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Purification | Polishing of His-tagged Proteins using Vivapure® prior to Crystallization

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Abstract

The 3D structure of a protein provides crucial insights into its function and allows for the design of specific drugs in the fight against numerous diseases. X-ray crystallography is a widely used technique for the determination of protein structures. Proteins are first crystallized before collecting diffraction data, which is used to solve the location of each atom in the polypeptide chain. To obtain high quality diffraction data, it is crucial that proteins are produced to high purity prior to crystallization. A method for scouting of ion exchange chromatography (IEC) purification conditions is described, using Vivapure® membrane adsorbers. In comparison to the use of FPLC systems, Sartorius membrane adsorber technology offers the ability to rapidly determine the optimal purification conditions in parallel, whilst minimizing buffer requirements and remaining relevant when scaling up to preparative purification.

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Introduction

Multi-milligram quantities of highly purified (~99% purity) protein are required for protein-crystallization trials (see McPherson 1998 for a review). In recent years purification of recombinant proteins has been greatly facilitated by the engineering of His-tags at the amino or carboxyl termini of proteins, allowing simple purification by immobilized metal affinity chromatography (IMAC). A simple purification scheme involving immobilized Ni^{2+}-chromatography and gel filtration may therefore provide protein of sufficient purity for crystallization. However, it is often necessary to introduce a further purification step to "polish" the protein sample, maximizing the chances of obtaining protein crystals.

Ion exchange chromatography (IEC) is frequently used for simple, high recovery purification of proteins (see Harris and Angal 1989 for a review). Protein separation by IEC is primarily dependent on the charge properties of the proteins in a sample, unlike IMAC where separation is dependent primarily on the presence (or absence) of solvent accessible histidine and cysteine residues on proteins. IEC may therefore enhance the selectivity and resolution of this purification scheme (IMAC and GF), providing efficient polishing of protein prior to crystallization trials.

The first step in establishing an IEC purification procedure for a particular protein is the identification of conditions for efficient binding (and elution) of target protein to ion exchange media, or conditions for efficient binding of only the contaminants. In practice this means performing a series of test adsorption and elution experiments over a range of pH using different ion exchange media. Appropriate binding conditions can then be scaled up for preparative purification or polishing of protein samples.

We have investigated the use of ion exchange membrane devices for the purification of a sample of a bacterial enzyme involved in inositol biosynthesis. We had previously identified conditions for the crystallization of enzyme at around 95% purity but had found difficulty in producing well-ordered crystals. This study aimed to further purify this enzyme to improve the chances of obtaining crystals of sufficient quality for structure determination. We demonstrate here the use of centrifugal Vivapure® ion exchange membrane adsorbers (Figure 1) for rapid comparison of anion (Q) and cation (S) exchange membranes under different adsorption and elution conditions. We show that conditions identified for efficient target protein adsorption and elution with centrifugal Vivapure ion exchange membrane devices also allow efficient adsorption and elution with the equivalent high binding capacity ion exchange FPLC membrane adsorbers MA 300S and MA 300Q (Sartorius, Goettingen, Germany), providing simple scale-up to preparative purification.

Figure 1

Vivapure® ion exchange membrane are available for samples up to 500 uL or 20 mL, offering a broad range of binding capacities.

Materials and Methods

Scouting Purification

A 12 mg sample of enzyme, purified by immobilized Ni^{2+}-chromatography, was desalted using a Vivaspin® 15 centrifugal ultrafilter (30 kDa MWCO, Sartorius) into distilled water. 50 µL aliquots containing 0.5 mg of desalted protein were diluted ten-fold into a range of binding buffers (Table 1).

These samples were loaded directly on to centrifugal Vivapure® anion (Q) or cation (S) exchange membrane adsorbers (one for each binding condition) and spun at 500 *g* in a microcentrifuge for 20 seconds. Each device was then washed with 0.5 mL of the appropriate binding buffer.

