GE Healthcare

Instructions 71-7080-00 AE **Hydrophobic interaction chromatography**

Phenyl Sepharose CL-4B

Phenyl Sepharose™ CL-4B is a separation medium for hydrophobic interaction chromatography (HIC). Substances are separated on the basis of their different hydrophobicity.

The phenyl group is covalently coupled to a cross-linked 4% agarose matrix by ether linkage, giving a hydrophobic medium with minimal leakage and no ionic properties.

Sepharose

Table 1. Medium characteristics

Ligand density:	40 µlmole/ml drained medium	
Available capacity*:	HSA (M_r 68 000)	15-20 mg/ml drained medium
	ß-lactoglobulin (M_r 18 000)	3-5 mg/ml drained medium
Bead structure:	4% cross-linked agarose	
Bead size range:	45–165 µm	
Mean bead size:	90 µm	
Max linear flow rate**:	150 cm/h at 25°C, HR 16/10 column, 5 cm bed height	
pH stability***.		
Long term:	3–12	
Short term:	4–12	
Chemical stability:	Stable in all commonly used aqueous buffers.	
Physical stability:	Negligible volume variation due to changes in pH or ionic strength.	
Autoclavable:	121 °C for 30 min.	
Storage:	20% ethanol or 0.1 M NaOH at 4 °C to 30 °C	

* The binding capacity was estimated in 0.01 M phospate buffer, pH 6.8, 1 M $(NH_4)_2SO_4$

** Linear flow rate = $\frac{\text{volumetric flow rate (cm}^3\text{)}}{\text{column cross-sectional area (cm}^2\text{)}}$

*** The ranges given are estimates based on our knowledge and experience. Please note the following: *pH stability, long term* refers to the pH interval where the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance. *pH stability, short term* refers to the pH interval for regeneration, cleaning-in-place and sanitization procedurse, see later.

Contents

1. Preparing the medium

Phenyl Sepharose CL-4B is supplied pre-swollen in 20% ethanol. Since ethanol markedly reduces hydrophobic interaction it is essential to thoroughly wash the medium to remove all traces before applying the sample. Wash the medium with at least 10 volumes of distilled water. Decant the medium. Prepare a slurry with binding buffer, see below, in a ratio of 75% settled medium to 25% buffer. The binding buffer should not contain agents which significantly increase the viscosity. The column may be equilibrated with viscous buffers at reduced flow rates after packing is completed.

2. Packing Sepharose CL-4B

1. Equilibrate all material to the temperature at which the chromatography will be performed.

2. De-gas the medium slurry.

3. Eliminate air from the column dead spaces by flushing the end pieces with buffer. Make sure no air has been trapped under the column net. Close the column outlet with a few centimeters of buffer remaining in the column

4. Pour the slurry into the column in one continuous motion. Pouring the slurry down a glass rod held against the wall of the column will minimize the introduction of air bubbles.

5. Immediately fill the remainder of the column with buffer, mount the column top piece onto the column and connect the column to a pump.

6. Open the bottom outlet of the column and set the pump to run at the desired flow rate. This should be at least 133% of the flow rate to be used during subsequent chromatographic procedures. However, the maximum flow rate, see Table 1, is typically employed during packing.

Note: If you have packed at the maximum linear flow rate, do not exceed 75% of this in subsequent chromatographic procedures.

7. Maintain the packing flow rate for 3 bed volumes after a constant bed height is reached.

3. Using an adaptor

Adaptors should be fitted as follows:

1. After the medium has been packed as described above, close the column outlet and remove the top piece from the column. Carefully fill the rest of the column with buffer to form an upward meniscus at the top.

2. Insert the adaptor at an angle into the column, ensuring that no air is trapped under the net.

3. Make all tubing connections at this stage. There must be a bubble-free liquid connection between the column and the pump, and column and the sample application system (LV-3 or LV-4).

4. Slide the plunger slowly down the column so that the air above the net and in the capillary tubings is displaced by eluent. Valves on the inlet side of the column should be turned in all directions during this procedure to ensure that air is removed.

5. Lock the adaptor in position on the medium surface open the column outlet and start the eluent flow. Pass eluent through the column at the packing flow rate until the medium bed is stable. Re-position the adaptor on the medium surface as necessary.

4. Equilibration

Before applying the sample, equilibrate the column with at least 2 column volumes at chosen binding conditions until the baseline is stable.

A common binding buffer for hydrophobic interaction chromatography is 0.05 M phosphate buffer, 1.7 M $(NH_4)_2SO_4$, pH 7.0.

5. Binding

The binding of proteins to hydrophobic media is influenced by:

- The structure of the ligand e.g. carbon chain or an aromatic ligand. A phenyl group is e.g. less hydrophobic than an octyl group.
- The concentration and salting out effect of the binding buffer (see the Hofmeister series in Table 2). Those salts which cause salting-out e.g. ammonium sulphate, also promote binding to hydrophobic ligands. The sample is applied in a solution of high concentration of salt. A salt concentration between 0.5-2.0 M ammonium sulphate is commonly used*. The column should be equilibrated at the same concentration.
- Temperature. Hydrophobic interactions usually decrease with decreasing temperature.

