第六章 親和層析法

壹、 原理概述

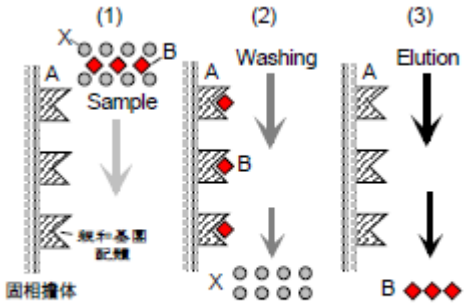


圖 3.8 親和層析法的作用機理

(壹) 固定相為親和基團：

一、 親和層析法的固定相為一固相擔体，上有 專一性親和基團 (A)，流動相為溶離緩衝液。

二、 當樣本通過管柱時，與親和基團有專一性的分子 (B) 結合到固定相上，非專一性分子 (X) 則隨流動相洗出管柱。

三、留在定相上的分子 (B)，可用酸或鹼溶離，或用專一性游離分子溶離。

(貳) 親和法四項要素： 請參考圖 3.9

一、 對所要純化的物質 (B)，需有專一性 配体 ligand (A)，而 A, B 之間要有專一性的親和力，其解離常數 (Kd) 約在 10-4~10-8。

二、 配体 (A) 能方便而大量取得，且能經由耦合反應接到固相擔体上，成為固定相。

三、 擔体具有可與配体連結的基團，且 非專一性吸附力低，通透性良好。

四、 A-B 結合成的 複合体 (complex)，可以方便地解離，而不傷害 A 或 B。(A 似釣竿概念，B 即魚)

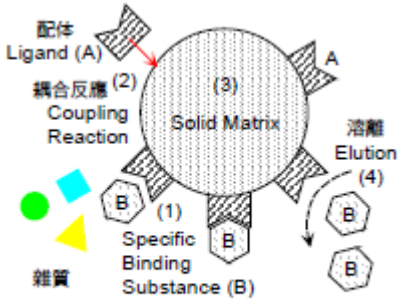


圖 3.9 親和層析法的各項要素

(參) 親和吸著劑

一、 固相擔体：

材料種類很多，舉凡 洋菜糖 (agarsoe)、纖維素、玻璃砂、幾丁質、合成聚合物 均可使用；但用在蛋白質，仍以聚糖類為最佳。 以 Sepharose 為例，可自行用 CNBr 活化，使糖分子接上 -O-C≡N (cyanate ester) 基，再與配体上的胺基反應。

二、 親和性介質：

表 3.3 可與各種配体基團反應的介質 (Pharmacia~Cytiva)：

表 3.3 可與各種配体基團反應的介質 (Pharmacia)：

配体基團	親 和 性 介 質	反應基團	反應方式
$-NH_2$	CNBr-activated Sepharose 4B	-C=N	直接反應
	CH-Sepharose 4B 或其活化型	-COOH	加 EDC*
		N-OH-succinimide	直接反應
	Epoxy-activated Sepharose 6B	oxirane	直接反應
-COOH	AH-Sepharose 4B	$-NH_2$	加 EDC*
-OH	Epoxy-activated Sepharose 6B	oxirane	直接反應
-SH	Epoxy-activated Sepharose 6B	oxirane	直接反應
	Thiopropyl-Sepharose 6B	-S-S-R	DTT 活化
	Activated Thio-Sepharose 4B	-G-S-S-R	直接反應

* EDC = *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide HCl

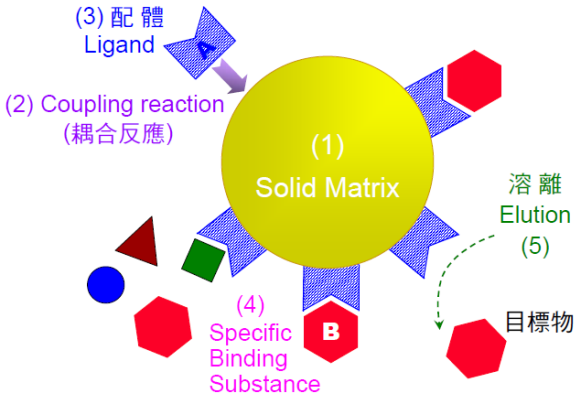
三、 共價層析法：

使用 Thio-Sepharose 時，樣本蛋白質以共價鍵(雙硫鍵)結合到親和介質上，然後再以 cysteine 或 mercaptoethanol 溶離下來；此法可以用來純化 papain 或如 urease 等含 -SH 基的蛋白質，特稱為共價層析法 (covalent chromatography)。

四、 耦合反應：

(一) 介質與配体 ligand 的耦合反應 都相當簡便，介質先經緩衝液洗過後，加入配体溶液反應後，再加入填塞分子，除去介質上未完全反應的基團，裝入管柱流洗後即可使用。

(二) 注意耦合緩衝液及樣本液中，不能含有會競爭耦合反應的分子；例如使用 CNBr 活化的 Sepharose 時，不可用 Tris 或 glycine 緩衝液 (有-NH2 基)。



五、 注意 spacer arm：

有些親和層析法使用 spacer arm 來降低配体的立体障礙，但是 spacer arm 多為六到八碳的碳氫鏈，有相當強的非極性，若表現出疏水性層析的作用 (見下節)，則可能對純化效果有正或負面的影響。

六、 現成的親和吸著劑：

利用以上各種介質，可自行接上有用的配体，進行親和層析法；但商品售有很多已經接好配体的成品，使用上更方便，例舉於表 3.4。

表 3.4 各種親和性介質及其專一性基團：

配 体	親和性分子	說 明
抗体	對應之抗原	免疫吸著劑，大多自行合成
基質或抑制劑	對應之酵素	酵素的專一性結合
Protein A	部分 IgG	單株抗体純化
Con A	醣蛋白	對 α-D-葡萄糖、甘露糖基有專一性
Heparin	凝血蛋白等	Heparin Sepharose CL-6B
Oligo (dT)	mRNA	Oligo (dT)-cellulose
Cibacron-Blue	$NAD(P)^+$ 結合酵素	Blue Sepharose CL-6B
AMP 或 ADP 等	同上	5'AMP-, 2', 5'ADP-Sepharose 4B
單糖及其衍生物	Lectin	用來純化 lectin