The ion exchange membrane inserts were transferred to fresh 1.5 mL microfuge tubes, and the tubes containing flow-through and wash were stored on ice. 250 µL of elution buffer (ie. 1 M NaCl in the appropriate binding buffer) was subsequently added before re-spinning (500 *g*, 20 seconds). Binding and elution efficiency were followed by analysis of the sample, flow-through, wash and eluate fractions separated by SDS-PAGE fractions separated by SDS-PAGE.

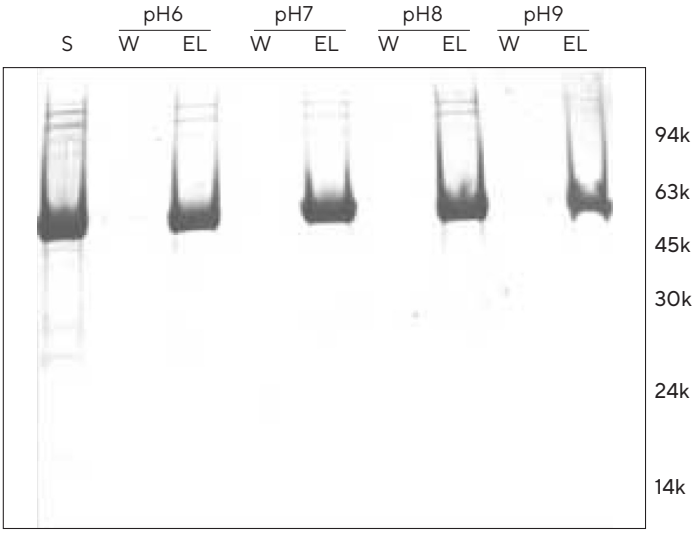
The binding buffer conditions selected for preparative-scale purification of enzyme were 20 mM MES pH 6.0 for cation-exchange (MA-300S) and 20 mM Tris pH 8.0 for anion exchange (MA-300Q).

Table 1

Buffers For Anion (Q) Exchange		Buffers For Cation (S) Exchange	
pH	Composition	pH	Composition
6	20 mM histidine	4	20 mM citrate
7	20 mM imidazole	5	15 mM acetate
8	20 mM Tris	6	20 mM MES
9	20 mM Tris	7	25 mM MOPS
		8	20 mM Tricine

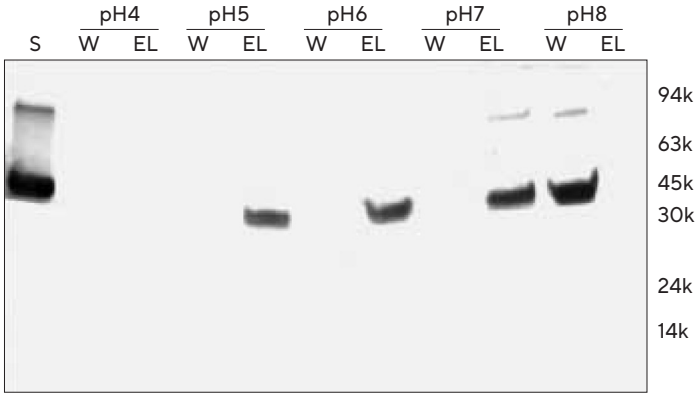
Note: Buffer compositions for scouting purification with centrifugal anion or cation exchange Vivapure® devices. Binding buffer compositions are listed in the table. Elution buffers used were 1 M NaCl in the appropriate binding buffer.

Figure 2



Note: Scouting buffer conditions for protein adsorption to Vivapure® anion (Q) exchange membrane adsorbers. Sample (S), wash (W) and eluate (EL) fractions were analyzed by Tris-Tricine SDS PAGE.

Figure 3



Note: Scouting buffer conditions for protein adsorption to Vivapure® cation (S) exchange membrane adsorbers. Sample (S), wash (W) and eluate (EL) fractions were analyzed by Tris-Tricine SDS PAGE.

Preparative Purification

Preparative-scale chromatography was performed under the control of an AKTA FPLC-XL. 20 mL samples were prepared by addition of concentrated buffers to 5 mg of desalted enzyme. The prep-scale ion-exchange devices were equilibrated in 100 mL of the appropriate buffer at 10 mL/min. Samples were loaded at 4 mL/min, and the ion-exchanger devices washed with the appropriate buffer prior to protein elution using a linear sodium chloride concentration gradient. Eluate fractions were analyzed by SDS-PAGE (Figures 4 and 5).

