from negative to positive as the carboxyl and amino groups on the amino acid residues are protonated. The positively charged protein falls back into solution and is eluted from the column.

Concentration of a Multimeric Membrane protein complex

Materials and methods

Anion exchange spin column (Vivapure Q & D spin columns)

Two Vivapure Q Mini L or Vivapure D Mini L (Product numbers:Vivapure Q= IX01QL1, Vivapure D= IX01DL1) spin columns, were equilibrated with 100 µl of 25 mM Tris-HCl loading buffer pH 8.0 at 500 g for 5 min in a 45º fixed angle rotor. 100 µg BSA (1 mg/ml; pI 4.6) made up in loading buffer pH 8.0 were added to the spin columns, which were centrifuged again at 500 g for 5 min. For MALDI/TOF-mass spectrometry, 1 mg/ml BSA containing 400 mM NaCl in loading buffer pH 8.0 was added to the spin columns. The spin columns were washed with 2 x 500 µl water at 500 g for 5 min to remove any unbound protein and contaminating salts. The bound protein was eluted from the Vivapure Q spin columns with 100 µl 0.01 % formic acid diluted in deionised water (final pH = 3.2) or the Vivapure D spin columns with 100 µl 0.125 % formic acid, (pH 2.3), and centrifuged at 500 g for 5 min. To ensure maximum recovery, the spin columns were centrifuged again at 12,000 g for 15 sec. An estimate of percentage recovery of protein was made using the dye binding method of Bradford (Bradford, 1976). Quantitative recovery of the protein was estimated using SDS-polyacrylamide gel electrophoresis. The resultant gel being stained using Coomassie Brilliant Blue R-250. The mass spectra were obtained using a Kratos Kompact MALDI 3 (V4.0.0) mass spectrometer. BSA samples, derived from the Vivapure Q spin column, were applied with sinapinic acid matrix to the target probe. Data from 50 laser shots were acquired and averaged to produce the spectra. BSA (M_r 66,000) was used as the external calibration standard.

Cation exchanger spin columns (Vivapure S & C spin columns)

Cytochrome c (100 µg; 1 mg/ml; pI 10.6) dissolved in 25mM sodium acetate buffer pH 5.5 was loaded on to each of two Vivapure S Mini L (Product number: IX01SL1) and two Vivapure C Mini L (Product number: IX01CL1) spin columns, equilibrated in 25 mM sodium acetate buffer pH 5.5. The spin columns were washed with 2 x 500 µl water at 500 g for 5 min to remove any unbound protein and contaminating salts.

Concentration of a Multimeric Membrane protein complex

The Vivapure S spin columns were eluted with 100 µl 0.05 % ammonium hydroxide made up in deionised water and the Vivapure C spin columns were eluted with 100 µl 0.25 % ammonium hydroxide made up in deionised water . The final eluates were analysed in the same way as the resultant eluates from the Vivapure Q and D spin columns.

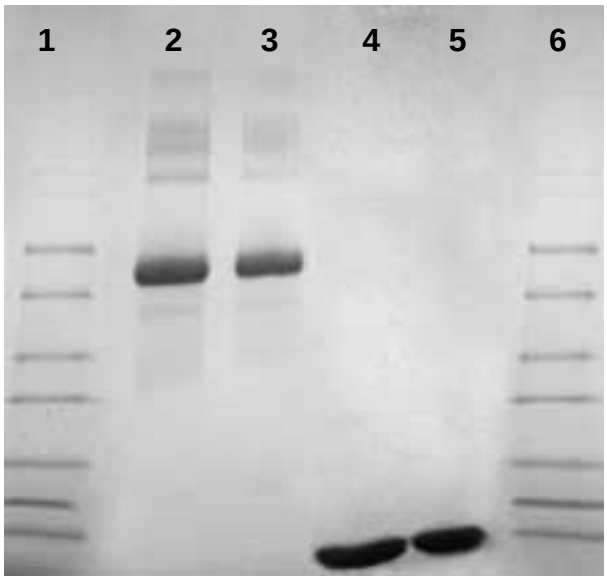
Results

Table 1: Recovery of proteins from Vivapure spin columns

Vivapure spin column	Q	D	S	C
Recovery (%)	97 %	94 %	96 %	93 %

Fig 2. SDS-polyacrylamide gel showing proteins eluted from Vivapure spin columns by altering the buffer pH. The molecular weight markers are myosin (207 kDa), b-galactosidase (121 kDa), BSA (66 kDa), ovalbumin (51.2 kDa), carbonic anhydrase (33.6 kDa), soybean trypsin inhibitor (28.6 kDa), lysozyme (21.1 kDa) and aprotinin (7.5 kDa).

Lane 1, Molecular weight markers.
Lane 2, Ovalbumin eluted from Vivapure Q with 0.01 % formic acid.
Lane 3, Ovalbumin eluted from Vivapure D with 0.125 % formic acid.
Lane 4, Cytochrome c eluted from Vivapure S with 0.05 % ammonium hydroxide.
Lane 5, Cytochrome c eluted from Vivapure C with 0.25 % ammonium hydroxide.
Lane 6, Molecular weight markers.



