Phenyl Sepharose™ High Performance
Butyl Sepharose High Performance

Phenyl Sepharose High Performance and Butyl Sepharose High Performance are members of the GE range of hydrophobic interaction chromatography (HIC) resins for intermediate purification and polishing of proteins in packed bed. These resins are particularly well suited for polishing step purification giving high resolution due to the small particle size.

Phenyl and Butyl Sepharose High Performance, are based on rigid, cross-linked, spherical agarose with a particle size of ~ 34 µm. The functional groups are attached to the matrix via uncharged, chemically stable ether linkages resulting in a hydrophobic resin with minimized ionic properties. Some characteristics of Phenyl and Butyl Sepharose High Performance are listed in Table 1.

BioProcess™ chromatography resins are developed and supported for production-scale chromatography. BioProcess resins are produced with validated methods and are tested to meet manufacturing requirements. Secure ordering and delivery routines give a reliable supply of resins for production-scale. Regulatory Support Files (RSF) are available to assist process validation and submissions to regulatory authorities. BioProcess resins cover all purification steps from capture to polishing.

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Read these instructions carefully before using the products.

Safety

For use and handling of the products in a safe way, refer to the Safety Data Sheets.

1 Characteristics

The Sepharose High Performance base matrix is a cross-linked agarose derivative with good flow properties and high resolution, making it ideal for process-scale applications, particularly the polishing stages of a separation when high resolution is required. The high physical and chemical stabilities of the matrix prevent bed compression and the formation of fines, and allow efficient maintenance procedures for increased life span.

Figure 1 shows a pressure/flow curve for Phenyl Sepharose High Performance in a BPG 100/500 column recorded in an open bed mode to a final bed height of 10 cm, using water as packing solution. Due to the wall support offered by columns with smaller diameters, for example, BPG100, the flow rate has to be lowered if columns with larger diameters are to be used to keep the pressure constant. In a large-scale column, a flow rate of 100 cm/h at a 20 cm bed height is recommended.

Table 1 lists the main characteristics for Phenyl and Butyl Sepharose High Performance.

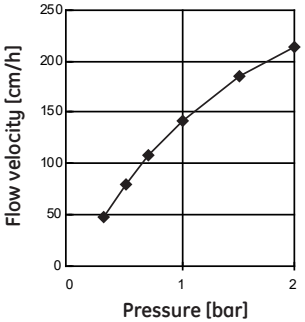

Fig 1. Pressure/flow curve for a Sepharose High Performance matrix

Table 1. Characteristics of Phenyl and Butyl Sepharose High Performance

	Phenyl Sepharose High Performance	Butyl Sepharose High Performance
Matrix	Cross-linked agarose, spherical	
Ligand concentration	~ 25 µmol phenyl/mL resin	~ 50 µmol butyl/mL resin
Particle size, d_{50v} [1]	~ 34 µm	
Exclusion limit [Mr] globular proteins [2]	1×10^7	
Recommended maximum operating pressure	0.3 MPa (3 bar, 43.5 psi) [3]	
Recommended operating flow velocity	≤ 100 cm/h [4]	
Chemical Stability	Stable to commonly used aqueous buffers, 1 M acetic acid 8 M urea 6 M guanidine hydrochloride 30% acetonitrile 30% isopropanol 70% ethanol 2% SDS 1 mM HCl 0.01 M NaOH	
pH stability, operational [5]	3 to 12	3 to 13
pH stability, CIP [6]	3 to 12	2 to 14
Storage	20% ethanol, 4°C to 30°C	

1 Median particle size of the cumulative volume distribution

2 Exclusion limit measured on the base matrix

3 10 cm diameter, 20cm bed height

4 10 cm diameter, 20 cm bed height, at room temperature using buffers with the same viscosity as water

5 pH range where resin can be operated without significant change in function

6 pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function

2 Method design and optimization

The aim of designing and optimizing a method for the separation of biomolecules is to identify conditions that promote binding of the highest amount of target molecule, in the shortest possible time with highest possible product recovery.

The binding of proteins to a HIC resin is often promoted by the addition of salt. Thus, to get a high binding capacity, add a high concentration of salt to the feed stock and binding buffer. Since the promotion of binding by the salt is different between proteins, it is possible to enrich the target molecule by choosing the most favorable concentration and type of salt. Some examples of salt type and concentration are ammonium sulphate 0.4 to 1.7 M, sodium sulphate 0.4 to 1.5 M and sodium chloride 1.0 to 3.5 M. Other types of salt can also be used. Solubility of the target protein sets the upper limit of the salt concentration.

