Butyl-S Sepharose 6 Fast Flow

Butyl-S Sepharose™ 6 Fast Flow (Fig 1) belongs to the family of products referred to as hydrophobic interaction chromatography (HIC) media. GE Healthcare produces a wide range of HIC media that is well characterized for group separation or purification of a variety of biological macromolecules in laboratory- or process-scale operations. Butyl-S Sepharose 6 Fast Flow is the least hydrophobic medium in the series. Butyl-S Sepharose 6 Fast Flow is particularly useful during the initial stages of a separation process to remove the bulk of impurities without stringent requirements for conditioning of the sample (e.g. adjustment of pH, salt concentration etc.) It is a BioProcess™ medium that meets the demands of large-scale biopharmaceutical manufacturers for efficient and cost-effective protein purification. This includes validated manufacturing methods, secure supply and Regulatory Support Files (RSF) to assist process validation and submission to regulatory authorities.

Butyl-S Sepharose 6 Fast Flow was developed and optimized in cooperation with a process-scale manufacturer of biopharmaceuticals.

- Designed for the binding and elution of relatively strong hydrophobic molecules at comparatively low salt concentrations
- Used in purification of recombinant Hepatitis B virus surface antigen from CHO cells
- Minimal lot-to-lot variation
- Hydrophilic backbone gives low non-specific interactions
- Low risk of denaturation of relatively strong hydrophobic solutes

Media characteristics

Butyl-S Sepharose 6 Fast Flow is based on rigid, highly cross-linked, and porous agarose beads that permit high flow rates at moderate pressure (Fig 2), and high loading capacity. Table 1 summarizes its characteristics.

Fig 1. Butyl-S Sepharose 6 Fast Flow for both research and industrial applications.

Table 1 Some general characteristics of Butyl-S Sepharose 6 Fast Flow

Matrix	Highly cross-linked agarose, 6%
Type of ligand	Butyl-S: -S-$(CH_2)_3$-CH_3 attached via a spacer
Bead form	Rigid, spherical, macroporous
Mean particle size	90 µm diameter
Particle size distribution	45–165 µm
Degree of substitution	8.9–11.3 µmoles ligand/ml medium
pH stability working range cleaning range	 3–13 2–14
Chemically stability	Stable in commonly used aqueous buffers: 1 mM HCl, 1 M NaOH, 30 % isopropanol, 50% ethylene glycol, 70% ethanol, 3 M ammonium sulfate, 6 M guanidine hydrochloride, 8 M urea
Autoclavable	In 0.05 M sodium phosphate buffer pH 7.0 at 121 °C for 20 min
Flow velocity at 25 °C with 0.1 M NaCl as mobile phase	Approx. 400 cm/h at 1 bar (100 kPa, 14.5 psi), XK 50/30 column, bed height 15 cm
Operating temperature	4–40 °C
Delivering conditions	20% ethanol

Butyl-S Sepharose 6 Fast Flow is suitable for applications at all scales of operation ranging from laboratory- to process-scale chromatography.

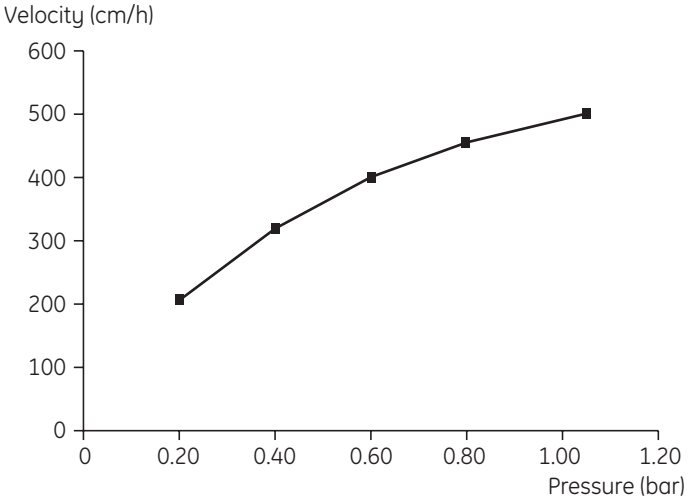

Fig 2. Typical pressure/flow curve for Butyl-S Sepharose 6 Fast Flow in an XK 50/30 column, bed height 15 cm; mobile phase 0.1 M NaCl.

