application note

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Purification of Axon Growth Inhibitory Proteins

A comparative study of an anion exchange membrane-based Viva spin coluon technology with a conventional agrose gel column in the purification of an inhibitory protein from embryonic chick lens.

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Background

Ion-exchange chromatography is widely used for the purification of a wide range 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 soluble proteins. The conventional method of ion-exchange employs a functional group, a strong or weak, anion or cation exchanger, bound to either sepharose or agarose beads and packed into a column. For instance, in anion exchange chromatography, proteins are loaded on to the column in low ionic strength, low pH buffer, to promote binding. The proteins are separated by elution with varying concentrations of NaCl in high ionic strength, high pH buffer. Different proteins elute from the beads as they are exchanged with the Na and Cl ions in the elution buffer. Elution can be accomplished using either a gradient of salt; eg 0.1 to 1 M NaCl or by stepwise elution.

In this study, a side-by-side comparision of the performance of a standard column-based agarose gel weak anion exchanger i.e. diethylaminoethyl (DEAE) and the Vivapure spin column for the separation of an inhibitory molecule present in the lens of 7 day chick embryos has been undertaken.

BETTER LIFE SCIENCE BY DESIGN

Purification of Axon Growth Inhibitory Proteins

Biology of retinal axon repulsion

Anion-exchange chromatography was used as a step in the purification of a soluble axon-growth inhibitory protein obtained from the lens capsule of the 7 day chicken embryo. The protein is important for the guidance of retinal axons towards the optic nerve disc where they bundle together to form the optic nerve. The retinal ganglion cell (RGC) axons, and their growth cones (highly sensitive hand-like projections at the growing tips of each axon) are repelled, or 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. This repulsion may also prevent RGC axons growing inappropriately into the vitreous humour and/or the pigmented epithelium.
Addition of the protein to retinal axons growing in culture results in a dramatic alteration in growth cone morphology –changing from a hand-like spread structure to a pointed collapsed stump; providing a convenient indicator of the molecules presence.

Both methods of anion exchange chromatography have been acessed for the following criteria:

I. Cost and equipment required
II. Time required
III. Recovery of active protein (measured as the percent of the total collapsed growth cones).
IV. Protein content and pattern of active eluates as indicated by 2-dimensional electrophoresis.

Purification of Axon Growth Inhibitory Proteins

Methods and Materials

Growth cone collapse assay

Retina were dissected from embryonic day 5 chick embryos since this is the peak time of retinal ganglion cell (RGC) axonogenesis. Eyes were removed from whole embryos dissected in Dulbecco`s minimal essential medium (DMEM) by peeling away the sclera and pigmented epithelial cell layer. The underlying retina was laid flat and cut into 0.5 mm2 using a single-sided 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.
Retinal growth medium consisted of Dulbecco`s mimimal essential medium supplemented with 2% E7 chick embryo extract, 50 mM gentamycin, insulin-transferrin-selenium mixture (ITS, Sigma, 1/100).
Selected retinal explant cultures were incubated for 1 hour at 37 °C with 100 litre of either, phosphate buffered saline (PBS) for control cultures, 10 litre of lens protein extract or fractions obtained from anion-exchange chromatography of the lens extract.

Quantitation of growth cone collapse

After incubation for 1 hour with the sample to be tested 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. This procedure was repeated to ensure that the growth medium was replaced gradually with fixative and that minimal disturbance to the retinal axons occurred. Fixed cultures were left for 1 hour at room temperature before scoring was undertaken under phase contrast microscopy. The degree of growth cone collapsed was measured by counting both spread and collapsed growth cones and expressing the number collapsed as a percentage of the total.

Purification of Axon Growth Inhibitory Proteins

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. To obtain sufficient lens protein the vitreous humour was extracted from dissected eyes simply by stabbing the eyes with watch makers forceps and pulling the vitreous out. The vitreous humour was collected on dry ice and frozen at -70 °C until required.
100 g of vitreous humour was obtained from embryonic day 7 embryos and was then homogenised, using a Polytron, 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 100,000g for 1 hour at 4 °C.
The supernatant was harvested and sterile-filtered through a 0.2 µm bottle top filter (Sartorius) and concentrated 10-fold in a Vivaspin 20 (100 kDa MWCO, PES; Vivascience, U.K). The concentrated sample was tested for activity and total protein. The remaining vitreous extract (VE) was subjected to anion exchange chromatography.