七、親和性層析法 (affinity chromatography)實務

基質親和性層析法

將酵素特異性結合的基質或配位體鍵結合到膠體上，可用於純化酵素

免疫親和性層析法

將抗體或抗原鍵結至膠體上，可用於純化抗體或抗原

金屬離子親和性層析法

將膠體結合二價金屬陽離子(Ni^{2+}, Zn^{2+}, Fe^{2+})，能純化表面含有組胺酸的蛋白質。

醣基親和性層析法

將 ConA 結合至膠體上，能分離純化醣蛋白

表 3.4 各種親和性介質及其專一性基團：

配 体	親和性分子	說　　明
抗体	對應之抗原	免疫吸著劑，大多自行合成
基質或抑制劑	對應之酵素	酵素的專一性結合
Protein A	部分 IgG	單株抗体純化
Con A	醣蛋白	對 α-D-葡萄糖、甘露糖基有專一性
Heparin	凝血蛋白等	Heparin Sepharose CL-6B
Oligo (dT)	mRNA	Oligo (dT)-cellulose
Cibacron-Blue	$NAD(P)^+$ 結合酵素	Blue Sepharose CL-6B
AMP 或 ADP 等	同上	5'AMP-, 2', 5'ADP-Sepharose 4B
單糖及其衍生物	Lectin	用來純化 lectin

Components of an affinity medium (親和層析的組成分)

The matrix (固相擔体)

The matrix is an inert support to which a ligand can be directly or indirectly coupled. The list below highlights many of the properties required for an efficient and effective chromatography matrix.

- Extremely low non-specific adsorption, essential since the success of affinity chromatography relies on specific interactions.
- Hydroxyl groups on the sugar residues are easily derivatized for covalent attachment of a ligand, providing an ideal platform for the development of affinity media.
- An open pore structure ensures high capacity binding even for large biomolecules, since the interior of the matrix is available for ligand attachment.
- Good flow properties for rapid separation.
- Stability under a range of experimental conditions such as high and low pH, detergents and dissociating agents.

Sepharose, a bead-form of agarose (Figure 55), provides many of the these properties.

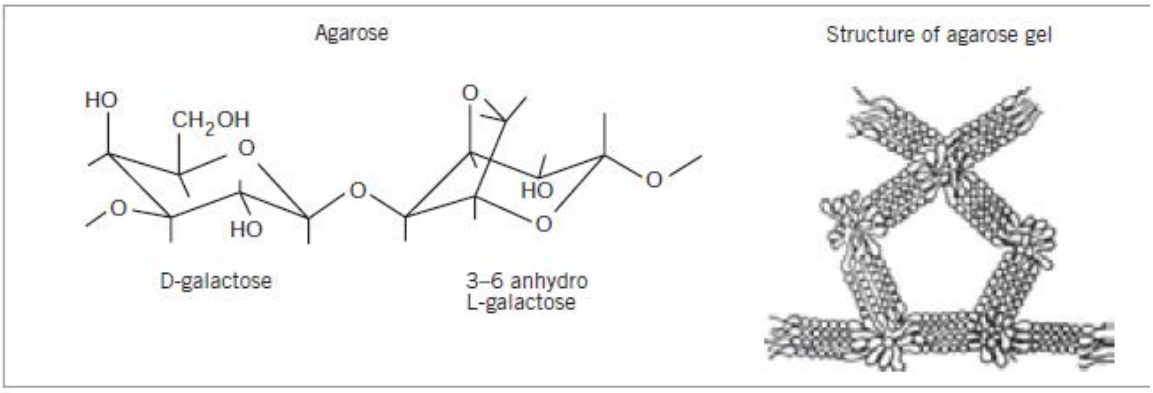


Fig. 55. Partial structure of agarose.

Table 6. Sepharose matrices used with GE Healthcare affinity media.

	Form	Mean particle size
Sepharose High Performance	6% highly cross-linked agarose	34 µm
Sepharose 6 Fast Flow	6% highly cross-linked agarose	90 µm
Sepharose 4 Fast Flow	4% highly cross-linked agarose	90 µm
Sepharose CL-6B	6% cross-linked agarose	90 µm
Sepharose CL-4B	4% cross-linked agarose	90 µm
Sepharose 6B	6% agarose	90 µm
Sepharose 4B	4% agarose	90 µm

The ligand (配體~釣竿)

The ligand is the molecule that binds reversibly to a specific molecule or group of molecules, enabling purification by affinity chromatography. The selection of the ligand for affinity chromatography is influenced by two factors: the ligand must exhibit specific and reversible binding affinity for the target substance(s) and it must have chemically modifiable groups that allow it to be attached to the matrix without destroying binding activity. The dissociation constant (kD) for the ligand - target complex should ideally be in the range 1 0-4 to 10-8 M in free solution.

Spacer arms

The binding site of a target protein is often located deep within the molecule and an affinity medium prepared by coupling small ligands, such as enzyme cofactors, directly to Sepharose may exhibit low binding capacity due to steric interference i.e. the ligand is unable to access the binding site of the target molecule, as shown in Figure 56a. In these circumstances a spacer arm" is interposed between the matrix and the ligand to facilitate effective binding. Spacer arms must be designed to maximize binding, but to avoid non-specific binding effects. Figure 56 shows the improvement that can be seen in a purification as the spacer arm creates a more effective environment for binding.

The length of the spacer arm is critical. If it is too short, the arm is ineffective and the ligand fails to bind substances in the sample. If it is too long, proteins may bind nonspecifically to the spacer arm and reduce the selectivity of the separation. As a general rule, use spacer arms when coupling molecules Mr < 1 000. Spacer arms are not generally needed for larger molecules. Table 7 shows the pre-activated media with different types of spacers arms that are available from GE Healthcare.

Ligand coupling

Several methods are available to couple a ligand to a pre-activated matrix. The correct choice of coupling method depends on the ligand characteristics. The use of commercially available, pre-activated media is recommended to save time and avoid the use of the potentially hazardous reagents that are required in some cases.

