VIVASCIENCE

Detergent removal from membrane proteins - Rapid removal of the detergent, n-octyl b-D-glucopyranoside from a membrane protein mimic using an innovative centrifugal Vivapure anion exchange membrane technology.

Dr. Mike D. Brownleader[1] and Dr. Alan R. Johnson[2].

1. Vivascience, Sartorius Group, Oldends Lane, Stonehouse, Gloucester GL10 3RQ, UK.

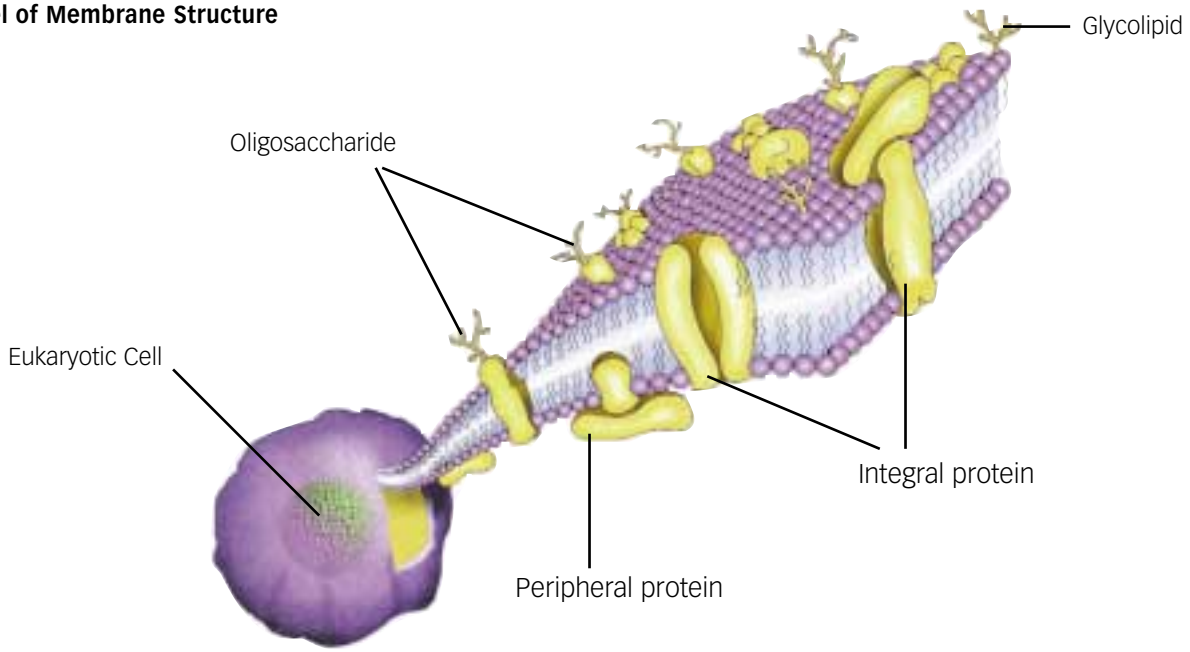
2. Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK.

Background

Ion exchange chromatography is routinely used to purify integral membrane proteins. Membrane proteins are classified as either peripheral (extrinsic) or integral (intrinsic) depending on how tightly they are associated with the membrane (Fig.1).

Peripheral membrane proteins are only loosely associated to the surface of the membrane and can normally be released by mild salt treatment. Integral membrane proteins, on the other hand, are extracted from the membrane using organic solvents such as chloroform or detergents such as n-octyl β-D-glucopyranoside, CHAPS, Triton X-100 or Triton X-114 which mimic the lipid of the natural environment. Towards the final stages of purification, when the protein concentration is less than 1 mg/ml, 0.1 % detergent is often more than adequate to retain solubility and bioactivity of purified membrane proteins (Scopes, 1994). If subsequent removal or reduction of detergent is required, for example in reconstitution or spectrophotometric experiments, detergents with high critical micelle concentrations tend to be removed by dialysis which can take several days. In some cases, researchers need to change the chemical composition of the detergent.

Fig. 1. Schematic Diagram showing the Fluid Mosaic Model of Membrane Structure



Detergent removal from membrane proteins - Rapid removal of the detergent, n-octyl b-D-glucopyranoside from a membrane protein mimic using an innovative centrifugal Vivapure anion exchange membrane technology.