Anion exchange chromatography

The remaining vitreous extract was split into two 95 ml samples. One sample was used for anion-exchange with conventional DEAE-agarose and the other was used with Vivapure D-type centrifugal anion-exchange membrane devices.

Conventional anion exchange chromatography using DEAE-agarose beads packed into a column

DEAE-agarose (90 ml) was packed into a column, washed with 10 ml of 1 M NaOH and equilibrated with de-ionised water and 20 mM phosphate loading buffer. Once equilibrated, 95 ml of VE was pumped on to the column at 5 ml/minute. The column was washed with 2 column volumes (CVs) of loading buffer (200 ml 20 mM phosphate buffer pH 6.5) and eluted with 2 CV, 0.2 M, 2 CVs, 0.5 M and 2 CV, 1M NaCl in 50 mM phosphate buffer at pH 8.0.

Purification of Axon Growth Inhibitory Proteins

Following one complete cycle of loading and elution the column was washed with NaOH and re-equilibrated for another cycle using the wash or breakthrough fraction which, after concentrating 5-fold and testing in the growth cone collapse assay, still contained retinal inhibitory activity. The procedure was repeated until the breakthrough fraction was depleted of biological activity.

Anion exchange chromatography using Vivapure D Maxi H spin columns.

In these devices the functional weak anion exchange group; diethylamine (R-CH2-N(C2H5)2) is bound to a membrane which is housed in a 50 ml centrifuge tube.
The equivalent number of Vivapure devices was used which equalled the capacity, in milliequilavents, of competitor's DEAE-agarose column. Four Vivapure D Maxi H spin columns were cleaned by loading with 2 ml NaOH and centrifuging the devices at 1,500 g in a Sorvall high speed centifuge for 2 minutes at 4 °C. The devices were then equilibrated with deionised water and 20 mM phosphate loading buffer. Once equilibrated, VE (12 ml) was added to the devices which were again centifuged as described above.
The membranes were eluted with 5 ml 0.2 M, 0.5 M and 1 M NaCl in 50 mM phosphate buffer pH 8.0. The procedure was repeated several times until all the VE had passed through the membranes and the breakthrough or wash fraction no longer contained any retinal biological activity. The total protein in each active sample was estimated using the BCA protein assay (Pierce and Warriner).

2-dimensional 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 or size separation in the second dimension by sodium dodecyl polyacrylamide electophoresis (SDS-PAGE) on a 10 % gel with a 4 % stacking gel. After electrophoresis, proteins on the SDS-PAGE gel were visualised using a protein silver stain.

Purification of Axon Growth Inhibitory Proteins

RESULTS

Cost and equipment required.

There were large differences in the equipment required and therefore costs associated with performing ion-exchange chromatography using conventional column-based methods when compared to the method of employing centrifugal membrane-bound ion exchangers (Table 1).

	Equipment required:
Conventinal DEAE agarose resin	Column, agarose or sepharose beads, pump, fraction collector, chart recorder, U.V. monitor, cold room or cold cabinet
Vivascience D-type centrifugal membrane	Membrane devices and centrifuge with appropriate rotor

Table 1: Comparison of equipment required to perform ion-exchange chromatography using conventional column based methods with innovative centrifugal membrane devices.

Purification of Axon Growth Inhibitory Proteins

Time taken to perform the experiment.

There were considerable differences in the amount of time required to perform the separation between the two methods, ranging from under an 1 hour when using centrifugal membrane anion exchangers to days when using conventional column-based methods (Table 2).

	Cleaning	Equilibration	Loading	Washing	Elution
DEAE Agarose resin	15 min	36 hrs	30 min	1 hr	3 hrs
Vivapure D spin column	2 min	4 min	2 min	4 min	6 min

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.

Protein and biological activity elution profile.

Both methods of anion exchange chromatography yielded active fractions containing equivalent amounts of biological activity. The protein content was also similar. Retinal biological activity was recovered in the 1 M and 0.5 M NaCl eluates (Table 3).