Low lot-to-lot variation

The medium contains a sulfur atom as a linker between the spacer arm and the butyl ligand (Fig 3). Figure 3 also shows the structure of Butyl Sepharose 4 Fast Flow. The main differences between these two HIC media lie in the length of their spacer arms, the concentration of the immobilized ligands, and the type of connector atom (O-ether or S-ether) linking each ligand to the Sepharose base matrix. The presence of sulfur as the connector atom in the Butyl-S Sepharose 6 Fast Flow offers a convenient and relatively accurate estimation of ligand concentration, even at low degrees of substitution. As a result, the lot-to-lot variations of different production batches of this medium are kept to a minimum.

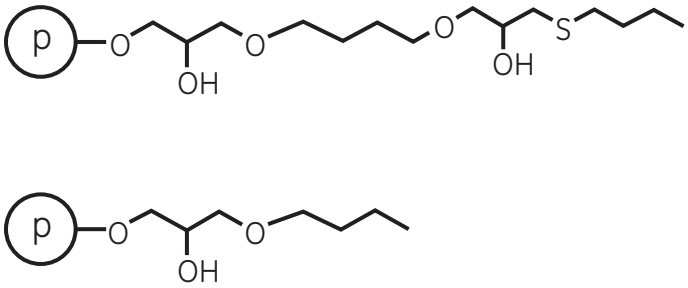

Fig 3. The general structures of Butyl-S Sepharose 6 Fast Flow (top) and Butyl Sepharose 4 Fast Flow (bottom).

The selectivities of Butyl-S Sepharose 6 Fast Flow and Butyl Sepharose 4 Fast Flow are compared in Figure 4.

Column:	Dimensions 10 mm × 10 cm. Packed bed volume = 5.9 ml
Buffer A:	0.02 M Tris-HCl, 1.7 M ammonium sulfate, pH 7.5
Buffer B:	0.02 M Tris-HCl, pH 7.5
Flow:	1 ml/min (76 cm/h)
Gradient:	0–100% B, 10 CV
System:	ÄKTAFPLC™

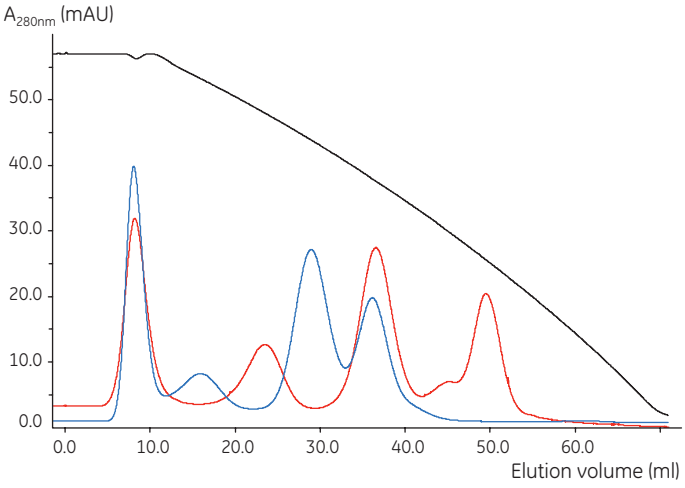

Fig 4. Selectivities of Butyl Sepharose 4 Fast Flow (red line) and Butyl-S Sepharose 6 Fast Flow (blue line) towards a cocktail mixture of 4 purified proteins that elute in the following order from both adsorbents: Cytochrome C, RNAse A, Lysozyme and -Chymotrypsinogen.

Figure 5 compares the selectivities of the HIC product portfolio based on Sepharose with Butyl-S Sepharose 6 Fast Flow as the least hydrophobic and Phenyl Sepharose 6 Fast Flow (high sub) as the most hydrophobic medium. The hydrophobicity covers a wide range with the individual proteins in the cocktail mixture eluting at different positions in the gradient.

