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Application Note

Date: Circa 2001

Keywords or phrases:
Membrane proteins, axon guidance, growth cone collapse assay, ion exchange purification, membrane adsorbers, Vivapure®, ultrafiltration, concentration, Vivaspin®

Efficient Purification of Adult Rat Brain and Chick Embryo Membrane Proteins Using Centrifugal Vivapure® D and Vivaspin® Devices

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Abstract

In this study, the effectiveness of a novel ion exchange method was evaluated for the purification of two membrane proteins that inhibit nerve growth in cell culture. This technique utilized Vivapure® centrifugal devices, which are fitted with membrane adsorbers incorporating various functional groups, instead of the agarose or sepharose beads typical of conventional column chromatography. Units featuring a weak anion exchanger - DEAE - were selected for rapid purification of each protein without the need for an expensive FPLC system. Following anion exchange, proteins were rapidly re-buffered and concentrated using Vivaspin® ultrafiltration devices. Finally, log dose response curves of protein activity and the degree of purification were determined for each target molecule, demonstrating the high yields and purities attainable when using Vivapure® D and Vivaspin® devices.

Introduction

It is well known that the central nervous system (CNS) of mammals, including humans, does not regenerate following injury. CNS axons fail to regrow and connect to their specific targets and it is this failure of axon regeneration that leads to the traumatic consequences of spinal cord lesions, including permanent paralysis and loss of function. Therefore, considerable efforts have been made to understand how axons are guided to their appropriate target cells during development. Various studies have already demonstrated axon guidance mechanisms effected by attractive and inhibitory molecules. Repulsion of axon growth can be via long-range diffusible or contact-mediated membrane proteins.

Growing axons produce hand-like structures at their tips, known as growth cones. The addition of inhibitory proteins to cultures of dorsal root ganglion (DRG) or retinal neurons induces a dramatic change in the morphology of growth cones from a spread structure to a collapsed stump. Thus, growth cone collapse assays can be used to detect the presence of these proteins and quantify the amount of inhibition present in a sample.

The hydrophobic properties of membrane proteins makes them insoluble in aqueous solvents. This tendency to aggregate poses a problem in research laboratories working to understand the structure and function of membrane proteins. To overcome this issue, the protein of interest must be purified in the presence of detergents and using methods which can tolerate these reagents, such as ion exchange chromatography. However, conventional column-based chromatography is expensive and time-consuming, with high capital equipment and consumable requirements. Furthermore, the ionic strength of chromatography eluates must be reduced and detergents removed from the purified protein prior to downstream analysis. This is typically performed as a time-consuming, two-step process with high buffer requirements. First, the sample is re-buffered to remove salts by buffer exchange or dialysis, and then detergent is removed during the incorporation into liposomes by dialysis.

In this study, we used Vivapure® D ion exchange devices in place of conventional resin-based chromatography to rapidly purify two inhibitory membrane proteins - one from adult rat brain and a second from chick embryo. Then, instead of conventional buffer exchange techniques, salts were removed from the purified proteins by diafiltration using Vivaspin® 20 ultrafiltration devices. To evaluate the effectiveness of this purification procedure, the activity, protein content and purity of each target protein was measured by growth cone collapse assay and 2D-PAGE. With Vivapure® D and Vivaspin® 20, rapid purification, re-buffering and concentration were possible, yielding high quantities of active membrane proteins.

Vivapure® Maxi ion exchange and Vivaspin® 20 ultrafiltration devices

Materials and Methods

Preparation of Membrane Protein Supernatants

For adult rat brain protein, 50 g of adult rat brain was homogenized in PBS containing a cocktail of protease inhibitors (leupeptin, PMSF and Pepstatin A). The homogenate was centrifuged at 100,000 *g* for 1 hour at 4°C. This process was repeated until all soluble proteins had been removed (via the supernatants). The pellet was then weighed and homogenized 1:2.5 (w:v) in 2% CHAPS, 25 mM phosphate buffer (pH 6.65) and centrifuged as above. A 20 mL sample of the supernatant was retained for purification by anion exchange.

For chick embryo protein, 200 g of embryonic day 7 chick embryos were eviscerated and homogenized in 2% CHAPS and PBS, containing a cocktail of protease inhibitors (leupeptin, PMSF and Pepstatin A). The homogenate was centrifuged at 100,000 *g* for 1 hour at 4°C and 40 mL of the supernatant retained for anion exchange purification.