Choosing the ligand and spacer arm

The ligand must selectively and reversibly interact with the target molecule(s) and must be compatible with the anticipated binding and elution conditions. The ligand must carry chemically modifiable functional groups through which it can be attached to the matrix without loss of activity (see Table 8). If possible, test the affinity of the ligand: target molecule interaction. Too low affinity will result in poor yields since the target protein may wash through or leak from the column during sample application. too high affinity will result in low yields since the target molecule may not dissociate from the ligand during elution. Use a ligand with the highest possible purity since the final purity of the target substance depends on the biospecific interaction. As discussed in Chapter 4, when using small ligands (Mr < 5 000) there is a risk of steric hindrance between the ligand and the matrix that restricts the binding of target molecules. In this case, select a pre-activated matrix with a spacer arm. For ligands with Mr > 5 000 no spacer arm is necessary.

Choosing the coupling method

Ligands are coupled via reactive functional groups such as amino, carboxyl, hydroxyl, thiol and aldehyde moieties. In the absence of information on the location of binding sites in the ligand, a systematic trial and error approach should be used.

Couple a ligand through the least critical region of the ligand to minimize interference with the normal binding reaction. For example, an enzyme inhibitor containing amino groups can be attached to a matrix

through its amino groups, provided that the specific binding activity with the enzyme is retained. However, if the amino groups are involved in the binding reaction, an alternative, non-essential, functional group must be used. Avoid using a functional group that is close to a binding site or that plays a role in the interaction between the ligand and target molecule. If a suitable functional group does not exist, consider derivatizing the ligand to add a functional group.

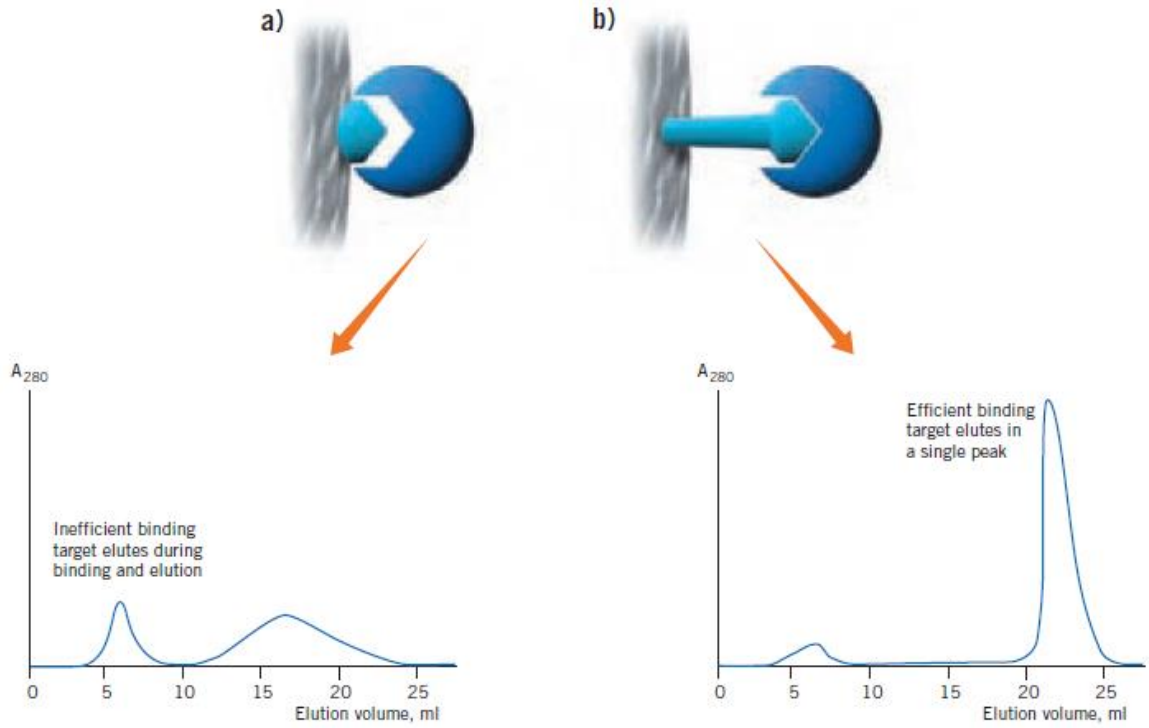


Fig. 56. Using spacer arms. a) Ligand attached directly to the matrix. b) Ligand attached to the matrix via a spacer arm.

Table 7. Examples of pre-activated media.

NHS-activated Sepharose High Performance	12-atom hydrophilic spacer arm to couple via amino groups.
NHS-activated Sepharose 4 Fast Flow	As above.
CNBr-activated Sepharose 4 Fast Flow	Coupling via primary amino groups.
EAH Sepharose 4B	10-atom spacer arms to couple via amino groups.
ECH Sepharose 4B	9-atom spacer arms to couple via carboxyl groups.
Epoxy-activated Sepharose 6B	12-atom hydrophilic spacer arm to couple through hydroxyl, amino or thiol groups.
Activated Thiol Sepharose 4B	10-atom spacer arm for reversible coupling through free thiol groups.
Thiopropyl Sepharose 6B	4-atom hydrophilic spacer arm for reversible coupling of proteins and small thiolated ligands through thiol groups. Also reacts with heavy metal ions, alkyl and aryl halides and undergoes addition reactions with compounds containing C=O, C=C and N=N bonds.

Table 8.