Concentration of a Multimeric Membrane protein complex

Fig 3. MALDI-TOF mass spectra of BSA. The molecular masses of the pseudo-molecular ion and the doubly-charged species are indicated in the figures (boxed). Fig. A represents the mass spectra of the original sample, BSA solution with 0.4 M NaCl. Fig. B represents the mass spectra of the final wash from a Vivapure Q mini spin column showing BSA has been effectively de-salted.

Fig. A

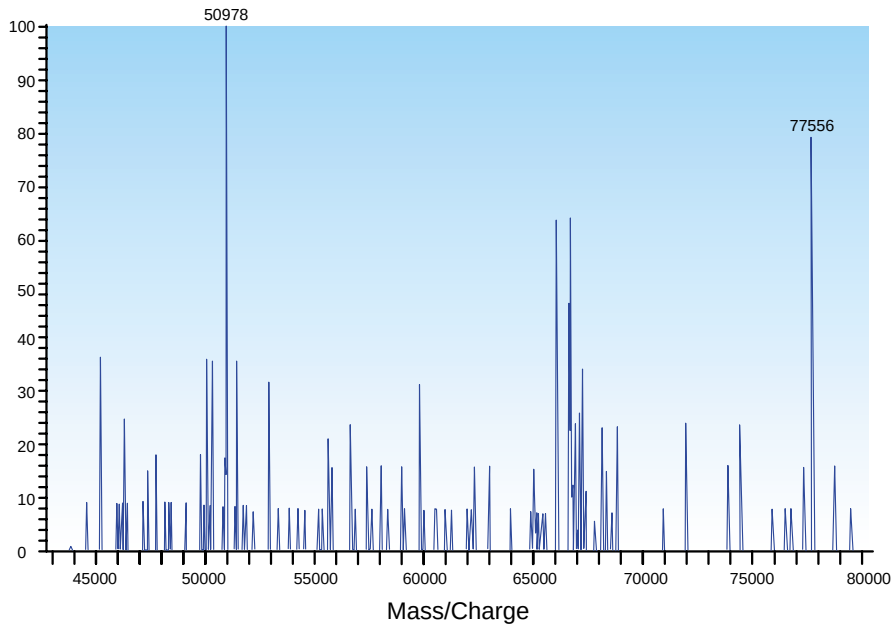
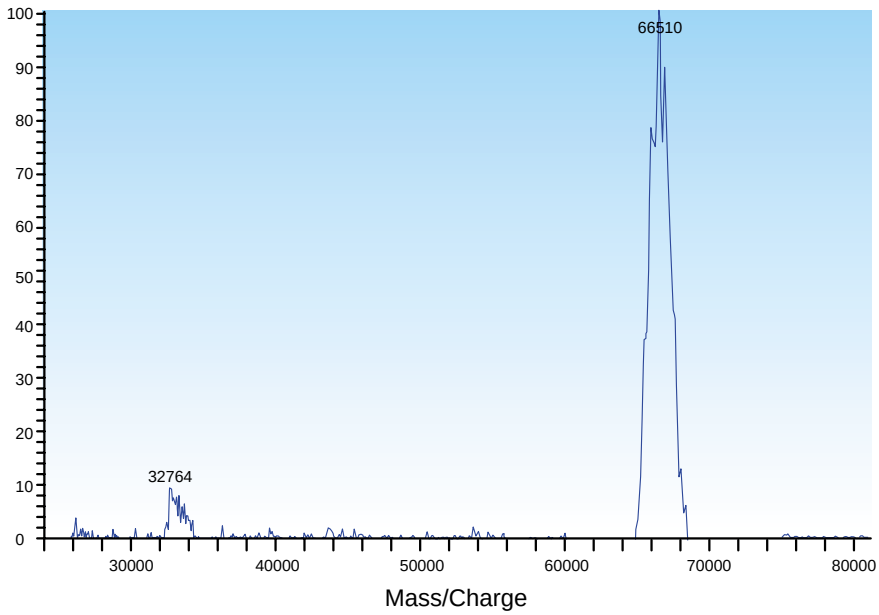


Fig. B



Concentration of a Multimeric Membrane protein complex

Conclusion

Formic acid or ammonium hydroxide can be used to elute proteins from Vivapure spin columns. Altering the pH of the buffer solution is an efficient method of elution and high recoveries of the purified protein can be achieved (Table 1). As can be seen from the SDS-PAGE gel image in Fig. 2, the protein has been eluted without degradation. Fig. 3B represents the mass spectrum of BSA (m/z 66,510) with the doubly-charged species having a m/z 32,764. Fig. 3A represents the mass spectra of a BSA solution containing 0.4 M NaCl where no visible peaks representing BSA were observed. The Vivapure spin columns are therefore rapid tools for de-salting proteins for physico-chemical analyses, generally regarded as intolerant to high levels of salt, such as mass spectrometry.