

Purification/Polishing of His-tagged proteins - Application of Centrifugal Vivapure Ion-exchange Membrane Devices to the Purification/Polishing of His-tagged Proteins for Crystallization.

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Background

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 Ni²⁺-chromatography and gel-filtration may therefore provide protein of sufficient purity for crystallization trials. However, it is often necessary to introduce a further purification step to "polish" the protein sample to maximize 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 different 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 used for scale up to preparative scale purification/polishing of protein samples.

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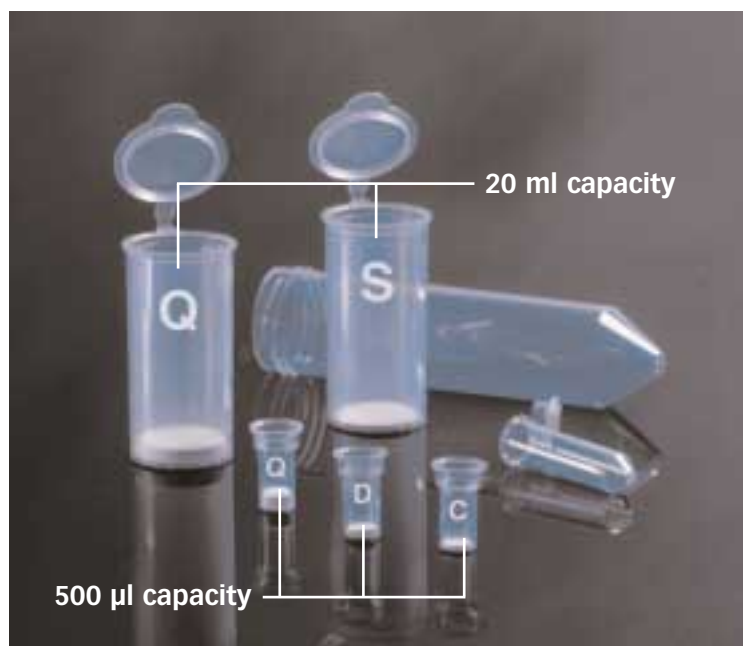
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We have investigated the use of ion exchange membrane devices for the purification of a sample of a bacterial enzyme involved in inositol biosynthesis. We had previously identified conditions for the crystallization of enzyme purified to around 95% 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 to allow structure determination. We demonstrate here the use of centrifugal Vivapure ion exchange membrane devices for rapid comparison of anion (Q), and cation (S) ion 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 scale purification.



Vivapure Ion Exchange membrane devices with various protein binding capacities

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Results and Discussion

Screening of Adsorption Conditions

A 12 mg sample of enzyme, purified by immobilized Ni²⁺-chromatography, was desalted using a Vivaspin 15 ultrafiltration concentrator (30 kDa cut-off) into distilled water. 50 µl aliquots containing 0.5 mg of desalted protein were then diluted ten-fold into a range of binding buffers (Table 1).

These samples were then loaded directly on to centrifugal Vivapure anion (Q) cation (S) ion exchange membrane devices (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 then 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. 1M NaCl in the appropriate binding buffer) were subsequently added before re-spinning (500 g, 20 secs.). Binding and elution efficiency were followed by SDS-PAGE analysis of the sample, flow-through / wash and eluate (Fig. 1 and 2).

Table.1

Buffers for centrifugal anion (Q) ion exchange membrane trial		Buffers for centrifugal cation (S) ion exchange membrane trial	
pH	Buffer (binding)	pH	Buffer (binding)
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: Elution buffers used were 1M NaCl in the appropriate binding buffer for all pH values.

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Fig. 1: Screening of buffer conditions for protein adsorption to centrifugal Vivapure anion (Q) ion exchange membrane devices. Tris-Ticine SDS-PAGE.