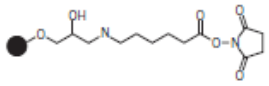
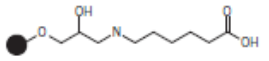
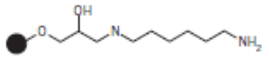
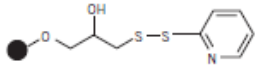
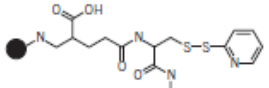
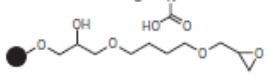
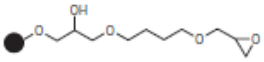
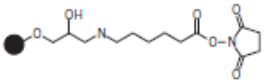
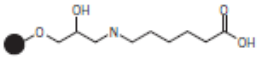
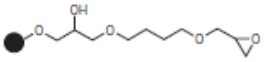
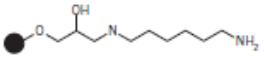
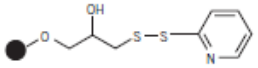
Chemical group on ligand	Length of spacer arm	Structure of spacer arm	Product
Proteins, peptides, amino acids			
amino	10-atom		HiTrap NHS-activated HP NHS-activated Sepharose 4 Fast Flow
	None	–	CNBr-activated Sepharose 4B CNBr-activated Sepharose 4 Fast Flow
	10-atom		ECH Sepharose 4B
carboxyl	11-atom		EAH Sepharose 4B
thiol	4-atom		Thiopropyl Sepharose 6B
	10-atom		Activated Thiol Sepharose 4B
	12-atom		Epoxy-activated Sepharose 6B
Sugars			
hydroxyl	12-atom		Epoxy-activated Sepharose 6B
amino	10-atom		HiTrap NHS-activated HP
	10-atom		ECH Sepharose 4B
	12-atom		Epoxy-activated Sepharose 6B
carboxyl	11-atom		EAH Sepharose 4B
Polynucleotides			
amino	None		CNBr-activated Sepharose 4B CNBr-activated Sepharose 4 Fast Flow
mercurated base	4-atom		Thiopropyl Sepharose 6B
Coenzymes, cofactors, antibiotics, steroids			
amino, carboxyl, thiol or hydroxyl			use matrix with spacer arm (see above)

Table 9 summarizes recommended ligand concentrations according to the experimental conditions.

Table 9.

Experimental condition	Recommended concentration for coupling
Readily available ligands	10–100 fold molar excess of ligand over available groups
Small ligands	1–20 µmoles/ml medium (typically 2 µmoles/ml medium)
Protein ligands	5–10 mg protein/ml medium
Antibodies	5 mg protein/ml medium
Very low affinity systems	Maximum possible ligand concentration to increase the binding

CNBr-activated Sepharose

Cyanogen bromide (CNBr) reacts with hydroxyl groups on Sepharose to form reactive cyanate ester groups. Proteins, peptides, amino acids or nucleic acids can be coupled to CNBr-activated Sepharose, under mild conditions, via primary amino groups or similar nucleophilic groups. The activated groups react with

primary amino groups on the ligand to form isourea linkages (Figure 61). The coupling reaction is spontaneous and requires no special chemicals or equipment. The resulting multi-point attachment ensures that the ligand does not hydrolyze from the matrix. The activation procedure also cross-links Sepharose and thus enhances its chemical stability, offering considerable flexibility in the choice of elution conditions.

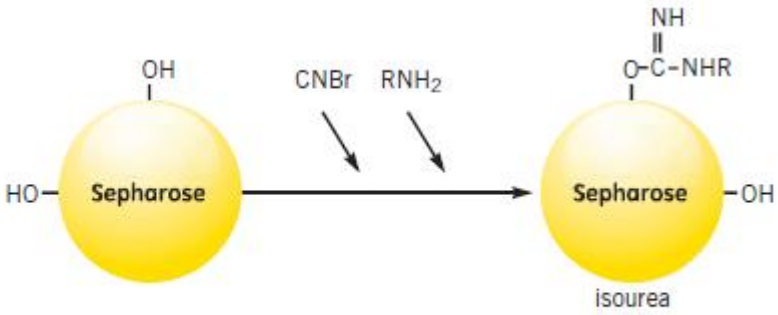


Fig. 61. Activation by cyanogen bromide and coupling to the activated matrix.

Options

Product	Spacer arm	Coupling conditions	Maximum operating flow	Comments
CNBr-activated Sepharose 4 Fast Flow	None	pH 7–9, 2–16 hours, +4 °C - room temp.	400 cm/h*	Supplied as a freeze-dried powder.
CNBr-activated Sepharose 4B	None	pH 8–10, 2–16 hours, +4 °C - room temp.	75 cm/h*	Supplied as a freeze-dried powder.

There are many examples in the literature of the use of CNBr-activated Sepharose. Figure 62 shows the separation of a native outer envelope glycoprotein, gp120, from HIV-1 infected T-cells. Galanthus nivalis agglutinin (GNA), a lectin from the snowdrop bulb, was coupled to CNBr-activated Sepharose 4 Fast Flow to create a suitable affinity medium.

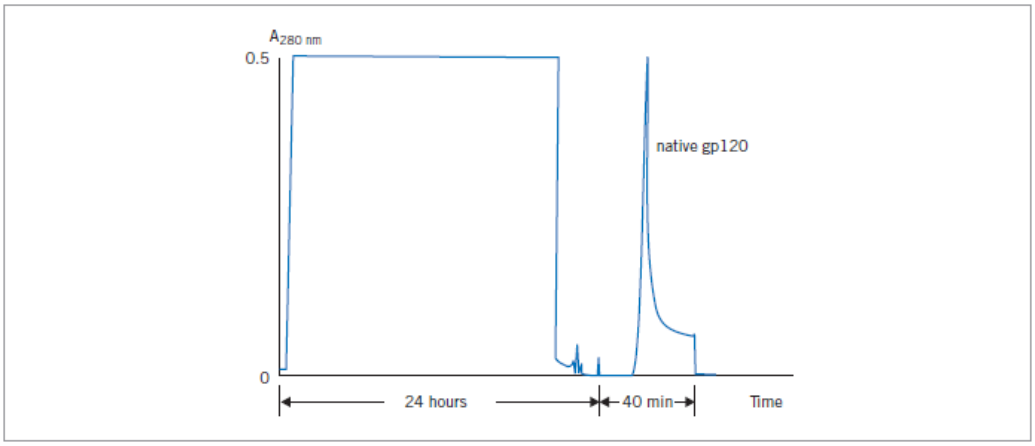


Fig. 62. Separation of native gp120 protein on GNA coupled to CNBr-activated Sepharose 4 Fast Flow. From Gilljam, G. *et al.*, Purification of native gp120 from HIV-1 infected T-cells. Poster presented at Recovery of Biological Products VII, Sept. 25-30, 1994, San Diego, CA, USA. Further details are available in the CNBr-activated Sepharose 4 Fast Flow datafile, from GE Healthcare.