Column: Dimensions 10 mm × 10 cm. Packed bed volume = 5.9 ml
Buffer A: 0.02 M Tris-HCl, 1.7 M ammonium sulphate, pH 7.5
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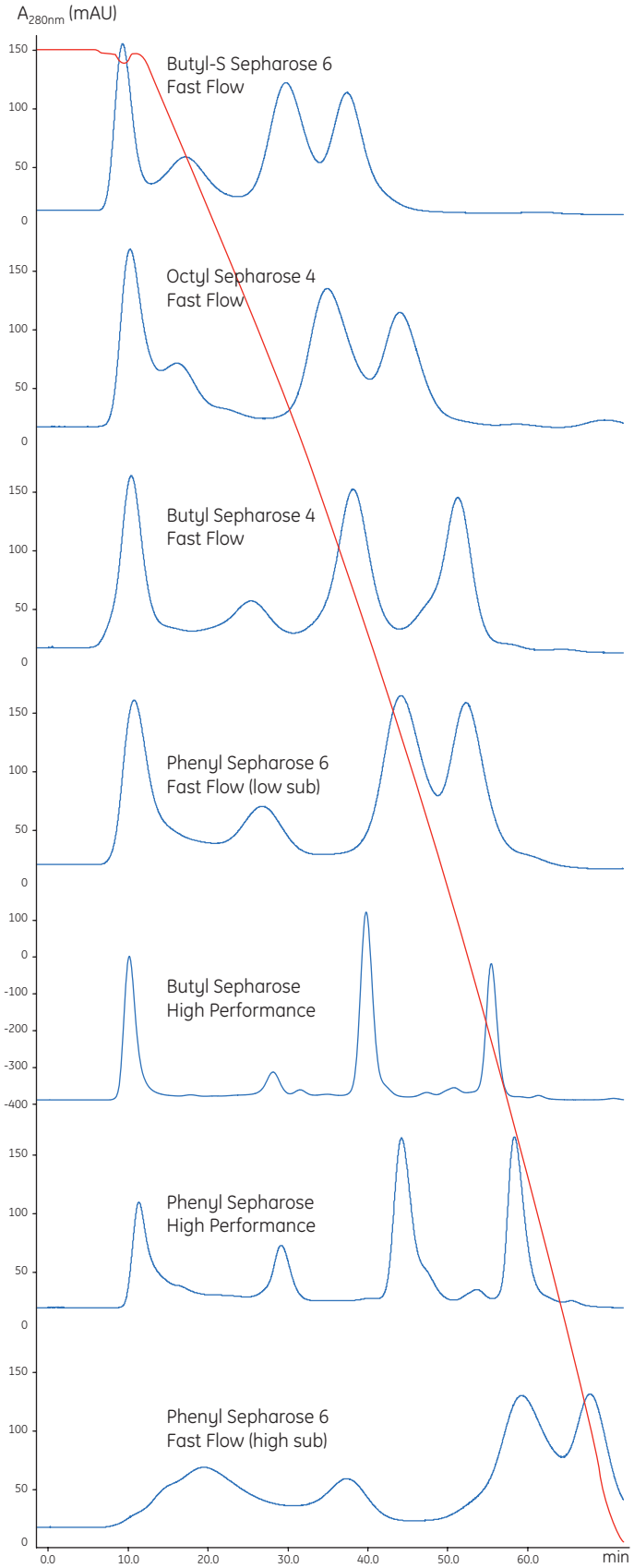


Cleaning-in-place

Cleaning-in-place (CIP) removes very tightly bound, precipitated, or denatured substances generated during previous production runs. In some applications, substances like lipids or denatured proteins may remain in the column bed and not be eluted by the regeneration procedure. CIP protocols should therefore be developed for the type of contaminants known to be present in the feedstream. Recommended procedures for removing specific contaminants are described below. CIP procedures can usually be carried out for hundreds of cycles without affecting column performance.

Suggested protocol to remove precipitated proteins:

- Wash the column with 4 bed volumes of 0.5–1.0 M NaOH solution at 40 cm/h, followed by 2–3 bed volumes of water.

Suggested protocol to remove tightly bound hydrophobic proteins, lipoproteins, and lipids:

- Wash the column with 4–10 bed volumes of up to 70% ethanol or 30% isopropanol. (Apply gradients to avoid the formation of air bubbles when using high concentrations of organic solvents.)
- Alternatively, wash the column with detergent in a basic or acidic solution, for example 0.5% non-ionic detergent in 1 M acetic acid. Wash at a flow rate of 40 cm/h. Residual detergent can be removed by washing with 5 bed volumes of 70% ethanol.