Vivapure Q pH trial

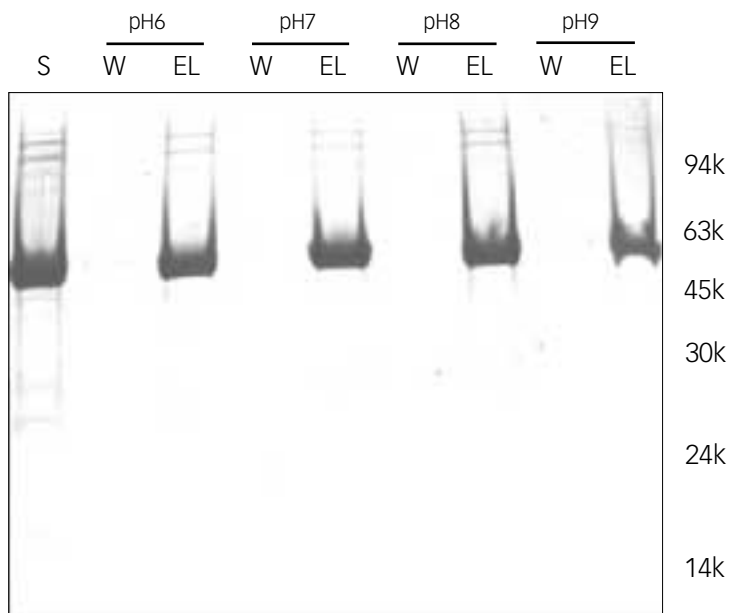
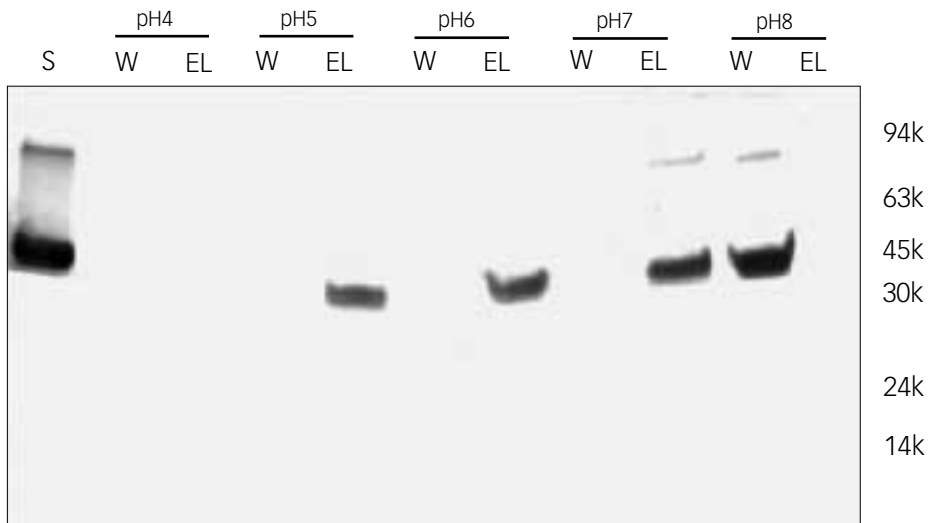


Fig. 2: Screening of buffer conditions for protein adsorption to centrifugal Vivapure cation (S) ion exchange membrane devices. Tris-Ticine SDS-PAGE.

Vivapure S pH trial



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Adsorption of enzyme to centrifugal Vivapure anion (Q) ion exchange membrane devices was efficient (>95%) over the pH range 6-8, but at pH 9.0 only partial-adsorption was observed. Elution was efficient at all pHs. Enzyme did not adsorb to the centrifugal cation (S) ion exchange membrane devices at pH 8, but binding was efficient at pH 5.0 and 6.0. At pH 4.0 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.

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).

