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# Comparative Study of Resin- and Membrane-Based Ion Exchange Technologies for Protein Purification

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## Abstract

Ion exchange (IEX) chromatography is widely used for the purification of both soluble and membrane-bound proteins, nucleic acids and for the removal of toxins and contaminants. This method gives reliable and reproducible separation of macromolecules. The conventional technique of IEX employs a functional group - a strong or weak, anion or cation exchanger - bound to either sepharose or agarose beads, which are packed into a column. In anion exchange chromatography, proteins are typically loaded on to the column in low ionic strength buffer with pH greater than the isoelectric point (pI) of the target molecule, to promote binding. The proteins are then separated by elution, usually by increasing the ionic strength of the mobile phase (elution buffer). Alternatively, elution may be effected by reducing the pH of the mobile phase. Different proteins elute from the beads as they are exchanged with $Cl^-$ counterions in the elution buffer, or when their pI is reached. Furthermore, elution can be accomplished either with a gradient or step-wise adjustment of the ionic strength or pH. In this study, the performance of a conventional column-based weak anion exchanger i(diethylaminoethyl, DEAE) was compared to Vivapure® D spin columns for the separation of an inhibitory molecule present in the lens of 7 day chick embryos.

# Introduction

Anion exchange chromatography was used as a step in the purification of a soluble axon-growth inhibitory protein obtained from the lens of 7 day chicken embryos. This protein is important for the guidance of retinal ganglion cell (RGC) axons towards the optic nerve disc, where they bundle together to form the optic nerve. The RGC axons and their growth cones (highly sensitive hand-like projections at the growing tips) are forced to grow in the opposite direction by the inhibitory molecule secreted from the lens, which diffuses through the vitreous into the retina, establishing a gradient of repulsion. This is one mechanism which may direct RGC growth cones towards the optic nerve head, the exit point for RGC axons leaving the retina on their journey to the optic tectum, where they make precise connections. Such repulsion may also prevent RGC axons growing inappropriately into the vitreous humour and | or the pigmented epithelium. Addition of the inhibitory protein to retinal axons growing in culture results in a dramatic alteration to growth cone morphology. The change from a hand-like spread structure to a pointed collapsed stump provides a convenient indicator of the presence of this molecule.

Two methods of anion exchange chromatography - a conventional resin-based column and the innovative membrane adsorber technology in Vivapure® centrifugal devices - have been acessed for the following criteria:

- Cost and equipment requirement
- Time requirement
- Protein yield and activity.

# Materials and Methods

### Growth Cone Collapse Assay

Retina were dissected from day 5 chick embryos, since this is the peak time of RGC axonogenesis. Eyes were removed from whole embryos and dissected in DMEM by peeling away the sclera and pigmented epithelial cell layer. The underlying retina was laid flat and cut into 0.5 $mm^2$ pieces using a razor blade. Three to four explants were plated in 24-well tissue culture dishes (Nunc) containing poly-lysine/laminin-coated glass cover slips and cultured for 36 hours in retinal growth medium (DMEM supplemented with 2% E7 chick embryo extract, 50 mM gentamycin and 1% insulin-transferrin-selenium mixture (ITS, Sigma)). Selected retinal explant cultures were incubated for 1 hour at 37 °C with 10 mL of either, PBS for control cultures, or 10 mL of lens protein extract or fractions obtained from anion exchange purification of the lens extract.

Following incubation, retinal explant cultures were fixed with 1 mL of 4 % paraformaldehyde, 20 % sucrose in PBS. 0.5 mL medium was removed and replaced with 0.5 mL of fixative and this procedure repeated once more, to ensure gradual replacement of growth medium with fixative and minimal disturbance to the retinal axons. Fixed cultures were left for 1 hour at room temperature before scoring under phase contrast microscopy. The degree of growth cone collapse was determined as a percentage of the total number of spread and collapsed growth cones.