Coupling through hydroxy, amino or thiol groups via a 12-carbon spacer arm

Epoxy-activated Sepharose 6B

Epoxy-activated Sepharose 6B is used for coupling ligands that contain hydroxyl, amino or thiol groups. Because of the long hydrophilic spacer arm, it is particularly useful for coupling small ligands such as choline, ethanolamine and sugars. The pre-activated matrix is formed by reacting Sepharose 6B with the bis oxirane, 1,4 bis-(2,3-epoxypropoxy-)butane. The partial structure is shown in Figure 67.

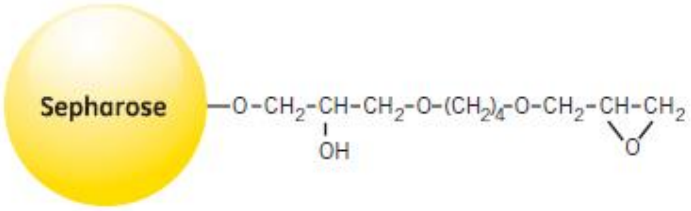


Fig. 67. Partial structure of Epoxy-activated Sepharose 6B.

Options

Product	Spacer arm	Substitution per ml matrix	Coupling conditions	Maximum operating flow	Comments
Epoxy-activated Sepharose 6B	12-atom	19–40 µmoles epoxy groups	pH 9–13, 16 hours - several days, +20 - +40 °C	75 cm/h*	Supplied as a freeze-dried powder.

Purification example

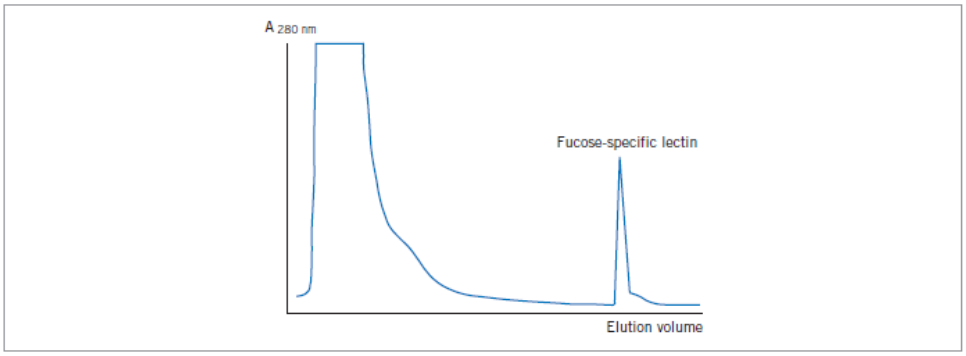


Fig. 68. Chromatography of a crude extract of *Ulex europaeus* on fucose coupled to Epoxy-activated Sepharose 6B, column volume 11 ml. Extract was applied in 0.9% NaCl. Fucose-specific lectin was eluted with 5 ml fucose (50 mg/ml).

Alternative coupling solutions:

Distilled water or aqueous buffers with sugars and carbohydrates are preferable. Carbonate, borate or phosphate buffers can be used. Sodium hydroxide may be used for solutions of high pH. Organic solvents such as dimethylformamide (up to 50%) and dioxane (up to 50%) may be used to dissolve the ligand. The same concentration of organic solvent should be included in the coupling solution.

Coupling procedure

1. Suspend the required amount of freeze-dried powder in distilled water (1 g freeze-dried powder gives about 3.0 ml final matrix volume).
2. Wash immediately for 1 hour on a sintered glass filter (porosity G3), using approximately 200 ml distilled water per gram freeze-dried powder, added in several aliquots.
3. Dissolve the ligand in the coupling buffer to a final concentration of 0.5–10 mg/ml (for protein ligands) or transfer solubilized ligands into the coupling buffer using a desalting column (see page 133). Adjust the pH of the aqueous phase.
4. Use a matrix:buffer ratio of 1:2, mix the matrix suspension with the ligand solution for 16 h at +25 to +40 °C in a shaking water bath.
5. Block remaining excess groups with 1 M ethanolamine for at least 4 h or overnight, at +40 to +50 °C.
6. Wash away excess ligand with coupling solution followed by distilled water, 0.1 M NaHCO3, 0.5 M NaCl, pH 8.0 and 0.1 M NaCl, 0.1 M acetate, pH 4.0.

Do not use Tris, glycine or other nucleophilic compounds as these will couple to the oxirane groups. Do not use magnetic stirrers as they may disrupt the Sepharose matrix.

雞蛋的卵白中，含有胰蛋白酶 (trypsin) 的專一性抑制劑 – 雞卵粘多醣蛋白(chicken ovomucoid protein, CHOM)。本實驗以丙酮沈澱 CHOM，離心取得蛋白質並乾燥後，以 Bradford method 測定其蛋白質含量。所得的 CHOM 將用在下一個實驗 P2，作為親和層析法的專一性配體，以便釣取胰蛋白酶；並且將在膠體電泳實驗 P3 檢查其純度。鴨蛋蛋白中也有類似的黏多糖蛋白 DKOM，除了可以結合 trypsin 之外，也可與 chymotrypsin 結合，其抽取純化方法與 CHOM 相同。

親和層析法有點像在釣魚。 釣竿是蟹殼幾丁質，釣餌是上次純化的 CHOM，用來釣胰臟抽取液中的胰蛋白酶 trypsin；這是因為 CHOM 對 trypsin 有專一性吸引力。蛋白質間的專一性吸引力，已成為最近生物化學探討的重要主題。若 A 與 B 兩分子之間，有專一性的親和力，而我們手中已有 A；若欲由樣本混合物中，將所含的 B 分子分離出來，則最方便的方法，是先把 A 固定在一固定相(solid phase) 上，當樣本混合物通過此固定相時，其中的 B 分子即被 A 吸附到固定相。俟洗去混合物中其它雜質後，破壞 A-B 之間的吸引力，溶離下 B，即可得到純質 B。親和層析法是很有效的純化方法，但並非任何物質均可應用此法，因為不一定能找到對它有親和力的分子。因此，進行親和層析法之第一條件為，尋找分子之間具有親和力的配對：

A ＋ B = AB [式 1]

而其解離常數，Kd ＝[A] [B] / [AB] ~ 10^{-4} 至 10^{-8}；親和力大小須適中。 [式 2]

