GE Healthcare

Instructions 71-7105-00 AE **Affinity media**

Thiopropyl Sepharose 6B

Thiopropyl Sepharose™ 6B contains reactive 2-thiopyridyl disulphide groups attached to Sepharose through a chemically stable ether linkage, see Fig. 1.

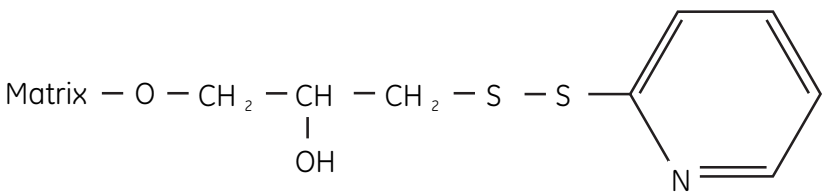

Fig 1. Partial structure of Thiopropyl Sepharose 6B.

Thiopropyl Sepharose 6B reacts with solutes containing thiol groups under mild conditions to form mixed disulphides. This reaction forms the basis of covalent chromatography and a procedure for reversible immobilization of thiol containing molecules. These techniques make it possible to:

- separate thiol-containing proteins and peptides from non-thiol-containing proteins and peptides
- in certain cases separate enzymes with active-site thiol groups from denatured enzymes
- store and protect thiol-containing proteins
- regenerate both the coupled biomolecule and the active bed material

Sepharose™

Thiopropyl Sepharose 6B is suitable for immobilizing low molecular weight substances. For immobilizing high molecular weight substances, we recommend Activated Thiol Sepharose 4B (17-0640-01).

Table 1. Medium characteristics.

Active group:	2-pyridyl disulphide
Active group concentration:	18–31 µmole 2-pyridyl disulphide/ml drained medium
Spacer:	2-hydoxypropanol
Coupling capacity:	approx 10–20 mg protein/ml drained medium, e.g. 14 mg ceruloplasmin (M_r 132 000) /ml drained medium
Bead structure:	6% agarose
Bead size range:	45–165 µm
Mean bead size:	90 µm
Max linear flow rate*:	75 cm/h at 25 °C, HR 16/10 column, 5 cm bed height
pH stability**	
Long term:	2–8
Short term:	2–8
Chemical stability:	Stable to all commonly used aqueous buffers and additives like detergents. Azides should be avoided.
Physical stability:	Negligible volume variation due to changes in pH or ionic strength.
Autoclavable:	Not recommended

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

** The ranges given are estimates based on our knowledge and experience. Data refer to the coupled product, provided that the ligand can withstand the pH or chemical environment. Please note the following:
pH stability, long term refers to the pH interval where the gel 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 and cleaning procedures.

Contents

1. Covalent chromatography

Covalent chromatography differs from other types of chromatography in that covalent bonds are formed between the medium and molecules in the mobile phase. Thiopropyl Sepharose 6B reacts with solutes containing thiol groups to form a mixed disulphide and release 2-thiopyridone, see Fig. 2. The solute is thus covalently linked to the medium from which it can be subsequently eluted by addition of a reducing agent, e.g. 2-mercaptoethanol, DTT or glutathione, see Fig. 2. Note that additional oxidizing reagents are not used and the risk of disulphide formation between molecules in solution is minimised.

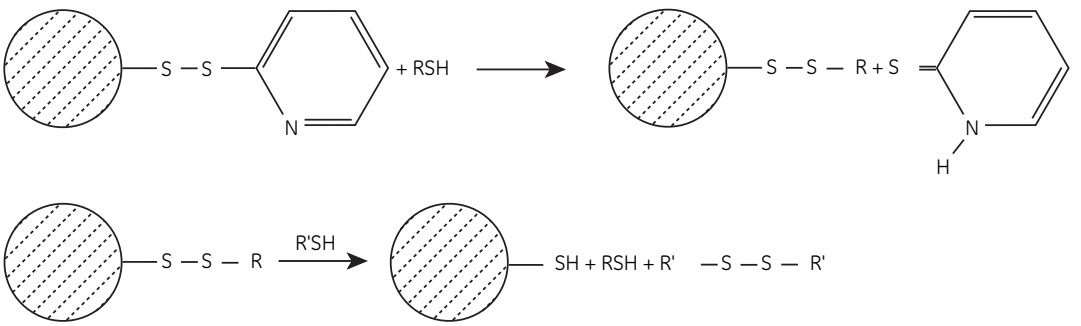

Fig 2. Reaction scheme for covalent chromatography of a thiolated substance (RSH) on Thiopropyl Sepharose 6B. R'SH represents a low molecular weight thiol such as dithiothreitol.