Preparative-Scale Purification

Preparative-scale chromatography was performed under the control of an Acta 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 100ml of the appropriate buffer at 10ml/min. Samples were loaded at 4 ml/min, and the ion-exchanger devices were then washed with the appropriate buffer and eluted with a linear sodium chloride concentration gradient. Eluate fractions were analyzed by SDS-PAGE (Figures 3 and 4). Efficient adsorption (and

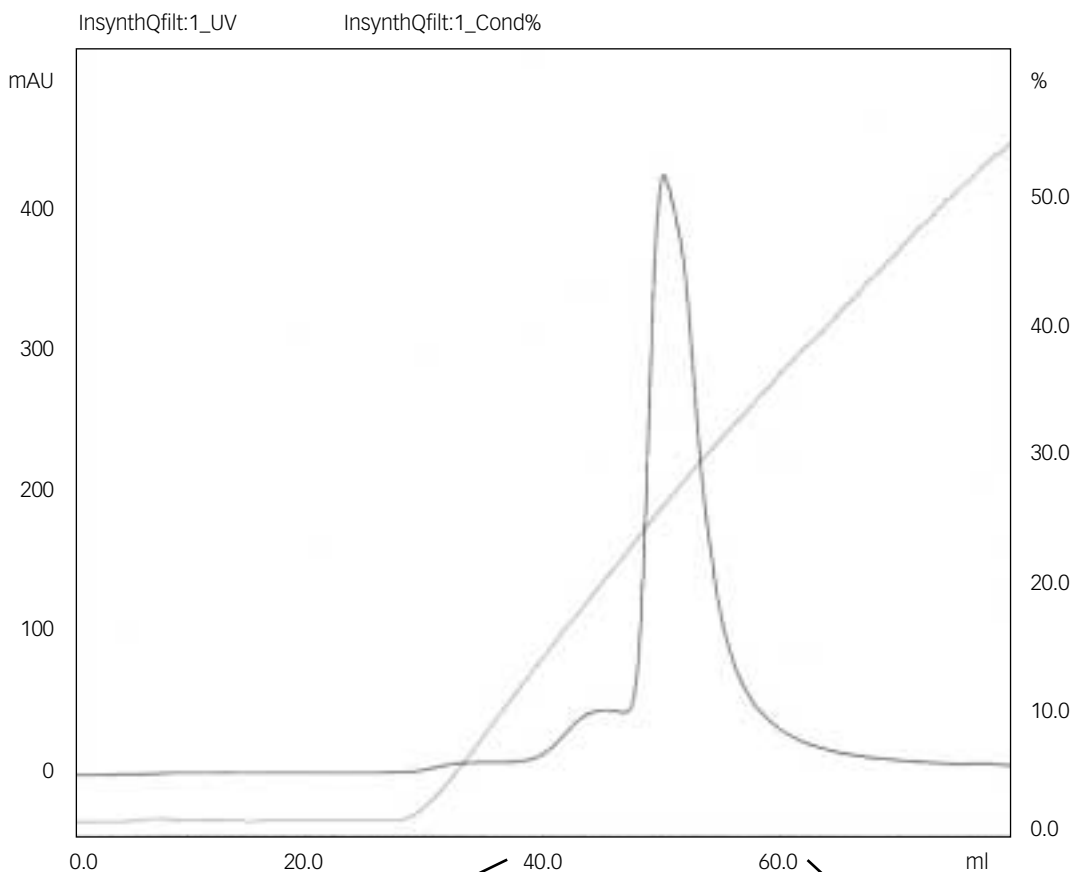
elution) of enzyme to both anion (MA300Q) and cation (MA300S) exchange devices was observed. Sample recovery was quantitative as assessed by absorbance measurements at 280 nm (sample) and from integrated peak areas from chromatograms (Table 2) (using the "Unicorn" software, Amersham Pharmacia Inc.). 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 3 and 4) 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 5).