Dr. Mike D. Brownleader[1] and Dr. Alan R. Johnson[2].

1. Vivascience, Sartorius Group, Oldends Lane, Stonehouse, Gloucester GL10 3RQ, UK.

2. Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK.

For example, detergents containing amin groups may interfere with protein detection based upon colorimetric assays.
We report a rapid and simple method using Vivapure anion exchange membrane centrifugal devices to remove detergent from a standard protein sample (Fig. 2).
N-octyl β-D-glucopyranoside, like many other detergents, is non-ionic and will not bind to an ion-exchange matrix. Since membrane proteins normally represent a very small proportion of total cellular protein, any level of contaminating detergent is going to have an inflated effect upon all down-stream analyses.

Methods

Removal of detergent

The centrifugal (0.5 ml) Vivapure Q devices were loaded with 0.5 ml 15 mM Tris/HCl pH 8.0 to equilibrate the quarternary ammonium-derivatized membrane. The devices were spun for 3 min at 500 g.
The inserts were then filled with 0.25 ml 2 mg/ml BSA (0.5 mg protein) containing 0.2 %, 0.5 %, 1.0 % or 2.0 % n-octyl β-D-glucopyranoside and the corresponding solutions were centrifuged at 500 g for 3 min.
The membranes were washed twice with 0.5 ml 15 mM Tris/HCl buffer pH 8.0. BSA was then eluted with 0.25 ml 0.5 M NaCl made up in 15 mM Tris/HCl pH 8.0 by centrifuging the devices at 500 g for 10 min.

Protein Estimation

Recoveries of proteins were assayed by protein concentration estimations using the BCA Protein Assay kit from Pierce and Warriner, Chester, UK. This kit is known to be compatible with up to 5 % n-octyl β-D-glucopyranoside. BSA (fraction V, essentially fatty acid free) was used as the protein standard.
All values represent the mean of triplicate data points.

Fig. 2. Centrifugal Ion Exchange Membrane Devices with various protein binding capacities.

Detergent removal from membrane proteins - Rapid removal of the detergent, n-octyl b-D-glucopyranoside from a membrane protein mimic using an innovative centrifugal Vivapure anion exchange membrane technology.

Dr. Mike D. Brownleader[1] and Dr. Alan R. Johnson[2].

1. Vivascience, Sartorius Group, Oldends Lane, Stonehouse, Gloucester GL10 3RQ, UK.

2. Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK.

Neuronal cultures

Ganglia were removed from chick embryo at day 7. Chick dorsal root ganglion (DRG) cultures were maintained on a permissive laminin-based substratum (EHS-laminin; Sigma, Poole, UK) made up in Dulbecco's minimal essential medium and allowed to grow on a cover-slip for up to 24 hours before addition of test material (Johnson et al, 1994).

Growth Cone Collapse Assay

The test material (0.1 ml) was incubated with the neuronal culture for 1 hour at 37˚C. The cultures were then fixed with 1 ml of 4 % (w/v) formaldehyde in PBS and 15 % (w/v) sucrose at 25˚C. One-half of the culture medium was removed and replaced with the same volume of fixative (0.5 ml) and the procedure was repeated until all the medium had been exchanged. Cultures were left for a minimum of 1 hour at room temperature before being assessed by phase-contrast microscopy (Johnson et al, 1994). The degree of growth cone collapse was measured by counting both spread and collapsed growth cones and expressing the number collapsed as a percentage of the total.

Results

Most membrane proteins will be purified in the presence of detergent. It may, however, be necessary to remove excess detergent prior to analyses of the protein. Frequent problems in polyacrylamide gel electrophoresis caused by detergents are poor resolution/mobility, aggregation and smearing of proteins (Findlay, 1995). In many cases, removal of the detergent will inactivate the protein but this is not of paramount importance if the ultimate analytical procedure is for example, amino acid sequencing by ESI-MS/MS.

Johnson et al (1994) homogenized chick embryo brain tissue using 2 % (w/v) (3-[(3-cholamidopropyl)-dimethy-lammonio]-1-propanesulfonate) (CHAPS) in the presence of a protease inhibitor cocktail. All subsequent biological procedures were performed at lower detergent concentrations such as 0.2 % (w/v) CHAPS.