Sanitization

Sanitization is the use of chemical agents to inactivate microbial contaminants. Sodium hydroxide (NaOH) is a commonly used sanitizing agent. A concentration of 0.5–1.0 M NaOH with a contact time of 30–60 min is effective for most microbial contamination.

Storage

We recommend that the medium be stored in 20% ethanol at a temperature of +4 to +30°C. Butyl-S Sepharose 6 Fast Flow is supplied in 20% ethanol.

Fig 5. The HIC product portfolio based on Sepharose was screened for selectivity and separation ability for Cytochrome C, RNAse A, Lysozyme and -Chymotrypsinogen (eluting in this order). Butyl-S Sepharose 6 Fast Flow is the least hydrophobic medium produced by GE Healthcare and is thus most suitable for the binding and elution of more hydrophobic solutes with high recovery and reduced risk of their denaturation.

Applications

A downstream process for purification of recombinant Hepatitis B virus surface antigen (r-HBsAg) from CHO cells.

Hepatitis B virus (HBV) is an infectious agent that causes acute and chronic hepatitis, cirrhosis, and primary hepatocellular carcinoma. It is estimated that approximately 5% of the world's population is infected by the Hepatitis B virus. The infection is especially prevalent in Southeast Asia, the Middle East, and Africa but it is also found to a lesser extent in the western world, mainly among drug abusers. In China alone, it is estimated that about 9% of its population are carriers of the HBV infection and that about 1 million are newly infected every year.

To curb this trend and possibly eradicate the HBV infection, a great effort is being undertaken (especially in China but also elsewhere) to produce relatively inexpensive and effective vaccines for the non-infected segment of the population. This has been achieved mainly by advances in recombinant DNA technology that led to the large-scale production of the recombinant vaccine using primarily genetically transformed yeast or Chinese hamster ovary (CHO) cell lines. Several alternative methods have been proposed for purifying the recombinant Hepatitis B surface Antigen (r-HBsAg) thus produced.

An example of a successful chromatographic purification process for r-HBsAg from the cell culture supernatant of CHO cells is presented schematically in Figure 6. The entire process comprises three chromatographic steps integrated with membrane filtration steps for buffer exchange and product concentration.