Low recovery can be remedied by adding some organic modifier, such as 30% isopropanol, in the elution buffer.

Design of Experiment (DoE) is an effective tool for investigation of the effect of several parameters on protein purity and recovery in order to establish the optimal elution protocol.

Suggested purification protocol

Step	Action
1	Add the salt dissolved in a neutral buffer to the feed stock until the predetermined concentration is reached. The exact salt concentration must determined for each target molecule.
2	Equilibrate the column with start buffer of the same salt concentration as in the feed.
3	Apply the sample to the column.
4	Wash out unbound sample using start buffer.
5	Elute the target protein by applying a gradient of descending concentration of salt. Typically, the gradient is 20 column volumes (CV).

Step	Action
6	After identifying the elution volume for the target protein, the slope of the gradient can be leveled out in order to increase the resolution. It is also possible to employ a stepwise gradient.

Optimization of throughput

Balancing product recovery against throughput is the major consideration when optimizing a method. The dynamic binding capacity for the target protein must be determined by frontal analysis using real process feedstock. Since the dynamic binding capacity is a function of the flow velocity applied during sample application, the breakthrough capacity must be defined over a range of different residence times (flow velocities) to show the optimum level of throughput.

3 Scaling up

After optimizing the method at laboratory-scale, the process can be scaled up.

Step	Action
1	Select the bed volume according to required binding capacity.
2	Select a column diameter to obtain a bed height of 10 to 25 cm.
3	While keeping bed height and flow velocity constant, increase bed diameter and volumetric flow rate.

To save sample and buffer, optimization is usually done with small column volumes. Some parameters, like the dynamic binding capacity, can be optimized using shorter bed height than those used in the final scale. As long as the residence time is constant, the binding capacity for the target molecule is the same.

Other factors, like clearance of critical impurities, can change when column bed height is changed. These factors must be validated using the final bed height. The residence time is approximated as the bed height (cm) divided by the flow velocity (cm/h) applied during sample loading.

4 Column packing

General guidelines

Columns with large diameter can be difficult to pack if the bed height is too low. Bed heights of at least 10 cm are therefore recommended. Scaling up is preferentially done by keeping the bed height constant while increasing the diameter and volumetric flow rate.

General packing procedures

The following parameters refers to large-scale packing:

Preferred packing solution: 10% to 20% ethanol

Resin slurry concentration: 50%

Packing pressure: 0.3 to 0.6 MPa (3 to 6 bar, 43.6 to 87.0 psi)

Packing flow velocity: 200 to 300 cm/h

Recommended columns

Laboratory-scale columns

Column	Size	I. d. (mm)	Bed volume, max (mL)	Bed height, max (cm)
Tricorn™	5/50	5	1.1	58
Tricorn	5/100	5	2.1	10.8
Tricorn	5/150	5	3.1	15.8
Tricorn	10/50	10	4.6	5.8
Tricorn	10/100	10	8.5	10.8
Tricorn	10/150	10	12.4	15.8
XK	16/20	16	30	15
XK	26/20	26	80	15
XK	50/20	50	275	15

Large-scale columns

- AxiChrom™
  Inner diameters of 50 to 200 mm, bed volumes max 16.7 L, bed heights max 50 cm
- AxiChrom
  Inner diameters of 300 to 1600 mm, bed volumes max 16.7 L, bed heights max 50 cm
- AxiChrom, i.d. 300 to 1600 mm, bed volumes max 1005 L, bed heights max 50 cm
- BPG variable bed, glass columns
  Inner diameters of 100 to 450 mm; bed volumes max 130 L, bed height max 58 cm
- FineLINE™ variable bed columns
  Inner diameters of 50 mm to 300 mm
- Chromaflow™ variable and fixed bed columns
  Inner diameters of 400 mm to 2000 mm

Packing laboratory-scale columns

Packing Tricorn 10/100 columns

To obtain a 10 cm bed height, pour 12 mL of a 75% resin slurry in 20% ethanol into the column to which a packing tube has been attached. Add 20% ethanol to the top of the packing tube and attach the adapter without trapping any air bubbles. Proceed with the packing as follows:

Step	Action
1	Pack the column at 2 mL/min (150 cm/h) until the bed is stabilized (approximately 2.3 min).
2	Increase the flow to 5.2 mL/min (400 cm/h) and pack for 5 min.
3	Stop the flow and remove the packing tube and connector. If needed, remove the excess of resin. Attach and adjust the adapter down to the surface of the resin.
4	Start the flow at 5.2 mL/min and pack for 1 min. Mark the bed height without turning the flow off.