Anion Exchange Chromatography

For adult rat brain protein, the 20 mL supernatant sample was loaded onto 2x Vivapure® D Maxi H devices (10 mL per spin column) by centrifugation at 150 *g* for 5 minutes at 4°C. The membranes were washed with 10 mL of 0.2% CHAPS, 25 mM phosphate buffer (pH 6.65) by centrifugation, as described above. Proteins were eluted step-wise with 0.2 and 0.5 M NaCl in 0.2% CHAPS, 50 mM phosphate buffer (pH 8). The 0.5 M NaCl eluates were pooled and retained for further processing and analysis.

For chick embryo protein, 4x Vivapure® D Maxi H spin columns were equilibrated with 5 mL of 0.2% CHAPS, 20 mM phosphate buffer (pH 6.65, wash buffer) and centrifuged at 500 *g* for 2 minutes at 4°C. To each device, 10 mL of the homogenate supernatant sample was added. The devices were centrifuged again, as described above, and washed with 10 mL of wash buffer per device. Proteins were eluted step-wise with 0.5 and 1 M NaCl in 0.2% CHAPS, 50 mM phosphate buffer (pH 8). The 1 M NaCl eluates were pooled and retained for further processing and analysis.

Diafiltration and Concentration

Un-purified rat brain and chick embryo supernatants were concentrated using Vivaspin® 20 devices (100 kDa MWCO PES). Anion exchange eluates were re-buffered into 2% CHAPS, 25 mM phosphate buffer by diafiltration and subsequently concentrated, using Vivaspin® 20 devices (100 kDa MWCO PES). The protein content of all supernatant and eluate samples was determined by BCA assay (Thermo Fisher Scientific).

Protein Incorporation into Liposomes

To remove detergent and mimic their natural environment, membrane proteins in the purified and concentrated ion exchange eluates were incorporated into liposomes. Concentrated eluates were mixed with lipids (phosphatidylcholine and phospatidylserine) and dialyzed overnight against PBS prior to use in the growth cone collapse assay.

Dorsal Root Ganglion (DRG) Cell Culture

Glass cover slips were pre-treated with 40 µL of 0.01% poly-L-lysine in PBS and 50 µg/mL laminin in DMEM, placed into 24 well cell culture plates, and covered with 1 mL growth medium comprising DMEM supplemented with 20 ng/mL nerve growth factor (NGF), 50 ng/mL gentamycin and 1% insulin, transferrin, selenium (ITS) mixture. Single DRGs, dissected from embryonic day 7 chick embryos, were placed into each well of the plates and incubated for 24 hours at 37°C in 7.5% CO2.

Growth Cone Collapse Assay

Membrane protein supernatant or anion exchange purified liposome-incorporated protein samples were added to each DRG cell culture and incubated for 1 hour at 37°C. To establish log dose response curves, 100 µL aliquots of each sample at 1:0, 3:1, 1:1 and 1:3 dilutions were tested. The cultures were fixed using 4% paraformaldehyde in PBS (pH 7.4) and scored by counting the number of spread and collapsed growth cones. Inhibitory activity was expressed as the number of collapsed growth cones as the percentage of the total.

Protein concentrations were plotted against collapse activity to determine the amount of protein necessary to induce 50% growth cone collapse following addition to growing neurons. These values were expressed as 1 collapse unit and used to establish the specific activity and degree of purification. Purity of the anion exchange eluates was also assessed by 2D-PAGE.

Results

For both proteins, the supernatant samples and pure eluates from Vivapure® D anion exchange spin columns showed potent growth cone collapse activity. Table 1 compares the total amount of collapse activity in 100 µL of each sample, the specific activity, and the level of purity obtained by anion exchange. The high level of purity for each protein was also evident from analysis of the Vivapure® D eluates by 2D-PAGE (Figure 1).

Conclusions

Ion exchange chromatography is widely used in many protein purification protocols, including those applied to membrane proteins. The conventional approach for this technique is to use Sepharose-bound functional groups packed into columns. However, this is slow, complicated, and labor-intensive. In this study, the efficiency of novel centrifugal devices fitted with membrane adsorbers has been demonstrated for the purification of two membrane proteins. Vivapure® spin columns possess the advantages of being easy to use while enabling ion exchange purifications to be performed rapidly. Despite their small size, the membrane adsorbers used in these devices also feature high ligand densities of 145 – 218 µEq/mL, ensuring that research scientists can benefit from high yields of active protein.

