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Hydrophobic Interaction Chromatography

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Abstract

Most proteins and large polypeptides have hydrophobic regions at their surface. These hydrophobic 'patches' are due to the presence of the side chains of hydrophobic or non-polar amino acids such as phenylalanine, tryptophan, alanine and methionine. These surface hydrophobic regions are interspersed between more hydrophilic or polar regions and the number, size and distribution of them is a specific characteristic of each protein. Hydrophobic Interaction Chromatography (HIC) is a commonly used technique that exploits these hydrophobic features of proteins as a basis for their separation even in complex biological mixtures ***(1) (2)***. In general the conditions under which hydrophobic interaction chromatography is used are relatively mild and 'protein friendly' resulting in good biological recoveries. Hydrophobic binding is relatively strong and is maintained even in the presence of chaotrophic agents, organic solvents and detergents. For these reasons this technique has a widespread use for the purification of proteins and large polypeptides.

Key Words : Non-polar , hydrophobic amino acids, phenyl sepharose, butyl sepharose, octyl sepharose , chaotrophic agents , glycerol gradients , detergents, biological activity.

1. Introduction

In hydrophobic interaction chromatography the separation of proteins primarily occurs by the reversible binding of the proteins hydrophobic surface regions to an interacting non-ionic group such as a hydrocarbon tail (octyl-, butyl- or hexyl-) or an aromatic ring (phenyl-) immobilized to an inert stationary mircoporous matrix such as cross-linked agarose or sepharose (*see* **Fig. 1**.).

Insert figure 1 here

It is vital to the technique that this support matrix does not carry any ionic charge and it is usually rendered inert by alkaline hydrolysis under reduced conditions. This treatment effectively desulphates the cross-linked agarose/sepharose matrix. Next an uncharged 'spacer arm' (usually a tricarbon hydrocarbon chain) is attached to the stable and inert matrix and the hydrophobic groups (e.g. octyl-, butyl- or phenyl- *see* **Fig. 1**) introduced by reaction with the corresponding glycidyl ether. The hydrophobic groups are now attached to the matrix via uncharged, chemically stable ether linkages. This results in a truly 'hydrophobic gel' with no ionic properties. These gels are unaffected by chaotropic agents such as urea and guanidine hydrochloride, most organic solvents such as methanol and isopropanol and detergents such as Triton X-100, sodium dodecyl sulphate (SDS) and sodium deoxycholate. Detergents are routinely used to release and solubilize

membrane-bound hydrophobic proteins but then must be kept in the running buffers of any chromatographic columns running to prevent protein aggregation and precipitation.

The ionic properties of both the protein and the running buffer also have an important effect on hydrophobic interaction and binding. A protein with no overall net charge (i.e. where the pH of the running buffer is the same as the pI value of the protein) will have maximum hydrophobic properties, whereas a charged protein will be potentially repulsed from the hydrophobic gel to a greater or lesser extent depending on the magnitude of its charge. For example, when the pH is increased, most proteins become negatively charged and therefore become more hydrophilic even under mildly alkaline conditions. The adsorption step often requires the presence of salting-out ions such as sodium chloride, potassium chloride or ammonium sulphate. These 'salting-out' ions decrease the availability of water molecules in solution, thus increasing surface tension and enhancing hydrophobic interaction ***(3) (4)***. High salt concentrations may also cause a partial denaturation and unfolding of the proteins with the resultant partially unfolded polypeptide chain(s) often exposing internal hydrophobic regions which will, in turn, enhance hydrophobic interaction and binding. In contrast, low salt concentrations or the use of chaotropic ions will actively reduce or prevent non-ionic or hydrophobic interactions.

Hydrophobic interaction chromatography has been also used to separate nucleic acids. This exploits the more hydrophobic nature of single stranded nucleic acids when compared with double stranded plasmid DNA ***(6) (7)***.

Hydrophobic interaction chromatography is ideal for use after an initial salt precipitation step where the high ionic strength of the sample will greatly enhance hydrophobic adsorption. Also, if the protein of interest is eluted with a positive glycerol and a negative salt gradient, it can then be subjected to ion-exchange chromatography with no need to change either the buffer/pH. In addition, eluting a protein with/into glycerol has been known to stabilize many proteins. Lastly, the diversity of potential eluting conditions from the hydrophobic matrices, allows for the resolution of even the most complex protein mixtures.