### Preparation of Lens Protein Extract

The inhibitory protein present in the lens is secreted into the vitreous humour which acts as a convenient sink for this protein. Lens protein was extracted from dissected eyes of day 7 chick embryos simply by stabbing the eyes with watchmaker forceps and pulling the vitreous out. The vitreous humour was collected on dry ice and frozen at -70 °C until required.

100 mg of vitreous humour were homogenized, using a Polytron® homogenizer, in 200 mL of 20 mM phosphate buffer containing a cocktail of protease inhibitors including PMSF (1/100), leupeptin (1/5000) and Pepstatin A (1/1000). The vitreous homogenate was ultracentrifuged at 100,000 *g* for 1 hour at 4 °C.

The supernatant was sterile-filtered through a Sartolab® RF unit with 0.2 µm PES membrane and concentrated 10-fold using a Vivaspin® 20 device (100 kDa MWCO PES). The concentrated sample was tested for activity and total protein content, and the remaining vitreous extract (VE) purified by anion exchange chromatography.

### Anion Exchange Chromatography

Two 95 mL samples of VE were purified by conventional DEAE-agarose or using Vivapure® D membrane adsorber devices.

For purification by DEAE-agarose, 90 mL of resin was packed into a column, washed with 10 mL of 1 M NaOH and equilibrated with 20 mM phosphate loading buffer. 95 mL of VE was pumped onto the column at 5 mL/min. The column was washed with approximately 2 column volumes (CV) of loading buffer (200 mL 20 mM phosphate buffer, pH 6.5) prior to step-wise elution with 2 CV each of 0.2, 0.5 and 1 M NaCl in 50 mM phosphate buffer, pH 8.0.

The column was washed with NaOH and re-equilibrated for isolation of inhibitory protein from the flow-through and wash fractions of the initial purification cycle (which, after concentrating 5-fold and testing in the growth cone collapse assay, still contained RGC inhibitory activity). This procedure was repeated until the flow-through was depleted of biological activity, thus maximizing the yield of inhibitory protein.

In Vivapure® D devices the functional weak anion exchange group diethylamine ($R\text{-}CH_2\text{-}N(C_2H_5)_2$) is bound to a membrane which is housed in a 50 mL centrifuge tube. To enable direct comparison against the conventional anion exchange method, four Vivapure® D Maxi H spin columns were selected for purification by membrane adsorption, ensuring the same ligand density (mEq) relative to the sample volume. The devices were cleaned by loading with 2 mL NaOH and centrifuging at 1,500 *g* for 2 minutes at 4 °C. The devices were then equilibrated with 20 mM phosphate loading buffer before applying 12 mL of VE to each device by centrifugation, as described above.

Elution was performed step-wise, using 5 mL each of 0.2, 0.5 and 1 M NaCl in 50 mM phosphate buffer, pH 8.0. As for the DEAE-agarose procedure, purification was repeated several times until the whole VE sample had been applied to the devices and the flow-through and wash fractions no longer contained any RGC inhibitory activity. The total protein in each active sample was estimated by BCA assay (Thermo Fisher Scientific).

### 2D Polyacrylamide Gel Electrophoresis

Proteins were separated in the first dimension by isoelectric focussing (IEF) on an 11 cm immobiline strip gel containing a pH gradient of 3-10. This was followed by molecular weight separation in the second dimension using SDS-PAGE with a 10 % gel (4% stacking gel). After electrophoresis, proteins were visualised by silver staining.

# Results

## Cost and Equipment Requirements

There were large differences in the equipment required and therefore costs associated with performing ion-exchange chromatography using conventional column-based methods or centrifugal membrane adsorbers (Table 1).