In addition, we have described the use of Vivaspin® ultrafiltration devices for salt removal from high ionic strength chromatography eluates. Compared to conventional techniques such as desalting or dialysis, which can take several hours or even days to complete, diafiltration with Vivaspin® enables efficient de-salination or re-buffering of samples in only a matter of minutes.

When combined into a purification workflow, both Vivapure® and Vivaspin® can enable rapid isolation, diafiltration and concentration of many target proteins, accelerating research progress.

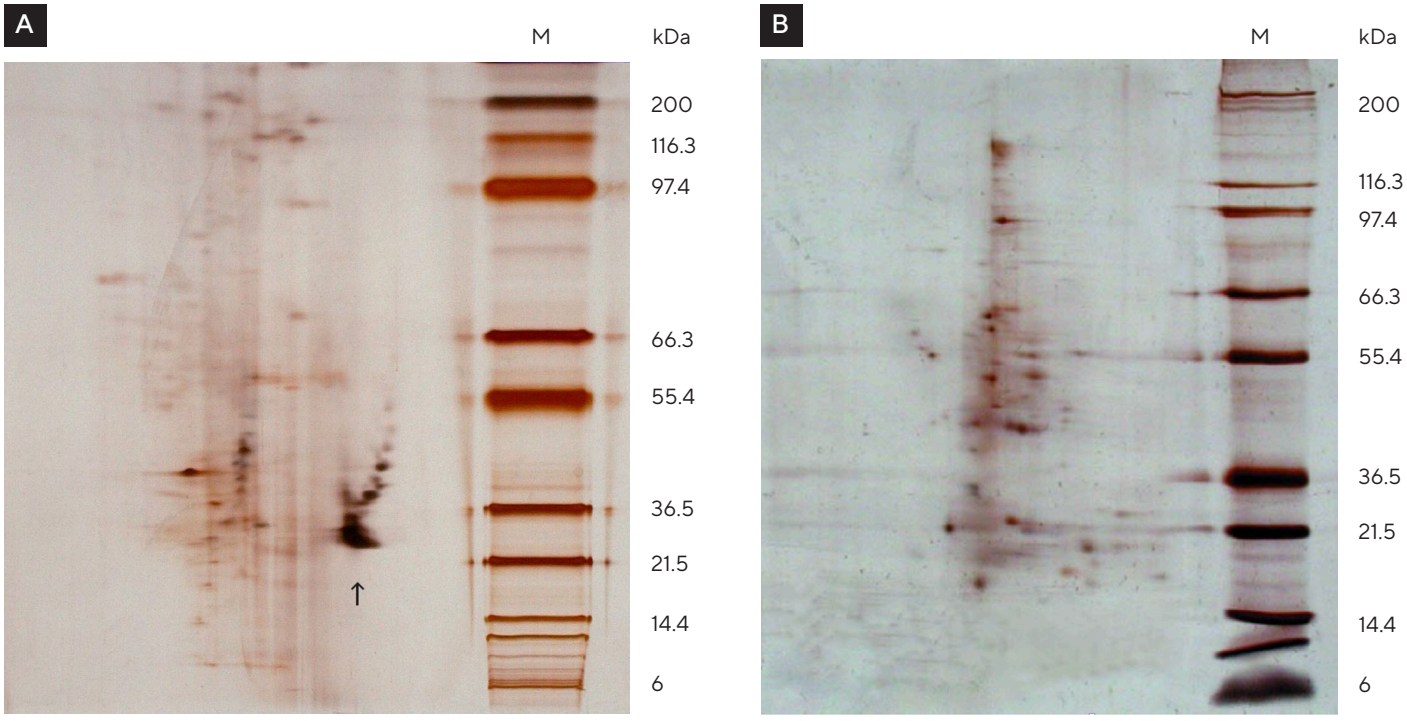
Table 1.

Activity and purity of two solubilized membrane proteins, compared to samples purified by anion exchange using Vivapure® D spin columns.

Sample	Collapse activity	Protein per Collapse Unit	Specific activity (collapse units)	Increase in purity
Adult rat brain protein				
- Supernatant	95%	358 µg	3.1/mg	na
- 0.5 M NaCl eluate	89%	9.8 µg	101/mg	39x
Chick embryo protein				
- Supernatant	96%	390 µg	2.5/mg	na
- 1 M NaCl eluate	78%	11.9 µg	84/mg	33.6x

Figure 1.

2D-PAGE of two membrane proteins purified using Vivapure® D Maxi H devices. A: adult rat brain protein (see arrow); and B: chick embryo membrane protein. M = molecular weight marker. Silver stain.



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Status: 11 | 2021