Step	Action
5	Stop the flow and adjust the adapter 2 mm below the mark.

Packing XK 16/20 columns

To obtain a 10 cm bed height, fill the column tube with a 75% resin slurry. Attach the adapter directly on the column as close to the upper edge as possible. The packing requires five steps:

Step	Action
1	Pack the column at 1 mL/min (30 cm/h) until the bed is stabilized (approximately 20 min).
2	Increase the flow until a constant pressure of 0.5 MPa (5 bar, 72.52 psi) is reached, and pack for 5 min or until the bed height is stable.
3	Stop the flow and adjust the adapter down to the surface of the resin.
4	Pack for another 1 to 2 min at the same flow rate and mark the bed height without turning the flow off.
5	Stop the flow, adjust the adaptor 10 mm below the mark, and pack at 10 mL/min for 10 column volumes.

Packing large-scale columns

For packing instructions, refer to *www.gehealthcare.com/purification_techsupport*.

5 Evaluating the packing

Test column efficiency to check the quality of the packing. Tests must be made directly after packing and at regular intervals during the working life of the column plus when separation performance is seen to deteriorate.

The best method of expressing the efficiency of a packed column is in terms of the height equivalent to a theoretical plate (HETP) and the asymmetry factor (A_s). These values are easily determined by applying a sample such as 2.0 M NaCl in water with 0.5 M NaCl in water as eluent. A solution of acetone (1%) in water can also be used as a test substance, but can interact with the hydrophobic resin.

Note: *The calculated plate number will vary according to the test conditions and it should only be used as a reference value. It is important that test conditions and equipment are kept constant so that results are comparable. Changes of solute, solvent, eluent, sample volume, flow velocity, liquid pathway, temperature, etc. will influence the results.*

For optimal results, the sample volume must be at maximum 2.5% of the column volume and the flow velocity of 30 cm/h. If an acceptance limit is defined in relation to column performance, the column plate number can be used as one of the acceptance criteria for column use.

Method for measuring HETP and A_s

To avoid dilution of the sample, apply it as close to the column inlet as possible.

Conditions

Sample volume	1.0% of bed volume
Sample concentration	2% v/v acetone in water
Fluid velocity	30 cm/h
UV	280 nm

Calculate HETP and A_s from the UV curve (or conductivity curve if NaCl is used as sample) as follows:

$$HETP = L/N$$

and $N = 5.54(V_e/W_h)^2$

where L = Bed height (cm)

N = Number of theoretical plates

V_e = Peak elution distance

W_h = Peak width at half peak height

V_e and W_h are in the same units.

To facilitate comparison of column performance, the concept of reduced plate height is often used. The reduced plate height is calculated as:

$$HETP / d50_v$$

where $d50_v$ is the median particle size of the cumulative volume distribution using the same unit as for HETP.

As a guideline, a value of $h < 3$ is normally acceptable at the optimal test conditions presented above.

The peak must be symmetrical, and the asymmetry factor as close as possible to 1 (values between 0.8 and 1.5 are usually acceptable). A change in the shape of the peak is usually the first indication of bed deterioration due to use.

Peak asymmetry factor calculation

$$A_s = b/a$$

where a = 1st half peak width at 10% of peak height

b = 2nd half peak width at 10% of peak height

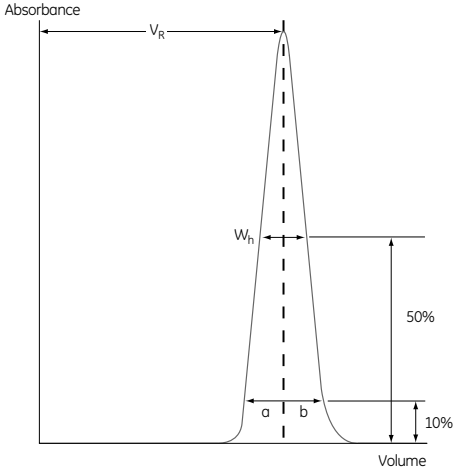

Fig 2. UV trace for acetone in a typical test

6 Resin and column maintenance

Regeneration

For best performance from the resin, wash bound substances from the column after each chromatographic cycle.

Wash with 2 bed volumes of water, followed by 2 to 3 bed volumes of starting buffer.