It is acknowledged that usually if the ion exchange buffers do not contain any detergent, there is a high probability that once the washing process commences, the proteins will denature or otherwise become insoluble in the ion exchange column and therefore not be recovered. Integral membrane proteins are, by definition, of low solubility in water, and in the absence of detergents will precipitate from solution. Therefore, a minimal amount of detergent, if possible less than the critical micelle concentration, should be retained in all buffers being used. In this report, we identified model experimental conditions that would remove all residual traces of detergents that could hinder any subsequent structural characterization of the target protein. Performance of the centrifugal Vivapure ion exchange membrane devices was measured by three criteria: their speed, quantitative protein recovery and ability to remove all traces of detergents.

Speed of Sampling

Ion exchange chromatography is often a time-consuming method requiring extensive periods of equilibration, washing and elution of the ion exchange resin and relatively specialized laboratory equipment such as a peristaltic pump and a fraction collector. Most purification regimes demand temperatures of 4˚C to minimize proteolytic degradation of the target protein, particularly as protease activation is often associated with membrane disruption. When working with a membrane bound enzyme, it is always desirable to complete the purification of the protein in a short period of time, as the activity of the solubilized

Detergent removal from membrane proteins - Rapid removal of the detergent, n-octyl b-D-glucopyranoside from a membrane protein mimic using an innovative centrifugal Vivapure anion exchange membrane technology.

Dr. Mike D. Brownleader[1] and Dr. Alan R. Johnson[2].

1. Vivascience, Sartorius Group, Oldends Lane, Stonehouse, Gloucester GL10 3RQ, UK.

2. Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK.

Protein Recovery

The purified protein has to be pulled away from the detergent so that it can be adsorbed on to the anion exchange matrix. Excellent protein recovery at all chosen detergent concentrations was observed (Table 1).

Additionally, the multi-layer centrifugal ion exchange membrane device has an effective adsorption area of 6.8 cm^2 which binds/recovers 0.5 mg BSA and therefore exhibits extremely low non-specific adsorption.

Table 1. Data showing that protein recovery is independent of detergent concentration

Detergent conc. (% w/v)	A562 Test solution	A562 Sample eluate	% recovery
0	0.468	0.460	101.7
0.2	0.419	0.425	98.6
0.5	0.440	0.450	97.8
1.0	0.420	0.435	96.6
2.0	0.467	0.455	102.6

Detergent removal from membrane proteins - Rapid removal of the detergent, n-octyl b-D-glucopyranoside from a membrane protein mimic using an innovative centrifugal Vivapure anion exchange membrane technology.

Dr. Mike D. Brownleader[1] and Dr. Alan R. Johnson[2].

1. Vivascience, Sartorius Group, Oldends Lane, Stonehouse, Gloucester GL10 3RQ, UK.

2. Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK.

Removal of detergent

Quantitative removal of detergent was assessed using an in vitro nerve growth cone assay which can detect picomoles of extraneous material. Explanted embryonic 7 day dorsal root ganglia have extended long neurites on laminin-coated glass. Under normal culture conditions, the tips of nearly all neurites have well-defined growth cones. The DRG growth cone morphology is very sensitive to exposure to extremely low levels of detergent (<< 0.01 % n-octyl β-D-glucopyranoside). High levels of 2 % n-octyl β-D-glucopyranoside will trigger complete collapse of neurite outgrowth extending from neural explants in tissue culture as shown in Fig. 3A. A growth-inhibitory protein derived from the posterior half sclerotome by Keynes et al (1990) induced the collapse of DRG growth cones (Fig. 3B).

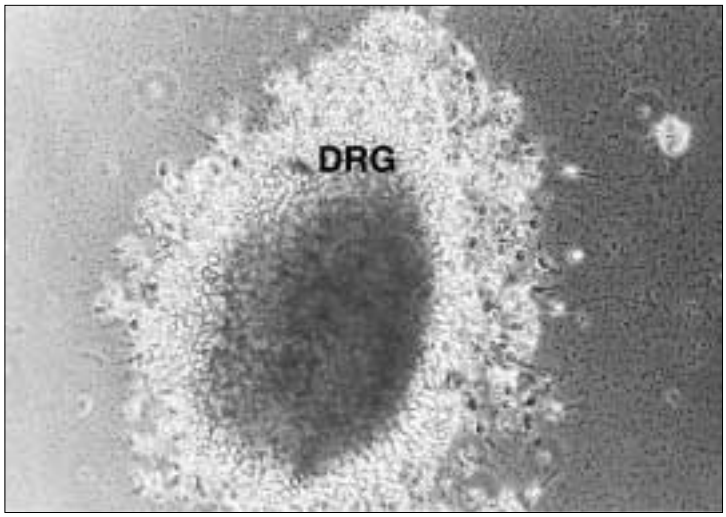


Fig. 3A. DRG Culture following addition of 2 % n-octyl β-D-glucopyranoside (DRG; dorsal root ganglion)