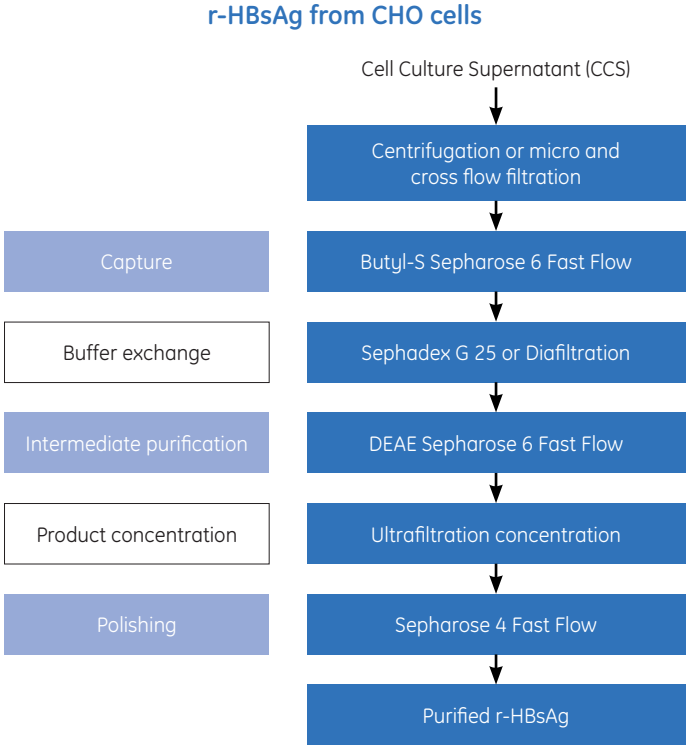
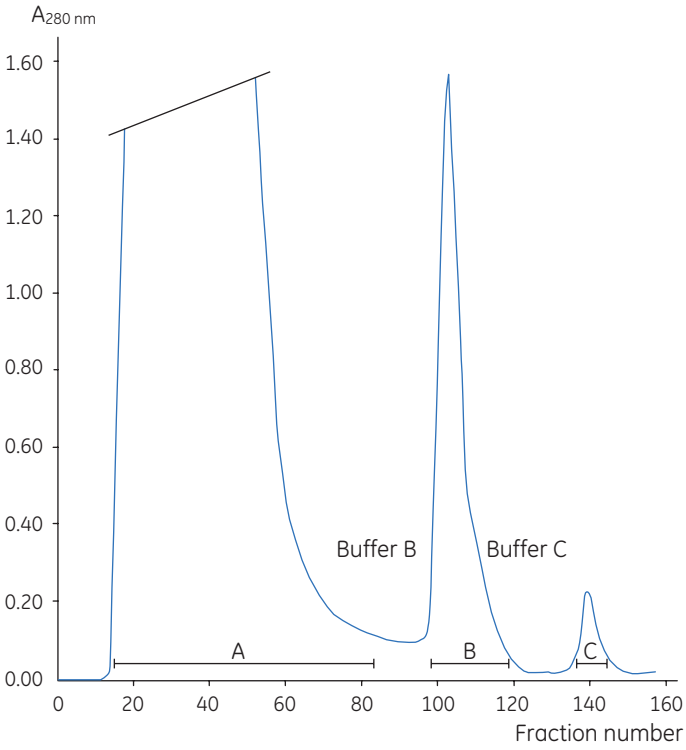
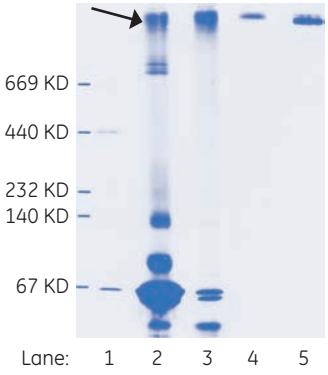


Fig 6. Downstream purification process for r-HBsAg outlining the essential steps used for the large-scale production of recombinant hepatitis B vaccine.

HIC medium:	Butyl–S Sepharose 6 Fast Flow
Column:	XK 50/20 (packed bed volume = 130 ml)
Sample:	300 ml of concentrated CCS (containing ca. 12 mg of rHBsAg). In the CCS was dissolved ammonium sulfate to ca. 0.6 M and the pH adjusted to 7.0.
Buffer A:	20 mM sodium phosphate, 0.6 M ammonium sulfate, pH 7.0
Buffer B:	10 mM sodium phosphate, pH 7.0
Buffer C:	30% iso-propanol dissolved in Buffer B
Flow:	2 l/h (100 cm/h)
Analytical Electophoresis:	Gradient PAGE (on 4–30% gradient gels) performed under non-denaturing conditions.





Lane 1 LMW reference proteins (RP)
Lane 2 Cell culture superantant (CCS)
Lane 3 Pooled fraction B (see chromatogram)
Lane 4 Purified r-HBsAg
Lane 5 Reference r-HBsAg (obtained from a commercial source and used as vaccine)

The arrow shows the position to which the r-HBsAG migrates in the gel. Due to its big size (a particle with a diameter of 22 nm), this band barely enters the pores of the gel.

Fig 7. Purification of r-HBsAg from the cell culture supernatant (CCS) of genetically modified CHO (Chinese hamster ovary) cells grown in Dubelco's Modified Eagle's Medium containing 5% of foetal calf serum. The chromatogram shows the elution pattern obtained after the initial purication step (capture) of the r-HBsAg using Butyl-S Sepharose 6 Fast Flow. At least 90% of the impurities are eluted in fraction A and 90% of the applied rHBsAg is localised to fraction B.