2. Preparing the medium for covalent chromatography

Thiopropyl Sepharose 6B is supplied freeze-dried in the presence of additives, which must be washed away at neutral pH before coupling. Distilled water is recommended for swelling and washing.

Weigh out the required amount of freeze dried powder (1 g freeze-dried powder gives about 3 ml final volume of medium) and suspend it in distilled water. The medium swells immediately and should be washed for

15 minutes with distilled water on a sintered glass filter. Use approximately 200 ml per gram freezedried powder, added in several aliquots.

Prepare a slurry with binding buffer, see "**Binding**" 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.

3. Packing Sepharose 6B

1. Equilibrate all material to the temperature at which thechromatography 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.

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.
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.

The column is now packed, equilibrated and ready for use.

Sample examination

We recommend you analyse the thiol content of the sample by thiol titration to make sure that the capacity of the medium is not exceeded.

Thiol titration is conveniently done by spectrophotometric determination of the released 2-thiopyridone (absorbance coefficient = 8.08×10^3 M^{-1} cm^{-1} at 343 nm) when a small amount of the sample, 1-5 mg in 1-3 ml binding buffer, reacts with 2-pyridyl disulphide, 2-PDS. The conditions can be chosen to suit the sample in question. Buffers between pH 3 to 8, formate, acetate, phosphate or Tris in the concentration range of 50–400 mM, with or without strong denaturants such as 8 M urea or 6 M guanidine HCl, can be used. Under standard conditions at pH 7.5, a reaction time of a few minutes is usually enough for complete reaction.

Sample preparation
The sample should be transferred to the binding buffer, see "**Binding**" below. Low molecular weight thiol compounds which might subsequently interfere with the coupling reaction can easily be removed from the sample by buffer exchange on Sephadex™ G-25, e.g. pre-packed PD-10 columns, or on HiTrap™ Desalting columns.

4. Binding

All buffers should be de-gassed to avoid oxidation of free thiol groups. Tris, phosphate or acetate buffer systems with the addition of 0.1–0.5 M NaCl may be used, e.g. 0.1 M Tris-HCl, pH 7.5, 0.5 M NaCl. 1 mM EDTA may be added to remove trace amounts of heavy metal ions, which may catalyze oxidation of thiols.

If monitoring non-bound substances at 280 nm, the contribution of released thiopyridone to the absorbance value has to be substracted. The absorbance of 2-thiopyridone at 280 nm and 343 nm is roughly equal. (Adsorption coefficient at 343 nm = 8.08×10^3 M^{-1} cm^{-1}).

To ensure maximum yield, the sample should be in contact with the medium for at least one hour in a standard procedure. Never exceed a flow rate of 10 cm/h during sample application. Too high flow rate will decrease the final yield and give dilution. After the sample has been loaded, wash the medium with binding buffer until the base line is stable.

5. Elution

Covalently bound biomolecules are eluted with 20–50 mM 2- mercaptoethanol or 10–25 mM dithiothreitol in Tris-HCl, phosphate or ammonium acetate buffer, pH 7–8, containing 1 mM EDTA. 2-thio-pyridone formed from the remaining 2- thiopyridyl groups will also be eluted during the elution procedure. The 2-thiopyridone is easily removed from the eluate by desalting on Sephadex G-25, e.g. prepacked PD-10 columns, or on HiTrap Desalting columns.

Alternatively, remaining 2-thiopyridyl groups may be removed before the bound molecule of interest is eluted. This is done by using a low concentration, 5 mM, of either 2-mercaptoethanol or dithiothreitol in the binding buffer. Bound molecules of interest are then eluted by raising the concentration of thiol reducing agent, 20–50 mM 2-mercaptoethanol or 10-25 mM dithiothreitol.