Figure 4

Preparative purification profile, and SDS-PAGE of the sample (S) and eluate fractions (16-25) from MA300Q.

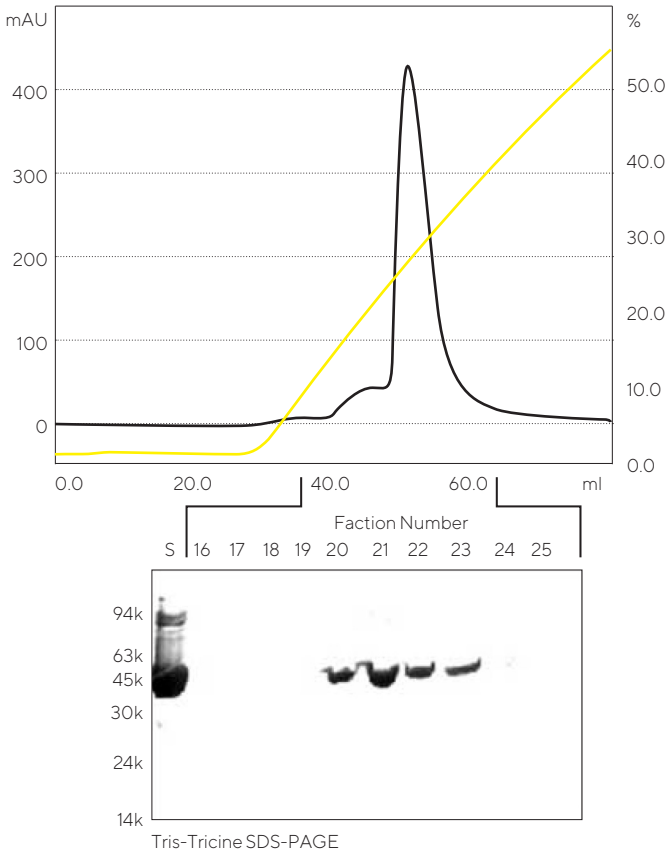


Figure 5

Preparative purification profile, and SDS-PAGE of the sample (S) and eluate fractions (23-31) from MA300S.

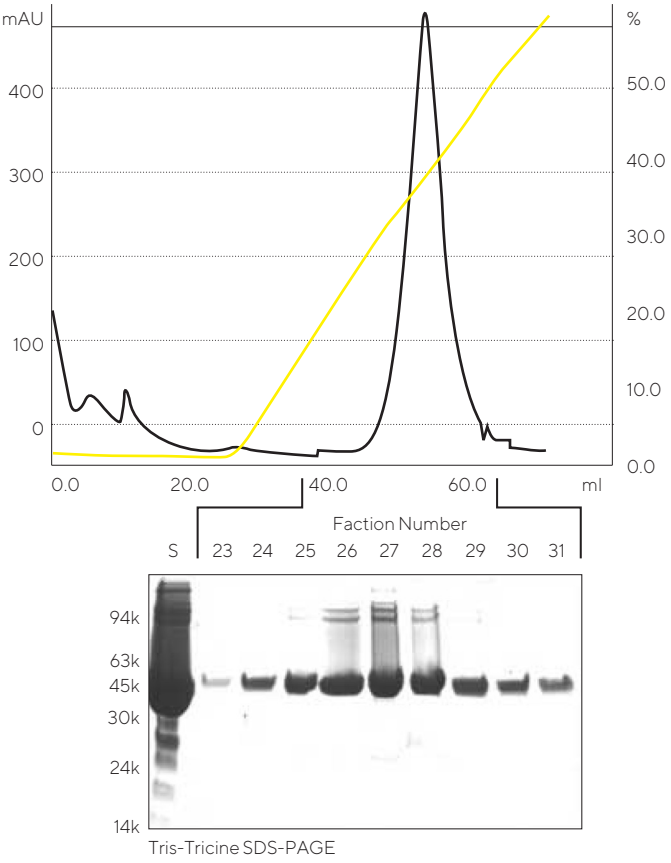
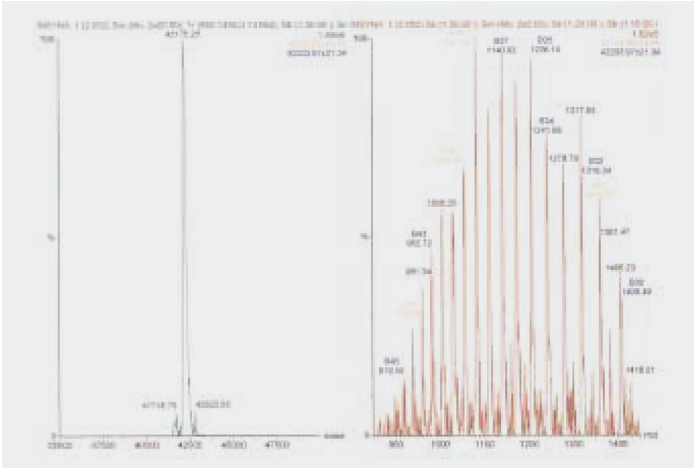