* When working with proteins which have a tendency to aggregate, start with a lower $(NH_4)_2SO_4$ concentration to avoid the risk of precipitation.

6. Elution

Bound proteins are eluted by reducing the hydrophobic interaction. This can be done by:

- reducing the concentration of salting out ions in the buffer with a decreasing salt gradient (linear or step)
- eluting with a non-polar organic solvent, for example 40–50% ethylene glycol, 30% isopropanol
- including detergent in the eluent

Table 2. The Hofmeister series. Increasing the salting-out effect strengthens the hydrophobic interactions, whereas increasing the chaotropic effect weakens them.

	← Increasing salting-out effect								
Anions:	PO_4^{3-}	SO_4^{2-}	CH_3COO^-	Cl^-	Br^-	NO_3^-	ClO_4^-	I^-	SCN^-
Cations:	NH_4^+	Rb^+	K^+	Na^+	Cs^+	Li^+	Mg^{2+}	Ba^{2+}	
	Increasing chaotropic effect →								

As a start, we suggest a linear gradient from 0 to 100% Elution buffer with:
Start buffer (A): 0.05 M phosphate buffer, pH 7.0 + 1.7 M $(NH_4)_2SO_4$
Elution buffer (B): 0.05 M phosphate buffer, pH 7.0

7. Regeneration

Depending on the nature of the sample, regeneration is normally performed by washing with 2-3 bed volumes of an aqueous solution of 30% isopropanol and with 3 bed volumes of distilled water, followed by re-equilibrating in start buffer. In some applications, substances such as denaturated proteins or lipids do not elute in this regeneration procedure. These can be removed by cleaning-in-place procedures.

8. Cleaning-in-place (CIP)

Remove precipitated proteins, tightly bound proteins, lipids and lipoproteins by washing the column, inreversed flow direction, with 2-3 bed volumes of 1 M NaOH. Stop the flow and let it stand for at least 4 hours or overnight. Wash with distilled water, at least 2-3 bed volumes, until the pH of the effluent is neutral. Re-equilibrate with at least 3 bed volumes of binding buffer.

Alternatively, wash the column with 4 bed volumes of up to 70% ethanol or 30% isopropanol or with 2 bed volumes of detergent in a basic or acidic solution. Use, for example, 0.1–0.5% non-ionic detergent (i.e. Triton X-100) in 0.1 M acetic acid. Wash at a low linear flow rate of approximately 40 cm/h, contact time 1-2 hours, reversed flow direction. When using high concentrations of organic solvents, apply increasing gradients to avoid air bubble formation. Detergents are difficult to remove and are recommended only if the other procedures fail to give satisfactory results. After treatment with detergent, wash with 5 bed volumes of 70% ethanol, or more, to remove residual detergents. Re-equilibrate the column with at least 3 bed volumes of binding buffer.

9. Sanitization

Sanitization reduces microbial contamination of the medium bed to a very low level. Wash the column in reversed flow direction with 2–3 bed volumes of 0.5–1 M NaOH. Stop the flow and let it stand for at least 5 hours or overnight. Wash with distilled water, at least 2-3 bed volumes, until the pH of the effluent is neutral.

Re-equilibrate with at least 3 bed volumes of sterile start buffer.

Column performance is normally not significantly changed by the cleaning-in place or sanitization procedures described above.

10. Storage

Store the medium in 20% ethanol or 0.1 M NaOH at 4–30°C.

11. Ordering information

Product	Pack size	Code No.
Phenyl Sepharose CL-4B	50 ml	17-0810-02
	200 ml	17-0810-01
Related Products	**Quantity**	**Code No.**
Phenyl Sepharose 6 Fast Flow (high sub)	25 ml	17-0973-10
Phenyl Sepharose 6 Fast Flow (high sub)	200 ml	17-0973-05
Phenyl Sepharose 6 Fast Flow (low sub)	25 ml	17-0965-10
Phenyl Sepharose 6 Fast Flow (low sub)	200 ml	17-0965-05
Phenyl Sepharose High Performance	75 ml	17-1082-01
HiPrep 16/10 Phenyl FF (high sub)	1	17-5095-01
HiPrep 16/10 Phenyl FF (low sub)	1	17-5094-01
HiTrap Phenyl FF (high sub)	5 x 1 ml	17-1355-01
HiTrap Phenyl FF (high sub)	5 x 5 ml	17-5193-01
HiTrap Phenyl FF (low sub)	5 x 1 ml	17-1353-01
HiTrap Phenyl FF (low sub)	5 x 5 ml	17-5194-01
HiTrap Phenyl HP	5 x 1 ml	17-1351-01
HiTrap Phenyl HP	5 x 5 ml	17-5195-01
HiTrap HIC Selection Kit	7 x 1 ml	28-4110-07
Handbook		
Hydrophobic Interaction and Reversed Phase Chromatography		11-0012-69

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