In one application, a series of solutions of thiols of increasing reducing power, 5–25 mM L-cysteine, 50 mM reduced glutathione, 20–50 mM 2-mercaptoethanol and 20–50 mM dithiothreitol, was used to sequentially elute different thiol proteins bound to the medium.

6. Regeneration

Thiopropyl Sepharose 6B can be regenerated by passing one to two bed volumes of a saturated solution (approx. 1.5 mM) of 2.2’ -dipyridyl disulphide at pH 8.0 through the medium. 2.2’ -dipyridyl disulphide is hard to dissolve in aqueous solutions.

Make a stock solution by adding 40 mg disulphide to 50 ml buffer at room temperature and stirring the suspension for several hours. Filter off insoluble material and adjust the pH.

The solution will be approximately 1.5 mM with respect to 2.2’ -dipyridyl disulphide.

Alternatively, the medium can be regenerated by mixing 1 volume of 2.2' -dipyridyl disulphide (30–40 mg/ml) in ethanol or isopropanol with 4 volumes of medium in borate buffer pH 8.0, containing 1 mM EDTA and refluxing for 3 hours. The medium is then washed with ethanol and re-equilibrated with starting buffer.

7. Cleaning

The medium may be cleaned by washing on a Buchner funnel with a non-ionic detergent, e.g. 0.1% Triton™ X-100 at 37 °C for one minute.

Immediately wash with at least 5 bed volumes of binding buffer.

8. Storage

Freeze-dried Thiopropyl Sepharose 6B should be stored below +8 °C. Swollen medium should be stored at neutral pH in the presence of a bacteriostat, i.e. 20% ethanol, at +4 to +8 °C. Do not use sodium azide as a bacteriostatic agent, since azide ions react with the 2-pyridyl disulphide groups.

The use of Thiopropyl Sepharose 6B for synthesis of adsorbents for affinity chromatography

The active thiol groups of Thiopropyl Sepharose 6B can be used for immobilizing many types of small ligands as such low molecular weight thiol-containing substances, derivatives of heavy metal ions, alkyl or aryl halides etc.

The medium has to be converted into the free thiol form, i.e. reduced, by removing the 2-thiopyridyl protecting groups with a reducing agent before ligands can be immobilized.

Preparation of the free thiol form of Thiopropyl Sepharose 6B

1. Prepare the freeze-dried powder as described above. Gently remove excess liquid on a glass filter.
2. Suspend the medium in a solution containing 1% (w/v) dithiothreitol or 0.5 M 2-mercaptoethanol in 0.3 M sodium bicarbonate, 1 mM disodium EDTA, adjusted to pH 8.4. Use 4 ml solution per gram freeze dried powder.