The first (capture) step in the purification of the r-HBsAg involves adsorption on a column packed with Butyl-S Sepharose 6 Fast Flow. Of some eight HIC media tested during process development this novel HIC medium gave the best performance with respect to high adsorption capacity combined with high recovery and good selectivity for the r-HBsAg. This first step alone resulted in the removal of at least 92% of the material absorbing at 280 nm (A_{280} absorbing material) applied to the column in the flowthrough fraction (Fig 7, fraction A). The major components in this fraction are LMW solutes (amino acids, pH indicators, etc.) as well as calf serum proteins (IgG, BSA, transferrin, fetuin, etc). The bound fractions (fractions B and C) account for about 6% of the applied A_{280} absorbing material, but for approximately 85% of the r-HBsAg activity. Figure 7 also shows the results of electrophoretic separation of fraction B (lane 3) compared with purified r-HBsAg (lane 4) and reference r-HBsAg (lane 5). Due to its large size (diameter 22 nm), the r-HBsAg band barely enters the pores of the gel.

Tandem columns, ideal for screening and removal of hydrophobic proteins

Columns coupled in series are useful for screening HIC media for a particular application or for removing very hydrophobic solutes from a biological sample. Since very hydrophobic proteins bind strongly to HIC media, most of these proteins require aquo-organic solvents for elution. Consequently, there is a risk of denaturation and difficulties regenerating the medium. In these situations a HIC medium of low hydrophobicity, such as Butyl-S Sepharose 6 Fast Flow, can be used to capture very hydrophobic target proteins at relatively low salt concentration (as in the r-HBsAg application above). Alternatively, the first HIC column in a series can serve as a scavenger to bind very hydrophobic contaminants in the sample, thereby protecting the second HIC column to bind the target protein.

Using a graded series of HIC media in columns is also a convenient approach to screening optimal binding conditions. This is achieved by applying the sample to a number of HIC columns connected in series and equilibrated with a suitable buffer. The least hydrophobic medium is in the first column and the most hydrophobic medium is in the last. After elution of the unbound material, the columns are disconnected and the bound material on each of the individual columns is eluted with a suitable desorption buffer. Analysis of the eluted fractions will help establish which of the HIC media is most suitable for purifying the target protein and also if the use of a scavenger column is of any advantage. This approach could be useful for the removal of lipids, lipoproteins, pigments, etc. from biological samples and for the purification of recombinant proteins produced by transgenic plants.

Overview of HIC

Separation of biomolecules on HIC media is based on an interplay between the hydrophobicity of the medium, the nature and composition of the sample, the prevalence and distribution of surface-exposed hydrophobic amino acid residues, and the type and concentration of salt in the binding buffer. Unlike reversed phase chromatography (RPC), which is a separation method closely related to HIC, the adsorption of biological solutes to HIC adsorbents is promoted, or otherwise modulated, by the presence of relatively high concentrations of anti-chaotropic salts such as ammonium sulfate, sodium sulfate, etc. (Fig 8). Desorption of bound solutes is achieved simply by stepwise or gradient elution with buffers of low salt content. In terms of its *modus operandi*, HIC is thus one of the simplest chromatographic separation methods in use today.

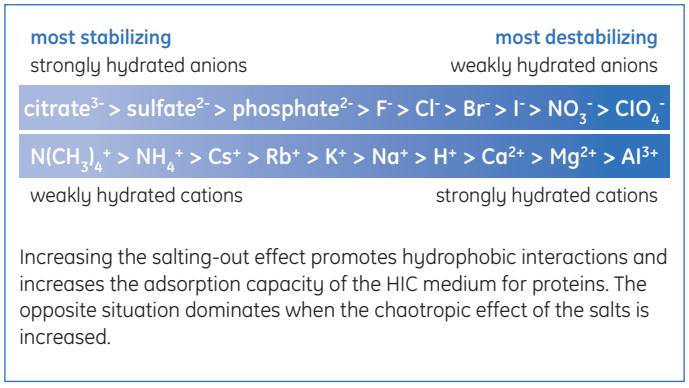


Fig 8. The Hofmeister series of some anions and cations arranged according to their effects on the solubility of protein in aqueous solutions.

HIC media available from GE Healthcare are produced as a graded series of hydrophobic adsorbents based on alkyl or aryl ligands attached to a hydrophilic base matrix, e.g. Sepharose. In each instance, the type and concentration of ligand has been optimized to cover the range of hydrophobicities of the proteins in a biological extract, varying from weak to moderate to strong hydrophobic proteins. This strategy results in HIC adsorbents for "all occasions" where the emphasis is on high recovery, purity, and reduced risk for denaturation of the target proteins in a biological extract.