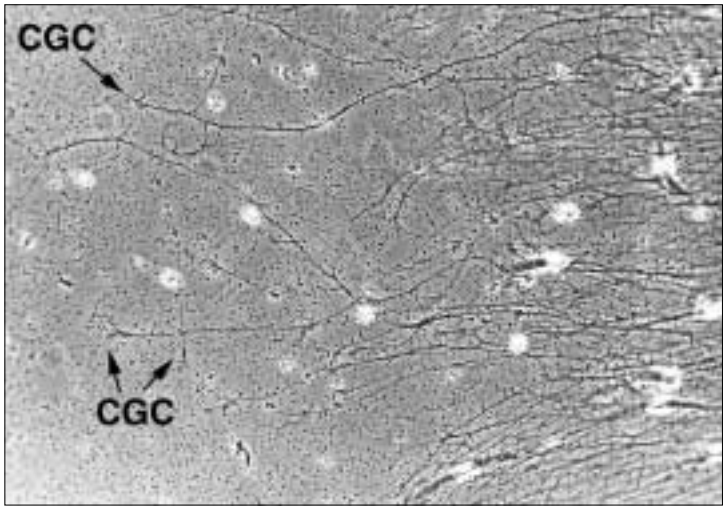


Fig. 3B. DRG Growth Cone Morphology following addition of an Inhibitory Protein from Embryonic Somites (CGC; collapsed growth cone)

Detergent removal from membrane proteins - Rapid removal of the detergent, n-octyl b-D-glucopyranoside from a membrane protein mimic using an innovative centrifugal Vivapure anion exchange membrane technology.

Dr. Mike D. Brownleader[1] and Dr. Alan R. Johnson[2].

1. Vivascience, Sartorius Group, Oldends Lane, Stonehouse, Gloucester GL10 3RQ, UK.

2. Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK.

Addition of the NaCl eluate from the ion exchange membrane devices had no effect upon normal growth cone development (Fig. 4A & B). Quantitative scoring of morphology of approximately 100-120 growth cones showed no evidence of collapsing activity indicating that there was no residual trace of n-octyl β-D-glucopyranoside in the protein sample.

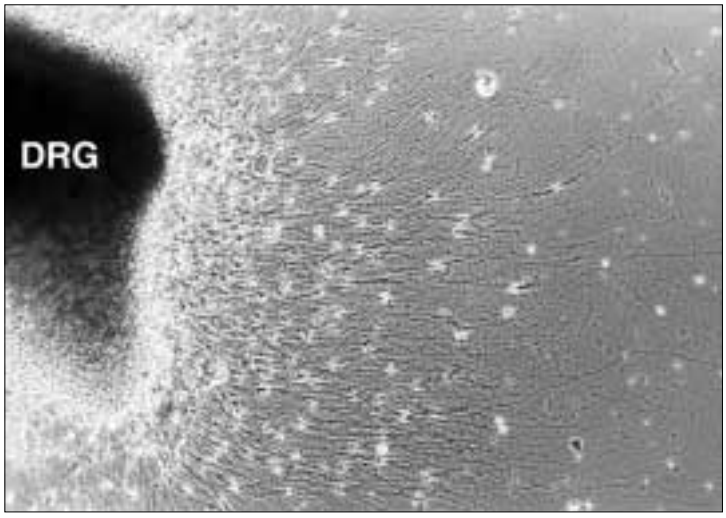


Fig. 4A. DRG Growth Cone following addition of the NaCl Eluate from the Centrifugal Vivapure Q Ion Exchange Membrane.