2. Materials

2.1 Typical Materials

1. Phenyl-Separose CL-4B (Pharmacia)
2. Octyl-Separose CL-4B (Pharmacia)
3. Loading buffer ; 100mM potassium phosphate buffer pH 7.2 .
4. Eluting buffer ; 100mM potassium phosphate buffer pH 7.2 with 2M ammonium sulphate.
5. Eluting buffer ; 100mM potassium phosphate buffer pH 7.2 with 2M KCl.
6. Eluting buffer ; 100mM potassium phosphate buffer pH 7.2 with 40% Glycerol.
7. Detergent ; 1% (w/v) Triton X-100

3. Methods

3.1 Typical Methodology

All stages are carried out preferably at 4 ^{0}C.

1. Filter, degas and dialyze the protein sample into potassium phosphate buffer (*see* **Notes 1** and **2**).
2. Pour the appropriate hydrophobic gel (*see* **Note 3**) into the column of appropriate dimensions (*see* **Note 4**).
3. Equilibrate the chromatography column with 4 to 5 column volumes of potassium phosphate buffer with either ammonium sulphate or KCl salts (*see* **Note 5**)..
4. Load the protein sample onto the top of the column (*see* **Note 6** and **7**).
5. Wash the column with 3 to 4 column volumes of potassium phosphate buffer with either ammonium sulphate or KCl salts (*see* **Note 8**).
6. Elute the bound protein with a negative gradient of 10 to 20 column volumes of potassium phosphate buffer (with no salt) and a positive gradient of glycerol in potassium phosphate buffer (*see* **Note 9**). Temperature also has an effect elution from hydrophobic gels (*see* **Note 10**).
7. Collect 1 ml fractions with a flow-rate in the range of 0.1- 0.2 mls per minute (*see* **Note 11**). .
8. Monitor the protein at 280 nm in a standard spectrophotometer (*see* **Note 12**).
9. Analyze the fractions for the biological activity of the desired protein or by SDS-PAGE.

10. Regenerate the hydrophobic resin by removing tightly bound hydrophobic protein or detergents by washing with 10 column volumes of potassium phosphate buffer with either KCl or ammonium sulphate. 20-30% ethanol may also be used in this buffer to remove tightly bound detergents.

11. Re-use the Phenyl or Octyl-Sepharose gels efficiently for up to 10-12 separation runs (see **Note 13**).

12. Store the hydrophobic gels in a potassium phosphate buffer with ethanol and sodium azide (anti-microbial agent) at 4 ^{0}C (*see* **Note 14**).

Figure 2 below shows the purification of a prolyl oligopeptidase from the soluble fraction of bovine brain using phenyl sepharose CL=4B ***(8).***

Insert figure 2 here

5. Notes

1. All buffers should be filtered and degassed as this prevents the build-up of gases such as CO_2 in the column if a pumping system is employed to regulate flow-rate.

2. The starting pH should ideally be between pH 6.5 and 7.5.

3. The choice of hydrophobic gel/ligand should be determined to a large extent by the degree of hydrophobicity of the protein to be purified and/or analyzed. Phenyl-Sepharose is often classified as a weak ‘hydrophobic gel’ and should therefore be used to bind strongly hydrophobic proteins such as membrane-bound enzymes and receptors. This is because of the nature of hydrophobic binding, which is a much stronger type of

interaction than ionic binding. Therefore, if a strongly hydrophobic protein was bound to a strongly hydrophobic gel, denaturing conditions would probably have to be employed to effect release/elution. It is of interest to note that pie-pie interactions have been noted to occasionally occur between the aromatic phenyl rings of the gel and the aromatic rings of the side chains of certain amino acids of proteins, and consequently protein binding to Phenyl-Sepharose may not always be purely through hydrophobic interaction. In the case of less hydrophobic proteins not binding/adsorbing to the Phenyl-Sepharose gel, a more hydrophobic matrix , such as Octyl-Sepharose may be used.

Octyl-Sepharose is often classified as a strong ‘hydrophobic gel’ and is routinely used to bind weakly hydrophobic proteins. These proteins may not bind until the ionic strength of the running buffer is increased to just below that required for precipitation (of the protein of interest), and the buffer pH adjusted to near the pI of the protein of interest, all of which will enhance non-ionic or hydrophobic adsorption. The binding capacity of both Phenyl-Sepharose and Octyl-Sepharose is reported by Pharmacia to be around 20 mg of protein per ml of gel. This compares very favourably with the binding capacities of most ion-exchange matrices.