To prevent a slow buildup of contaminants on the column over time, you maybe have to apply more rigorous cleaning protocols on a regular basis.

Cleaning-in-place (CIP)

CIP removes very tightly bound, precipitated or denatured substances generated during previous production runs. In some applications, substances such as lipids or denatured proteins can remain in the column bed and not be eluted by regeneration. You must therefore develop CIP protocols for the types of contaminants known to be present in the feed. Recommended procedures for removing specific contaminants are described below. CIP procedures can normally be carried out for hundreds of cycles without affecting column performance.

Suggested protocol for removal of precipitated proteins

- Wash the column with 4 bed volumes of 0.01 M NaOH at 40 cm/h, followed by 2 to 3 bed volumes of water.

Suggested protocol for removal of tightly bound hydrophobic proteins, lipoproteins and lipids

- Wash the column with 4 to 10 bed volumes of up to 70% ethanol or 30% isopropanol followed by 3 to 4 bed volumes of water. (Apply gradients to avoid air bubbles forming when using high concentrations of organic solvents.)
- Alternatively, wash the column with detergent in a basic or acidic solution, for example, 0.5% nonionic detergent in 1 M acetic acid. Wash at a flow velocity of 40 cm/h. Remove residual detergent with 5 bed volumes of 70% ethanol followed by 3 to 4 bed volumes of water.

CAUTION

70% ethanol can require the use of explosion-proof areas and equipment.

Suggested protocol for removal of other contaminants

- Wash the column with 4 to 10 bed volumes of up to 70% ethanol or 30% isopropanol followed by 3 to 4 bed volumes of water. (Apply gradients to avoid air bubbles forming when using high concentrations of organic solvents.)
- Alternatively, wash the column with detergent in a basic or acidic solution, for example, 0.5% nonionic detergent in 1 M acetic acid. Wash at a flow rate of 40 cm/h. Remove residual detergent with 5 bed volumes of 70% ethanol followed by 3 to 4 bed volumes of water.
- Wash the column with 4 bed volumes of 0.01 M NaOH at 40 cm/h, followed by 2 to 3 bed volumes of water.

Use the CIP protocols above as guidelines when formulating a cleaning protocol specific for the raw material used. The frequency of CIP depends on the raw material applied to the column, but it is recommended to use a CIP procedure at least every 5 cycles during normal use.

Depending on the nature of the contaminants, different protocols maybe have to be used in combination. If fouling is severe, the protocols maybe have to be further optimized. During CIP, reverse the flow direction through the column.

Sanitization

Sanitization is the reduction of microbial contamination in the column and related equipment to an acceptable minimum. A specific sanitization protocol must be designed for each process according to the type of contaminants present. The following is a recommended protocol.

- Wash the column with 0.01 M NaOH at a flow velocity of approximately 40 cm/h, contact time 30 to 60 min.

Storage

Store Phenyl Sepharose High Performance and Butyl Sepharose High Performance in 20% ethanol at 4°C to 30°C to avoid microbiological growth.

7 Ordering information

Product	Pack size	Product code
Phenyl Sepharose High Performance	75 mL	17108201
	1 L	17108203
	5 L	17108204
	60 L [1]	17108260
Butyl Sepharose High Performance	25 mL	17543201
	200 mL	17543202
	1 L	17543203
	5 L	17543204
	60 L[1]	17543260

[1] Pack size available upon request

Related product	Quantity	Product code
HiTrap™ HIC Selection Kit	7 x 1 mL	28411007
HiTrap Butyl HP	5 x 1 mL	17108203
HiTrap Butyl HP	5 x 5 mL	17108204
HiTrap Phenyl HP	5 x 1 mL	17135101
HiTrap Phenyl HP	5 x 5 mL	17519501
HiLoad™ 16/10 Phenyl Sepharose HP	1 (20 mL)	17108501
HiLoad 16/10 Phenyl Sepharose HP	1 (53 mL)	17108601
HiScale™ 16/20		28964441
HiScale 16/40		28964424
HiScale 26/20		28964514
HiScale 26/40		28964513
HiScale 50/20		28964445
HiScale 50/40		28964444
Tricorn 5/50 column		28406409
Tricorn 5/100 column		28406410
Tricorn 5/150 column		28406411
Tricorn 10/50 column		28406414
Tricorn 10/100 column		28406415
Tricorn 10/150 column		28406416

Related literature	Product code
Hydrophobic Interaction and Reversed PhaseChromatography Handbook	11001269

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71712900 AG 08/2018 a85