Figure 6



Note: Electrospray mass spectrum of the purified protein. Mass spectra were collected on a VG Platform Electrospray Mass Spectrometer (Micromass), with a sample desalted using a Vivaspin® 500 ultrafiltration device into deionized water. Twelve 10 second scans were accumulated for each sample over the m/z range 850-1500. Spectra were processed using the "Masslynx" software (Micromass Inc.).

Table 2

Yield of protein after purification by IEC.

	Peak Area (mAu + mL)	% Yield
Sample	5.7	N/A
MA 300Q eluate peak	5	88
MA 300S eluate peak	5.2	91

Results

Adsorption of enzyme to centrifugal Vivapure® anion (Q) exchange membrane devices was efficient (>95%) over the pH range 6-8, but at pH 9 only partial adsorption was observed. Elution was efficient at all pHs. Enzyme did not adsorb to the centrifugal cation (S) exchange membrane devices at pH 8, but binding was efficient at pH 5 and 6. At pH 4 the sample appeared to bind efficiently but was not eluted by 1M NaCl, suggesting that the protein was either very strongly adsorbed or had precipitated from solution. It is interesting to note that this enzyme is able to bind efficiently to both anion and cation exchangers at around pH 6. This may reflect the fact that the enzyme is predicted to have a net charge of –13 at neutral pH, while the His-tag may become sufficiently protonated at pH 6 to allow binding to the cation exchanger.

Efficient adsorption (and elution) of enzyme to both anion (MA300Q) and cation (MA300S) exchange devices was observed. Sample recovery was quantitative as assessed by absorbance measurements at 280 nm (sample) and from integrated peak areas from chromatograms (Table 2) (using the "Unicorn" software, Amersham Pharmacia Inc.). The molar extinction coefficient of the enzyme was calculated to be 36,960 $M^{-1}.cm^{-1}$ (iso-electric point 5.77), using the "Peptidesort" program (GCG, Inc.). The anion exchange experiment gave superior resolution (Figure 4 and 5) of enzyme from contaminants, in comparison to the cation exchange experiment. Electrospray mass spectroscopy of the purified enzyme indicated that the protein was essentially homogeneous and of the expected mass (Figure 6).

Conclusions

In summary, the centrifugal Vivapure® devices allowed rapid scouting of adsorption and elution conditions using different ion-exchangers. The optimal purification conditions could then be used directly for rapid preparative-scale purification of enzyme using the MA 300S and MA 300Q preparative IEC devices, yielding enzyme of high purity for crystallization trials.

Notes

FPLC membrane adsorbers MA 300S and MA 300Q are now discontinued. However, Sartorius continues to offer Sartobind® devices compatible with FPLC systems for preparative scale capture and polishing applications. For capture applications such as the one described in this Application Note, we recommend testing scale-up from Vivapure® using Sartobind® Nano 3 mL devices, with 8 mm bed height.

References

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