可以用圖 P2-1 說明親和層析法的進行步驟：

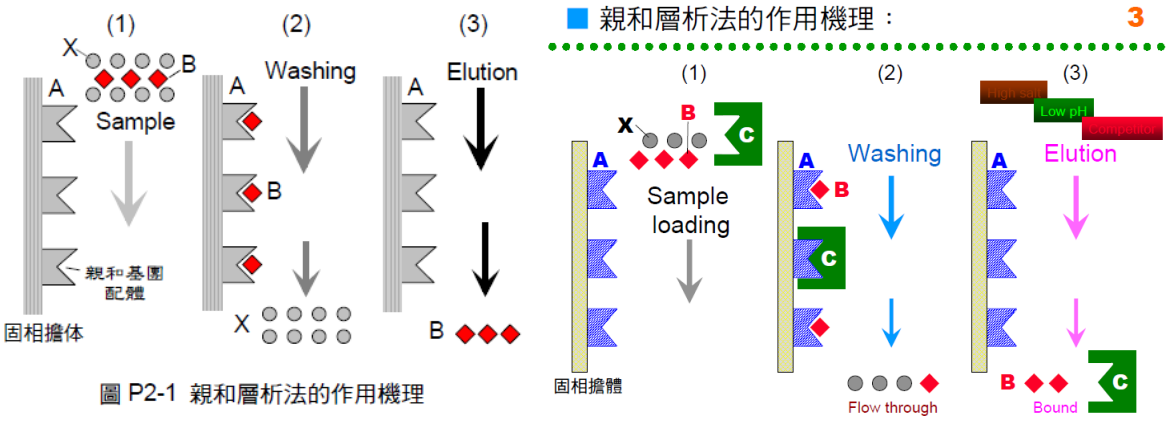


圖 P2-1 親和層析法的作用機理

1) 分子 A 已經被結合到固相擔体上(斜線部份)，樣本準備通過之，其中含有所要分離的 B 分子(◆) 以及雜質 (●)。
2) 當樣本通過此親和吸著劑時，只有 B 被吸著住，其餘雜質將直接流出。
3) 破壞 AB 分子之間的親和力，即可收穫得純質 B。

2.1 親和層析法的各項要素

要成功建立一個良好的親和層析法，必須考慮幾個重要因素，以圖 P2-2 說明之：

2.1.1 良好的固相擔体(solid support)

固相擔体的材質要有良好多孔性，通透性佳，構成的分子骨架本身穩定，無太大的非專一性吸附；最重要的是要具有相當活性的官能基(functional group)，以便與其它分子鍵結。通常用作擔体的材質有： 洋菜醣 (agarose)、聚丙烯醯胺(polyacrylamide)、幾丁質(chitin)、聚苯乙烯(polystyrene)。

2.1.2 Ligand 及其專一性結合分子

配體 (ligand, 即鍵結在固相擔体上分子之統稱) 與其目標分子之間，要有相當強的親和力(如上述)；例如抗原-抗体、酵素-基質、荷爾蒙-受体(receptor)、酵素-抑制因子(如 trypsin 與 CHOM)。一般把較容易得到的分子做為餌，結合到固相擔体上，成為親和吸著劑(affinity adsorbent)。

2.1.3 Ligand 與固相擔体間之的耦合反應

Ligand 與固相擔体之間，須有官能基可供鍵結反應：

固相擔体-X + Y-Ligand → 固相擔体-x-y-Ligand [式 3]

常用的耦合反應如下列幾種：

a) 以 carbodiimide 進行脫水反應，連結胺基與酸基。

b) 以 CNBr 活化 agarose 醣分子上的醇基，可接上蛋白質的胺基。

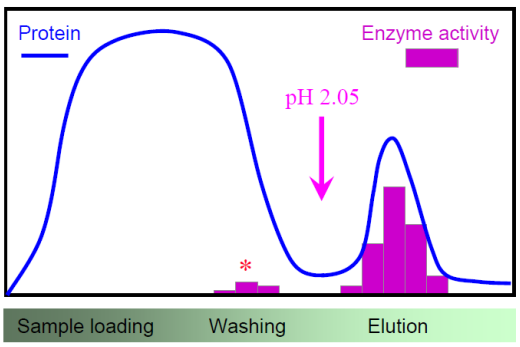
c) 固相接有 N-hydroxysuccinimide (可看作活化的酸基)，可與胺基反應。

d) 以 glutaraldehyde (分子上有雙醛基) 為中間架橋物，可連接兩個胺基。

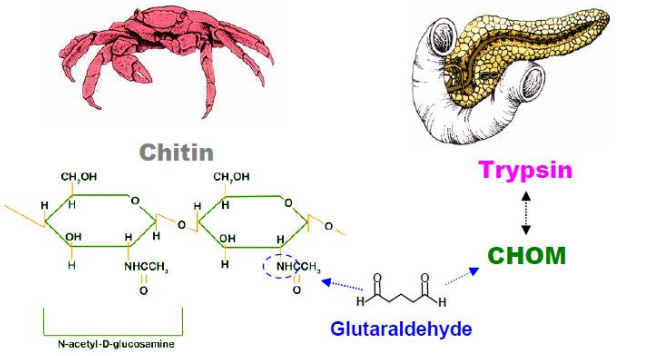
2.1.4 可溶離下所要分離之目標分子

可改變溶離的 pH、離子強度或其它方法；注意某些方法可能會使 ligand 或所欲純化之分子，受到不同程度的破壞。因此，所選用的親和性配對，兩者間的親和力也不能太大，否則很難把結合上去的蛋白質溶離下來。