4. The optimal column dimensions should favour larger cross sectional areas i.e. larger radius with smaller column heights. This allows for a better ‘concentration’ of the protein sample during the binding phase.

5. Ammonium sulphate salt is a stronger salt than KCl and may be used for less hydrophobic proteins as it will cause a greater degree of protein unfolding thus promoting more hydrophobic binding.

6. Protein concentration should be ideally in the 1 to 5 mg/ml concentration range so as not to exceed the binding capacity of the hydrophobic gel.

7. Sample volumes may be larger than the column volume as this is a binding chromatography and thus effectively 'concentrate's the sample.

8. This wash should remove any unbound or loosely bound protein. A low concentration of detergent (0.1% w/v Triton X-100) may be used in this wash if using a membrane-released sample which will have a complex mix of proteins

9. Negative salt gradients are routinely used to elute proteins from hydrophobic interaction columns. These conditions tend to reduce overall hydrophobic conditions and thus favour elution of the bound protein. Gradients of chaotropic ions , such as urea and guanidine HCl, are rarely used to elute proteins from hydrophobic gels as they often denature the protein with a resultant loss of biological activity. Positive gradients of polarity reducing agents such as glycerol or the water miscible solvents such as methanol or ethanol are routinely used to elute bound proteins from hydrophobic gels. A combination of a positive gradient of a polarity-reducing substance such as glycerol with a negative gradient of a 'strongly ionic' salt such as ammonium sulphate is one of the commonly used methods for the elution of bound proteins during hydrophobic interaction chromatography. However, the water-miscible solvents are rarely used for elution, as they may cause denaturation of proteins with subsequent loss of biological activity. Detergents (especially the milder non-ionic detergents such as Triton X-100), while not affecting the hydrophobic ligand/gel itself, do reduce the degree of hydrophobic binding and may therefore also be used for the elution of bound material. The concentrations of these polarity reducing agents typically used are ; glycerol (0-40%) ,methanol (0-30%),

ethanol (0-30%) and ethylene glycol (0-80%). All are routinely used to elute bound proteins from hydrophobic gels.

10. Temperature also has an effect on hydrophobic interaction. Lower temperatures reduce hydrophobic binding between two non-ionic groups. This is because the free energy of the interaction becomes more positive with decreasing temperature ***(5)*** thus making the interaction less likely to occur spontaneously. However, this reduction in the strength of hydrophobic interaction at lower temperatures is not routinely used as a means for effecting elution of bound material. There is up to a 20-30% reduction in the strength of hydrophobic binding when the temperature is reduced from 25 ^{0}C to 4 ^{0}C. The strength of hydrophobic interaction increases with temperature up to about 60 ^{0}C at which point the additional stability provided by electrostatic and Van der Waal's forces disappears. Above this temperature the strength of hydrophobic binding falls off rapidly.

11. The flow-rate has to be slow enough to allow for hydrophobic binding to occur efficiently as it is a thermodynamically ‘slow’ reaction.

12. If glycerol is used in the elution buffer then other analytical methods for monitoring protein must be employed as glycerol interfers directly with absorbance at 280nm.

13. After a certain number of separation and regeneration runs the binding capacity of the gels becomes reduced especially if detergents are used in the chromatography.

14. Most of the hydrophobic matrices are completely reusable, and may be regenerated by firstly removing any tightly bound protein with 6 M urea and then subjecting them to repeated washing with starting buffer (with salt). Other cleaning methods involve washing with strong bases such as 1M sodium hydroxide. Detergents

may be removed by sequential ethanol, butan-1-ol, ethanol, and distilled water washes. For long-term storage, the hydrophobic gels may be suspended in 20% (v/v) ethanol and 0.05% sodium azide and stored at 4 ^{0}C.

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References

1. Queiroz J.A. , Tomaza C.T. and Cabralb J.M.S. (2001) Hydrophobic interaction chromatography of proteins *Journal of Biotechnology* **87,** 143-159.
2. Eisenberg, D. and McLachlan, A.D., (1986) Solvation energy in protein folding and binding. *Nature* **319**, 199–203.
3. Porath, J. (1986) Salt-promoted adsorption: recent developments. *J. Chromatogr.* **376**, 331–341.
4. Porath, J.(1990) Salt-promoted adsorption chromatography. *J. Chromatogr.* **510**, 47–48.
5. Ochoa, J.L. (1978) Hydrophobic Interaction Chromatography. *Biochimie* **60**, 1–15.
6. Diogo, M.M., Queiroz, J.A., Monteiro, G.A. and Prazeres, D.M.F. (1999) Separation and analysis of plasmid denatured forms using hydrophobic interaction chromatography. *Anal. Biochem.* **275**, 122–124.