**Table 1.**

*Comparison of equipment required to perform ion exchange chromatography using conventional column based methods with innovative centrifugal membrane adsorbers.*

| Method | Equipment Required | Estimated Cost (2021) |
|---|---|---|
| Conventional column chromatography | Column | € 670.00 |
| | Resin (DEAE-agarose beads) | € 275.00 |
| | FPLC system | € 11,645.00 |
| | **Total** | **€ 12,590.00** |
| Vivapure® D membrane adsorbers | Spin columns (ready-to-use) | € 210.00 |
| | Centrifuge with appropriate rotor | € 3,900.00 |
| | **Total** | **€ 4,110.00** |

## Time Requirement

There were considerable differences in the amount of time required to perform the separation between the two methods, ranging from under 20 minutes when using centrifugal membrane adsorbers to days when using conventional column-based chromatography (Table 2).

**Table 2.**

*Comparison of time taken to perform anion exchange chromatography of an inhibitory embryonic lens protein by column-based or centrifugal membrane-based methods.*

| | Cleaning | Equilibration | Loading | Washing | Elution | Total Time |
|---|---|---|---|---|---|---|
| Conventional column chromatography | 15 min | 36 hr | 30 min | 1 hr | 3 hrs | 40 h 45 min |
| Vivapure® D membrane adsorbers | 2 min | 4 min | 2 min | 4 min | 6 min | 0 h 18 min |

## Protein Yield and Activity

Both methods of anion exchange chromatography yielded protein fractions containing equivalent levels of biological activity. Total protein content was also similar. RGC axon inhibitory activity was recovered in the 0.5 and 1 M NaCl eluates (Table 3).

**Table 3.**

*Protein and biological activity profile following purification of a chick embryo lens inhibitory protein by anion exchange chromatography.*

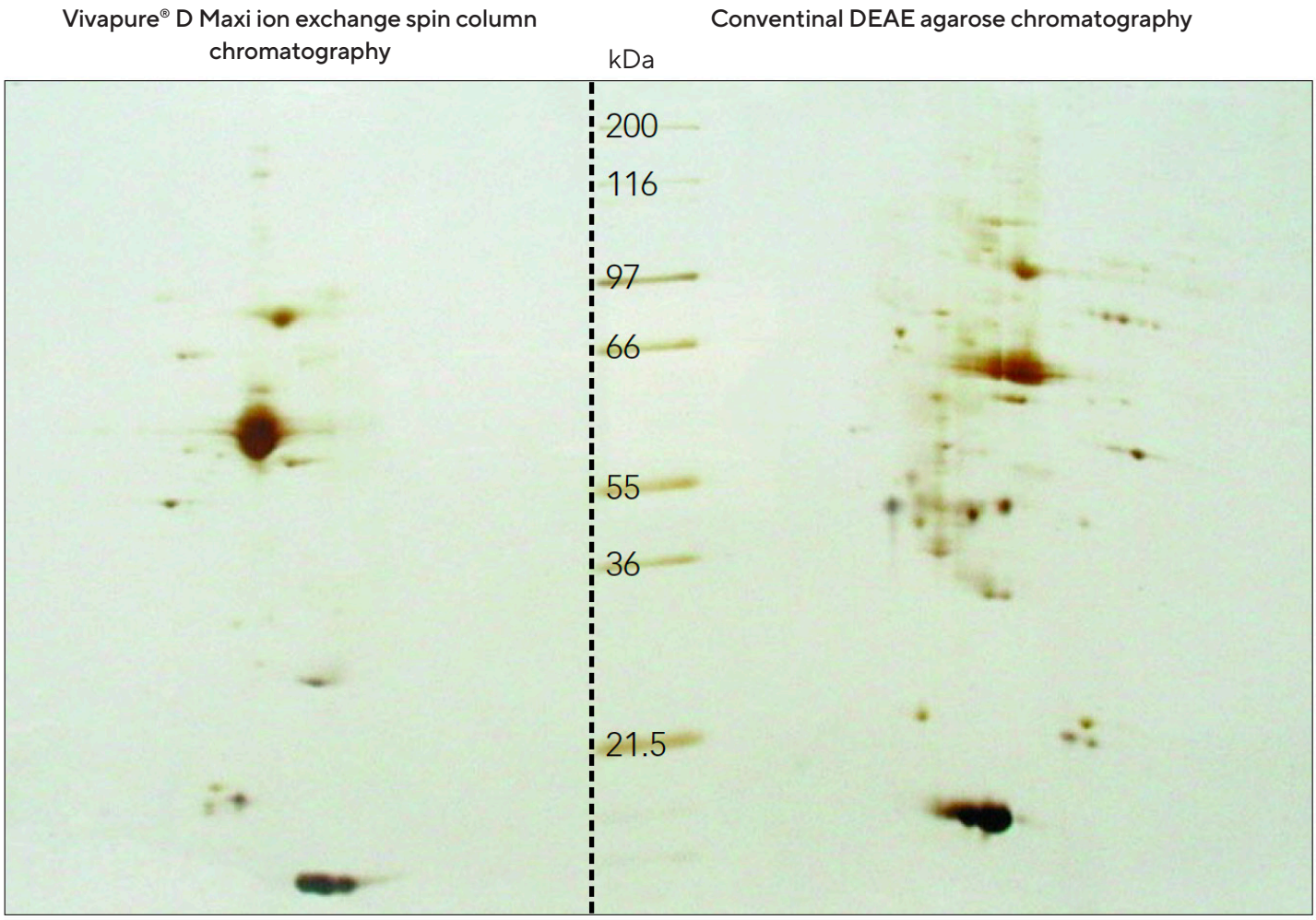
| | Fraction (NaCl eluate) | Growth cone collapse activity | Total protein concentration |
|---|---|---|---|
| Conventional column chromatography | 0.2 M | 1 % | nd |
| | 0.5 M | 39 % | 2.08 mg/mL |
| | 1 M | 56 % | 1.12 mg/mL |
| Vivapure® D membrane adsorbers | 0.2 M | 1 % | nd |
| | 0.5 M | 42 % | 3.26 mg/mL |
| | 1 M | 58 % | 0.86 mg/mL |