■ 典型的親和層析操作： 4



■ 親和層析法實例的原始材料： 5



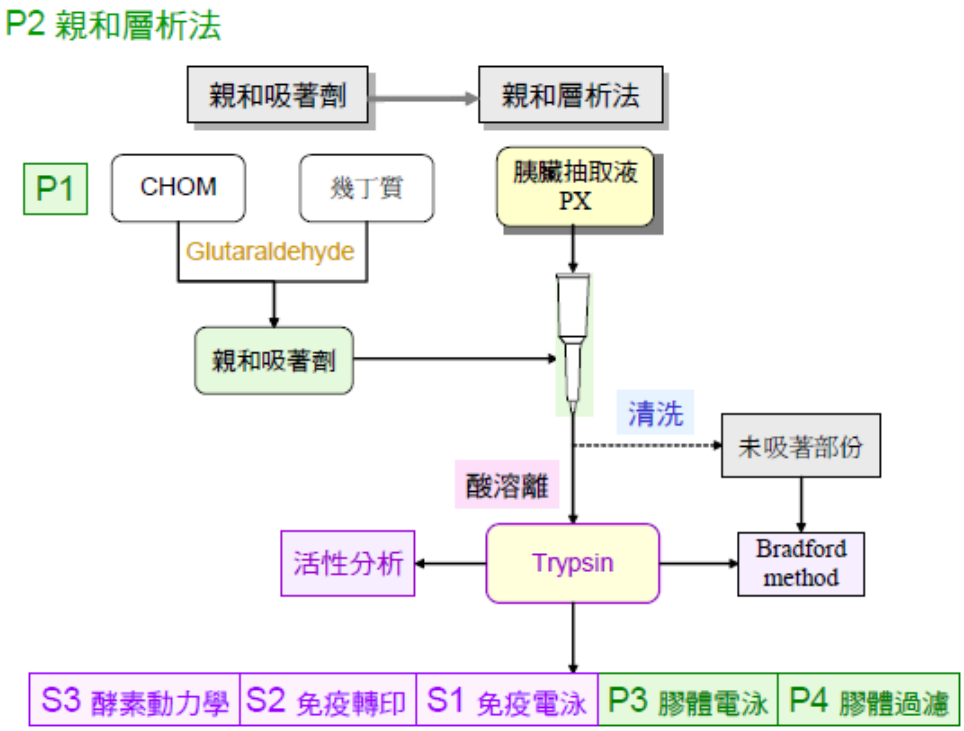


圖 P2-3 製備親和吸著劑與親和層析法流程

先做好親和吸著劑，裝入管柱後通入胰臟抽取液，再以酸溶離出所要的酵素。如此所的到的酵素純度很高，將應用在後面的許多實驗，是個非常關鍵的步驟。

2.2 實驗操作

本實驗先製備幾丁質親和吸著劑，然後再加入胰臟抽取液，進行親和層析法。所有操作前，請檢查所有試劑及儀器，並確實瞭解其使用及操作方法。

2.2.1 儀器設備

a) 小型塑膠管柱(0.5×5 cm) 或用注射針筒亦可。
b) 分劃收集器(Pharmacia 或 Gilson)：若無分劃收集器，以液體高度估計體積。
c) 小試管 20 支及試管架。
c) ELISA 光度計(Dynatech)。
d) 微量滴定盤(96-well microtiter plate)。

2.2.2 藥品試劑

a) Chitin (幾丁質)：蟹殼曬乾，磨粉過篩後，以強酸、強鹼處理得之。
b) CHOM (雞卵白粘多醣蛋白)：P1 實驗所純化的 CHOM 溶液。
c) Glutaraldehyde (戊二醛 30%)：具雙官能基的連結劑。
d) Tris-HCl 緩衝液(0.1 M, pH 7.5)。
f) 胰臟抽取液(PX) 每組 8 mL：取 pancreatin 5 g 溶於 200 mL Tris-HCl 緩衝液，充分攪拌後，加入 5 g 矽藻土，並以過濾得到清澈的 PX 溶液。
g) Coomassie Brilliant Blue G-250：蛋白質定量呈色用 (見 P1, 1.3)。
h) 甲酸液(0.1 M, pH 2.05)：改變 pH 以便溶離 trypsin 下來。
i) BAPNA 合成基質液(benzoyl-arginine p-nitroanilide)：可被 trypsin 水解呈黃色。配製法： 取 BAPNA 43.5 mg 加入 1 mL DMSO 懸濁均勻，等約 1 h 完全溶解，慢慢滴入 100 mL Tris (0.05 M, pH 8.2, 含 20 mM CaCl2) 同時快速攪拌。

2.2.3 親和吸著劑之製備

1) 取幾丁質 2 g，盡量除去水分，置於 15 mL 塑膠離心管中。
2) 加入 4 mL CHOM 液，均勻懸著之 (剩下的 CHOM 請保留起來)。
3) 加 0.1 mL 25% glutaraldehyde，封口後混合均勻。
4) 在室溫反應 30~60 min，不時輕輕上下倒轉，均勻混合。
5) 以傾倒法用蒸餾水洗若干次，最後加適量 Tris-HCl 緩衝液懸濁之。
6) 裝入管柱，俟沉降後用 10 mL 緩衝液流洗，讓 Tris-HCl 慢慢通過，洗約數分鐘後備用。

2.2.4 親和層析法之操作

1) 如上準備好親和層析管柱，在 Tris-HCl 緩衝液平衡完全後，塞住出口，吸去吸著劑上方的液体，但勿使吸著劑乾掉。
2) 小心加入 8 mL 之胰臟抽取液(PX)，不可弄亂吸著劑表面。
3) 打開出口，調整流速約每 4 s 一滴。

◆ 請事先作好收集試管的準備： 取 4 mL 水置入試管中，並在其液面高度做記號，然後以所收集流出液的高度，作為每一分劃的依據。

4) 馬上收集流出液，大約每 4 mL (或 80 滴) 收一支，約收 8 支試管；同時注意勿使吸著劑乾掉，當 PX 完全沒入吸著劑表面後，小心追加 Tris-HCl。

5) 收集 8 支試管後，關住出口；每支分別取樣 50 µL，以 Bradford method 檢測蛋白質的量，到後面的試管應該已經完全洗淨，若尚未洗淨，再洗數支試管。

◆ 若有必要，可以收集蛋白質濃度較高的幾隻試管，再通過一次親和管柱。

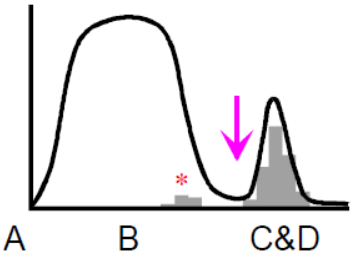


圖 P2-4 典型的親和層析圖譜

6) 再次吸去吸著劑上方之液体，改加入 0.1 M 甲酸液(pH 2.05)，打開出口，流速降為每 8 s 一滴，馬上收集流出液，每 2 mL 收一支，收集 4 支後暫停。