Factors affecting HIC

The main parameters to consider when selecting a HIC medium and optimizing its chromatographic performance are:

- The nature of the base matrix (e.g. agarose, organic co-polymers, etc.)
- Structure of the ligand
- Concentration of the ligand
- Characteristics of the target protein and other sample components
- Type of salt
- Concentration of salt
- Temperature
- pH

Of these parameters, the type and concentration of ligand as well as the type and concentration of salt added during the adsorption step (Fig 8) are of paramount importance in determining the outcome of a HIC event. In general, the type of immobilized ligand determines its adsorption selectivity toward the proteins in a sample while its concentration determines its adsorption capacity. Adsorption of proteins to HIC media is promoted by high concentrations of structure-forming salts such as ammonium sulfate, potassium phosphate, sodium sulfate, etc. This makes HIC particularly suitable to use after techniques that leave the sample in a high salt concentration (e.g. precipitation with ammonium sulfate, affinity-based separations, and ion-exchange chromatography) since the sample can often be applied directly. Elution of the bound solutes is usually performed with a linear or stepwise decrease in the salt concentration. Some strongly hydrophobic solutes might bind so strongly to the HIC medium that they require organic solvents or detergents for their elution.

The characteristics of the target protein (in a HIC context) are usually not known since minimal data are available in this respect. There are some published data regarding the hydrophobicity indices for a number of purified proteins based on amino acid composition, the number and distribution of surface-exposed hydrophobic amino acids, and the order of their elution from RPC columns but few, if any, have proved to be useful when purifying a protein in a real biological sample. For this and other reasons, the adsorption behavior of a protein exposed to a HIC medium has to be determined on a case-by-case basis.

The effect of pH is not well established. In general, an increase in pH above 8.5 weakens hydrophobic interactions whereas a decrease in pH below 5.0 results in an apparent increase in the retention of proteins on HIC adsorbents. In the range of pH 5–8.5, the effect seems to be minimal or insignificant. In regard to temperature, it is generally accepted that the binding of proteins to HIC adsorbents is entropy driven, which implies that the solute-adsorbent interaction increases with increase in temperature. In some instances, the reverse effect has been observed, indicating that temperature might have a differential effect on the conformational state of different proteins and their solubility in aqueous solutions. In practical work, one should be aware that a downstream purification process that is developed at room temperature might not be reproduced in the cold room, or vice versa. In other instances, temperature control is mandatory in order to obtain reproducible results from run to run.

Additives (e.g. water-miscible alcohols, detergents, etc.) present in low concentrations effectively compete with proteins for the binding sites of HIC ligands. This results in the weakening or abolition of protein-HIC ligand interactions leading to the displacement of the protein by the additive. Solutions of chaotropic salts also have a similar effect by virtue of their negative effects on the ordered structure of water close to the HIC ligand and proteins. Although additives can be used to improve selectivity during desorption of bound proteins, one must be aware of the risk that proteins could be denatured by exposure to such additives. On the other hand, some additives can be very useful for CIP of HIC columns exposed to very hydrophobic solutes.

References

Purification of recombinant hepatitis B surface antigen produced by transformed Chinese hamster ovary (CHO) cell line grown in culture.
Bioseparation 1 (1991) 397–408, Belew, M., Mei, Y., Li, B., Berglöf, J., Janson, J-C.

Ordering Information

Product	Pack size	Code no.
Butyl-S Sepharose 6 Fast Flow	25 ml	17-0978-10
Butyl-S Sepharose 6 Fast Flow	200 ml	17-0978-02
Butyl-S Sepharose 6 Fast Flow	1 l	17-0978-03
Butyl-S Sepharose 6 Fast Flow	5 l	17-0978-04

Note: All bulk media are supplied in 20% ethanol. For more information contact your local GE Healthcare representative.

Literature

Handbook	Code no.
Hydrophobic Interaction Chromatography C handbook, principles and methods	18-1020-90
Ion Exchange Chromatography & Chromatofocusing: Principle and Methods	11-0004-21

Brochure	Code no.
Solid Advantages of Hollow Fiber Cartridges & Systems	18-1164-98
Cassette Brochure – Fast. Reliable. Trouble-Free.	18-1172-15
Standard Membrane Systems	18-1177-26

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