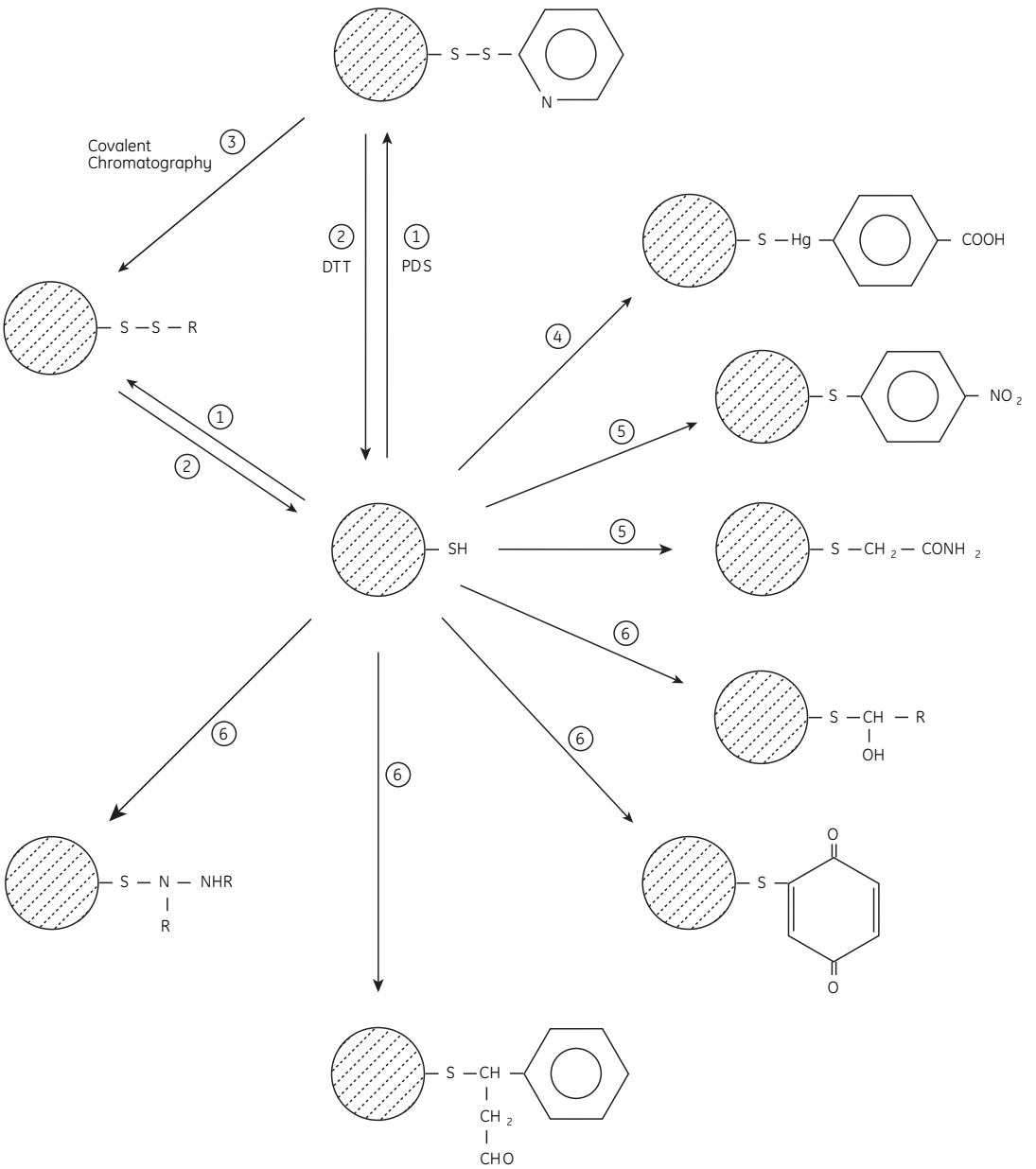

Fig 3. Reactions of immobilized thiol groups. 1; Mixed disulphide formation. 2; Reversible by reducing agents such as dithiothreitol (DTT). 3; Mixed disulphide formation with 2,2' -dipyridyl disulphide gives a 2-thiopyridyl derivative suitable for use in covalent chromatography. 4; Reactions with heavy metals and their derivatives e. g. p-chloromercuri-benzoate leads to mercaptide formation. 5; Treatment with alkyl or aryl halides gives thioether derivatives. 6; Additional reactions are possible with a wide variety of compounds containing C=O, C=C and N=N bonds.

3. Allow to react for 40 minutes at room temperature with gentle mixing. Do not use a magnetic stirrer.
4. Wash the medium thoroughly with 0.1 M acetic acid containing 0.5 M NaCl and 1 mM disodium EDTA. Use a total of 400 ml of solution per gram freeze-dried powder and carry out the washing in several steps.

   The resulting thiol-containing medium may be stored as a suspension in this washing solution. Storage under nitrogen is recommend to prevent oxidation of thiol groups.

Reactions of free thiol groups

Some reactions of free thiol groups are shown in Fig. 3. A more complete treatment of the properties of thiol groups may be found in reference 1.

9. Ordering information

Product	Quantity	Code No.
Thiopropyl Sepharose 6B	15 g	17-0420-01

10. Reference

1. Covalent coupling of unsaturated compounds to thiol agarose using y-radiation. A new method for preparation of adsorbents for affinity chromaotgraphy. *J. Solid-Phase Biochem. 2 (1977) 105-109,* Brandt, J., Svenson, A., Carlsson, J. et al.

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