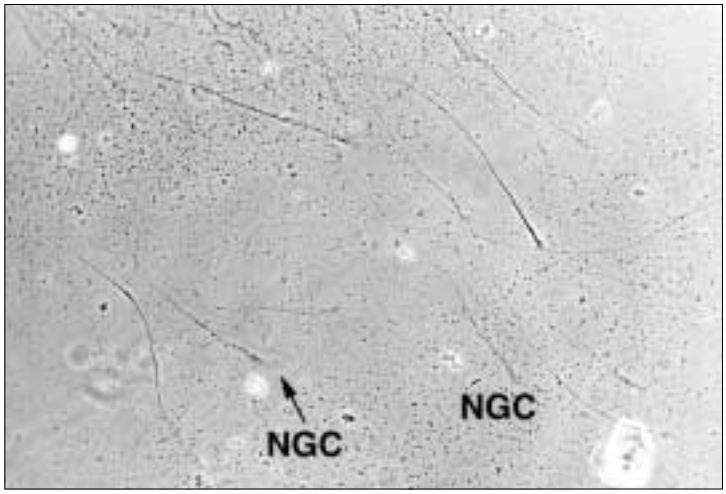


Fig. 4B. DRG Outgrowth following addition of NaCl Eluate from the Centrifugal Vivapure Q Ion Exchange Membrane Device (magnified image from Fig. 4a).

Detergent removal from membrane proteins - Rapid removal of the detergent, n-octyl b-D-glucopyranoside from a membrane protein mimic using an innovative centrifugal Vivapure anion exchange membrane technology.

Dr. Mike D. Brownleader[1] and Dr. Alan R. Johnson[2].

1. Vivascience, Sartorius Group, Oldends Lane, Stonehouse, Gloucester GL10 3RQ, UK.

2. Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK.

Discussion

A cross-linked regenerated cellulose membrane with large pore size (3-5 µm) and derivatized with functional ionic groups have been incorporated into two multi-layer centrifugal device formats comprising 0.5 ml and 20 ml volume inserts (Table 2). These devices allow rapid flow rates, confer high protein binding capacity approaching 2 mg protein/cm^2 effective adsorption area and possess high chemical and enzymatic stability (Broverman and Prestwich, 1995; Demmer and Nussbaumer, 1999).

There is considerable interest in the phenomenon of growth cone collapse in the inhibition of axonal regeneration in the higher vertebrate central nervous system. Collapse is characterized by a dramatic change in growth cone shape from a spread to a retracted morphology (Raper and Kapfhammer, 1990).

The mechanisms that lead to growth cone collapse are the subject of active investigation. Changes in neurite spreading and growth cone morphology were used in this report to monitor the ability of these Vivapure ion exchange membrane devices to rapidly remove n-octyl β-D-glucopyranoside from the reference protein solution. All the above observations support unambiguously the use of these centrifugal ion exchange membrane devices in the complete and rapid (< 15 min) removal of the detergent, n-octyl β-D-glucopyranoside, from a protein sample. The discovery that their performance does not suffer from interference by the presence of different concentrations of detergent in the sample suggests that these ion exchange membrane devices may have widespread application for all non-ionic detergent-solubilized membrane proteins

Table 2. Ionic groups coupled to modified cellulose membrane functional groups

Sulfonic Acid (S)	$R\text{-}CH_2\text{-}SO_3^-Na^+$	Strong cation exchanger
Quaternary ammonium (Q)	$R\text{-}CH_2\text{-}N^+\text{-}(CH_3)_3\,Cl^-$	Strong anion exchanger
Carboxylic Acid (C)	$R\text{-}COO^-$	Weak cation exchanger
Diethylamine (D)	$R\text{-}CH_2\text{-}N\text{-}(C_2H_5)_2$	Weak anion exchanger

References

Brovermann, S.A. and Prestwich, G.D. (1995) BioTechniques 19, 874-875.

Cox, E.C., Muller, B. and Bonhoeffer, F. (1990) Neuron 5, 733-743.

Demmer, W. and Nussbaumer, D. (1999) J. Chromatography 852, 73-81.

Findlay, J.B.C. (1995) Purification of Membrane Proteins, Ch 4. In: Protein Purificaytion Applications: A Practical Approach. (Harris, E.L.V. and Angal, S.) pp. 179, IRL Press, Oxford.

Johnson, A.R., Cook, G.M.W. and Keynes, R.J. (1994) Neuroprotocols 4, 121-128.

Keynes, R. J., Johnson, A. R., Picart, C. J., Dunin-Borkowski, O. M., Cook, G. M. W. (1990) Society for Neuroscience Abstracts 16, 77.6.

Raper, J.A. and Kapfhammer, J.P. (1990) Neuron 2, 21-29.

Scopes, R.K. (1994) In: Protein Purification: Principles and Practice (3rd Edition) pp. 380, Springer-Verlag, New York.