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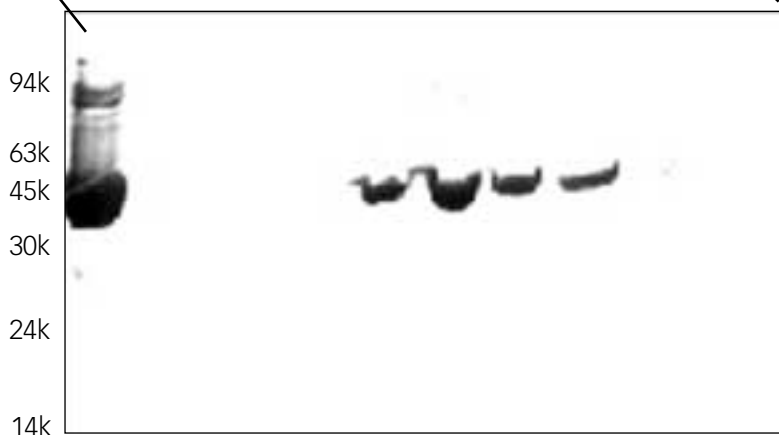
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Sample Fraction Number
 16 17 18 19 20 21 22 23 24 25

Fig.3



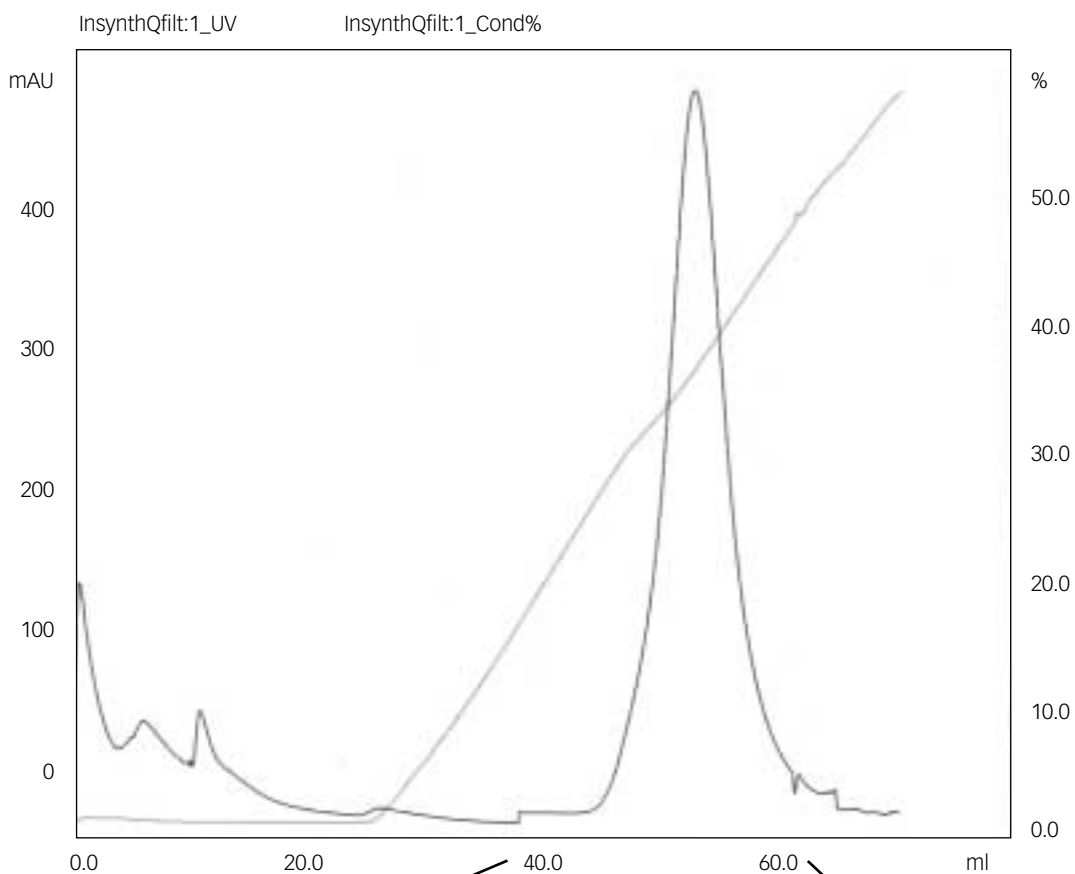
Tris-Tricine SDS-PAGE of MA300Q Eluate Fractions