7. Diogo, M.M., Queiroz, J.A., Monteiro, G.A., Martins, S.A.M., Ferreira, G.N.M. and Prazeres, D.M.F. (2000) Purification of a cystic fibrosis plasmid vector for gene therapy using hydrophobic interaction chromatography. *Biotechnol. Bioeng.* **68**, 576–583.
8. Dowling, O. (1998) The purification and characterization of a proyly oligopeptidase from the cytosolic fraction of bovine brain. *Ph.D. Thesis* Dublin City University, Ireland.

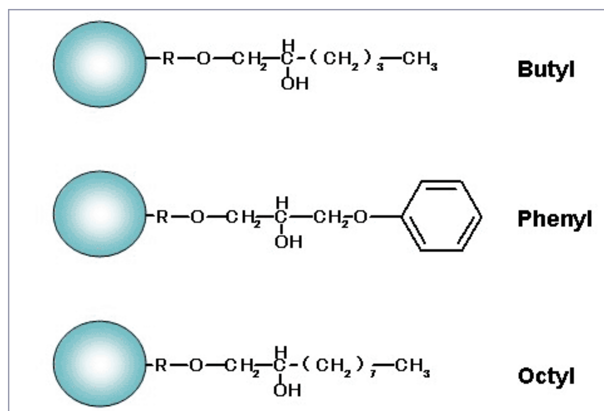

Fig. 1. Diagrammatic representation of Butyl- , Phenyl- and Octyl- chemical groups covalently linked to sepharose (o) matrix

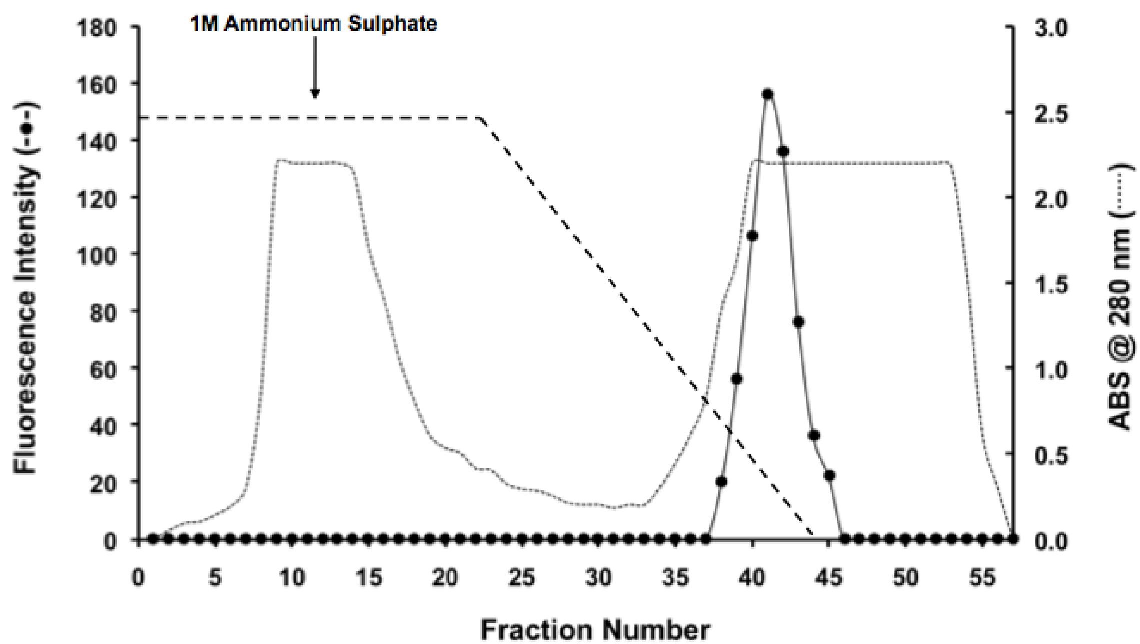

Fig. 2. Purification of a proyl oligopeptidase from bovine brain using phenyl sepharose ***(8).***