In both methods, the 1 M NaCl eluate contained the least total protein content but the highest inhibitory activity. Therefore, the protein composition of these samples were established by 2D-PAGE. For direct comparability, the IEF strips for each sample were electrophoresed side-by-side in the second dimension SDS polyacrylamide gel (Figure 1).

The protein composition of the 1 M NaCl eluates from the DEAE-agarose column and the Vivapure® D Maxi H spin column were very similar. However, the latter sample contained much less contaminating, inactive protein, indicating that higher resolution separation is achievable when using Vivapure® membrane adsorbers for protein purification.

**Figure 1.**

*2-dimensional gel electrophoresis of 1 M NaCl eluates from DEAE-agarose (right) and Vivapure® D (left). Bands visible between the two samples are Novex molecular weight markers.*



# Conclusion

There is a need for the purification and molecular characterization of novel, functionally important proteins. Protein purification is often a time consuming and expensive approach but can yield invaluable results in the identification of key players in developmental and physiological processes. The availability of innovative and reliable purification methods which can also reduce the cost and expense of these workflows will be of immense value.

The results presented here demonstrate that ion exchange technology, based upon functional groups bound to membranes, performs identically to the conventional agarose bead method in terms of protein binding and recovery of biological activity. Furthermore, we also found greatly reduced equipment requirements, and significant savings in cost (67%) and time (>99%). This will save the researcher days, if not weeks, of valuable time and budget, which can be used to perform other important experiments.

The simplicity and convenience involved in performing purification with Vivapure® ion exchange spin columns, which only require access to a centrifuge, enables researchers to perform necessary pilot experiments without committing to large capital investments. In addition, the application of these devices is not restricted to protein purification but also the rapid and inexpensive detoxification of wide ranges of samples, offering a cost-effective and efficient alternative to conventional ion exchange chromatography.

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