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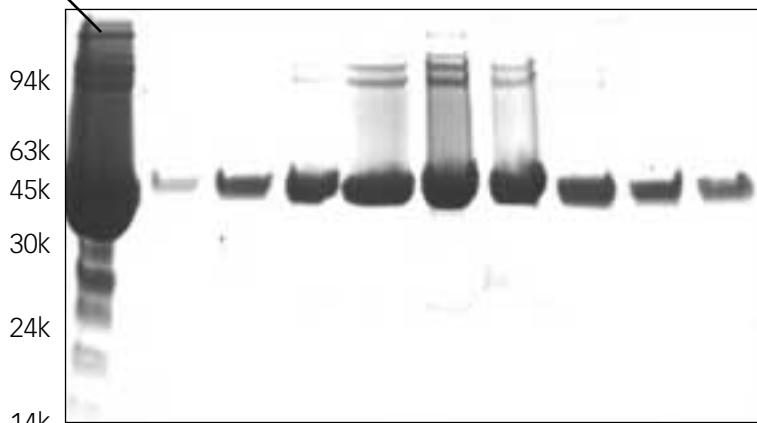
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Sample Fraction Number

 23 24 25 26 27 28 29 30 31

Fig.4



MA300S Gradient Elution
Tris-Tricine SDS-PAGE of Eluate Fractions

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Table 2. Yield of protein after ion exchange purification.

	Peak area (mAu x ml)	% yield
Sample	5.7	N/A*
MA 300Q eluate peak	5.0	88
MA 300S eluate peak	5.2	91

*N/A: Not Applicable

In summary, the centrifugal Vivapure devices allowed rapid piloting of adsorption and elution conditions using different ion-exchangers, which 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.

References

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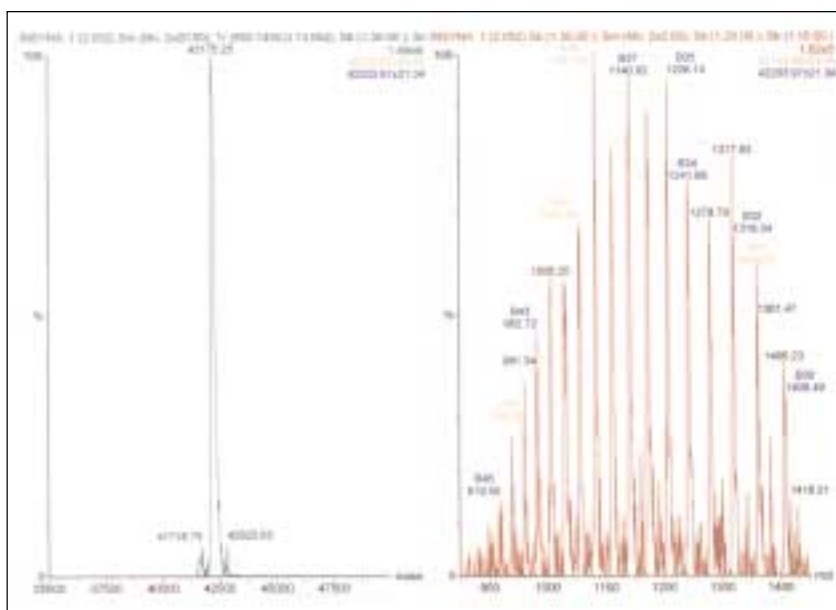


Fig 5. Mass spectra were collected on a VG Platform Electrospray Mass Spectrometer (Micromass), with a sample desalted using Vivaspin 500 ultrafiltration device into deionised water. Twelve 10 second scans were accumulated for each sample over the m/z range 850-1500. Spectra were processed using the "Masslynx" software (Micromass Inc.).