	Fraction (NaCl eluate)	% Collapse activity	Total protein (mg/ml)
DEAE Agarose resin	1 M	56	1.12
	0.5 M	39	2.08
	0.2 M	1	nd
Vivapure D spin column	1 M	58	0.86
	0.5 M	42	3.26
	0.2 M	1	nd

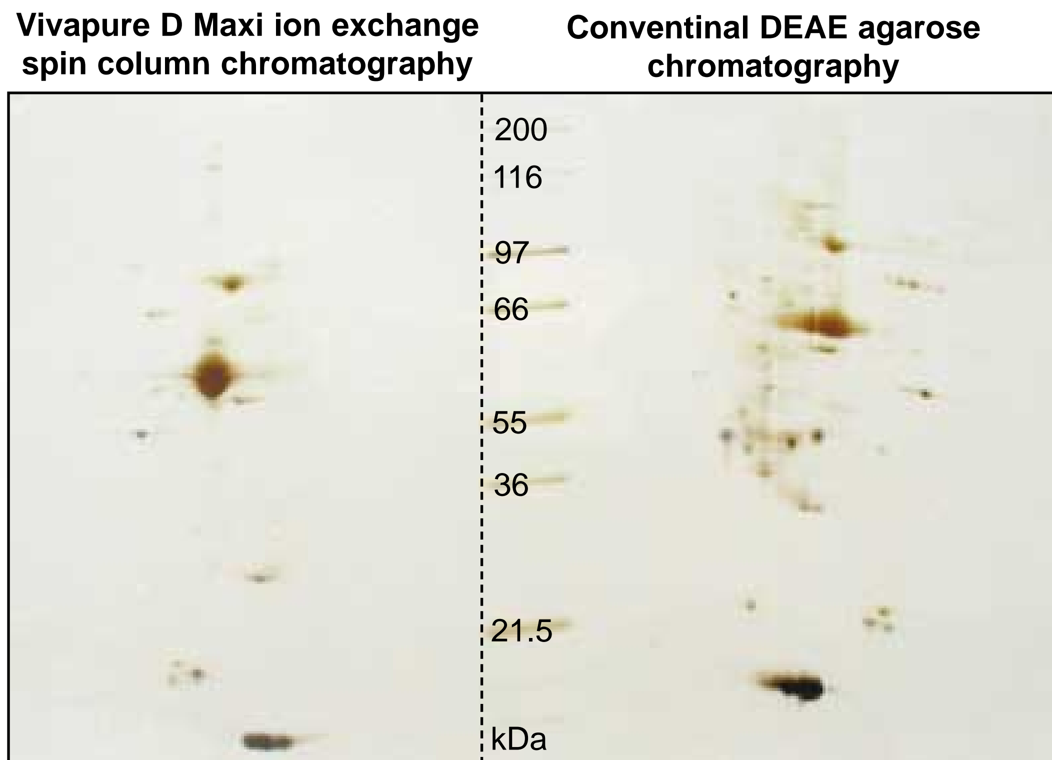
nd = not determined

Table 3: Protein and biological activity profile of anion exchange chromatography of a chick embryo lens inhibitory protein.

Purification of Axon Growth Inhibitory Proteins

In both cases, the 1 M NaCl eluate contained the least protein with the highest activity and the protein composition of these samples were established by subjecting them to 2-dimensional electrophoresis. The IEF strip gels for each sample were run side by side on the second dimension SDS-PAGE gel for direction comparison (Figure 1).

Figure 1: 2-dimensional gel electrophoresis of 1 M NaCl eluates of DEAE agarose (right) and Vivapure D-type anion exchange membrane (left). Bands visible between the two samples are Novex molecular weight markers.



The protein composition of the 1 M NaCl eluates from the DEAE agarose column and the Vivapure D Maxi H spin column are very similar but the latter sample contains much less contaminating, inactive protein indicating a much better separation is obtained using this method of anion exchange chromatography for protein purification.

Purification of Axon Growth Inhibitory Proteins

Discussion

There is a considerable research effort aimed at the elucidation of the molecular basis of axon guidance in the vertebrate nerve system, including the visual system. There are increasing attempts to purify as yet unknown factors which form the foundation of this phenomenon. There is, therefore, the concommitent need for the purification and molecular characterisation of these novel functionally important proteins. Protein purification is often a time consuming and expensive approach to this problem but can yield invaluable results in the identification of key players in developmental and physiological processes. Any method that can reduce both the cost and expense of these experiments will be of immense value. Clearly, the results presented here demonstrate that this new 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. However, the most interesting finding is the greatly reduced time, cost and equipment required to perform an ion exchange separation. This will save the researcher days, if not weeks of valuable time which can be used to perform other important experiments. Furthermore, the simplicity involved in the performance of experiments using just the membrane devices and only requiring access to a centrifuge enables the majority of researchers to perform necessary pilot experiments without committing large capital investment to projects in the form of pumps, fraction collectors, ultraviolet monitors and chart recorders. The application of these devices is not restricted to protein purification but will also allow the rapid and inexpensive detoxification of wide ranges of samples, offering a cheap and efficient alternative to existing ion exchange technology.