7) 每支試管取 50 µL 以 Bradford method 檢測蛋白質量，應在前面一兩支出現。

8) 同法取樣 50 µL，在微量滴定盤上加入 200 µL BAPNA 液，混合後稍稍加溫，樣本中若有 trypsin，則呈黃色反應；以 ELISA 光度計記下吸光值(A405)。

◆ 注意：前面所收集的 8 支試管也可以作 BAPNA 活性分析。

9) 取呈色最深的一或兩支試管(步驟 6 中) 置入一隻全新 15 mL 離心管，寫好組別後交出，下面實驗將以電泳檢定之(分子量及純度)。

◆ 若有兩隻呈色相同，可以混合後交出。

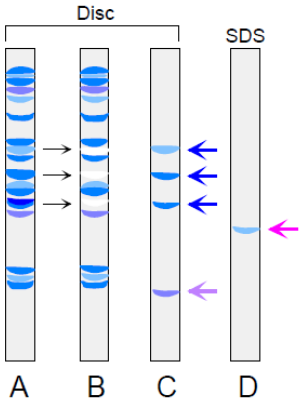


圖 P2-5 電泳分析結果

2.3 結果與報告

2.3.1 實驗結果

a) 以條列方式，把整個實驗過程及步驟，一一寫下來，請勿直接抄襲 2.2 節。

b) 收集所測得的吸光值作為 y 軸，並以各分劃為 x 軸，製作色析圖譜。可製作得兩條吸光曲線，一為蛋白質含量，另一為酵素活性，類似下面圖 P2-4。

2.3.2 附註說明：

a) 親和層析法的純化效果非常好，但非萬能，有許多限制與缺點，應當注意：

1) 並非所有的物質都可找到具有親和力的 ligand，也非所有的物質均能有效地誘導出抗体。請試著舉出自然界中，所有可能的專一性配對分子。

2) 經常要使用強烈的條件，去溶離下所要的物質(例如用 pH 2)，因而不免破壞物質的穩定性與活性。幸運的是，trypsin 在酸性環境下相當安定。

3) 有時非專一性吸附很難避免，純化效果會打折扣；如何避免之？

b) 圖 2-4 為親和層析法的典型溶離圖形。以本實驗為例，先洗去許多雜質，可能有些過量的酵素活性出現在後端(*)； 再用甲酸溶離 (箭頭處)，即可收得所要的 trypsin。為何甲酸可以把吸附上去的蛋白質溶離下來？

c) 每一個分離階段的樣本，都可以收集起來，再以電泳分析其蛋白質成份。圖 P2-5 是圖 P2-4 所收得樣本的電泳結果，並以兩種不同電泳來檢視所含的蛋白質，請嘗試解釋所得電泳圖譜的意義。

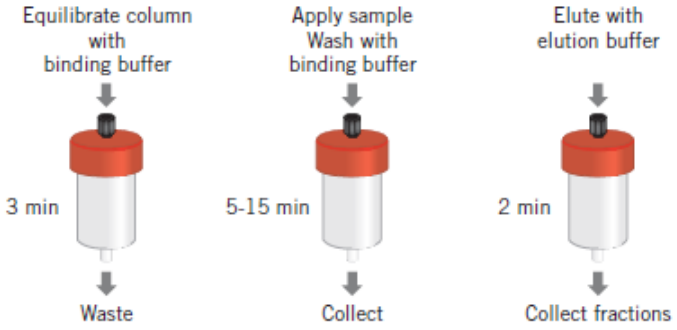


Figure 4 shows the simple procedure used to perform affinity purification on prepacked HiTrap columns.

Use high quality water and chemicals. Solutions should be filtered through 0.45 µm or 0.22 µm filters. Avoid using magnetic stirrers as they may damage the matrix. Use mild rotation or end-over-end stirring.

pH elution、Ionic strength elution (usually NaCl) 、Competitive elution

Enzymes usually elute at a concentration of 1 M NaCl or less

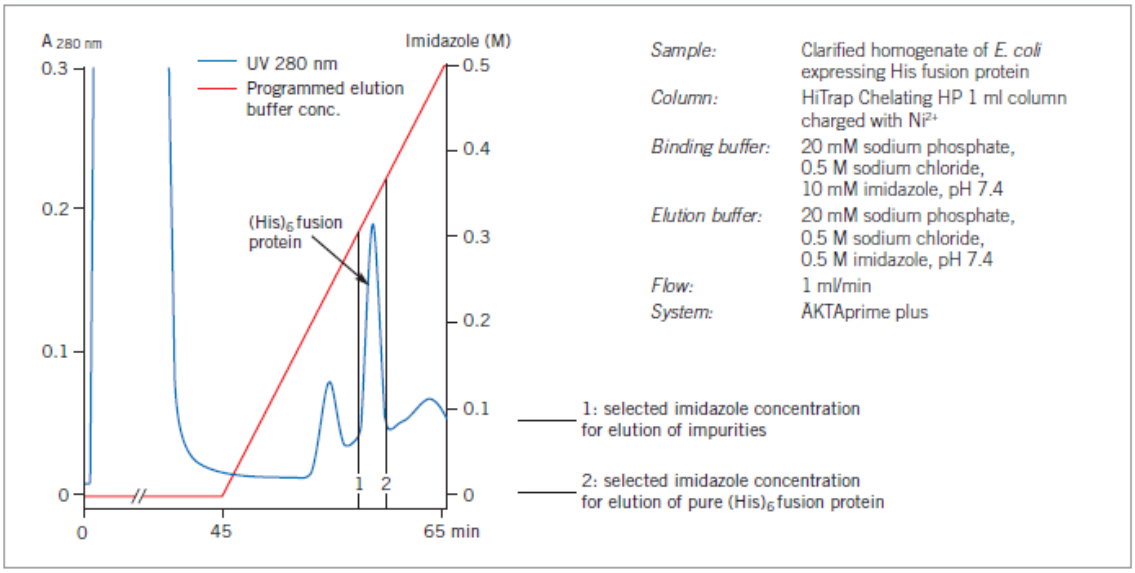


Fig. 7. Gradient elution of a $(His)_6$ fusion protein.

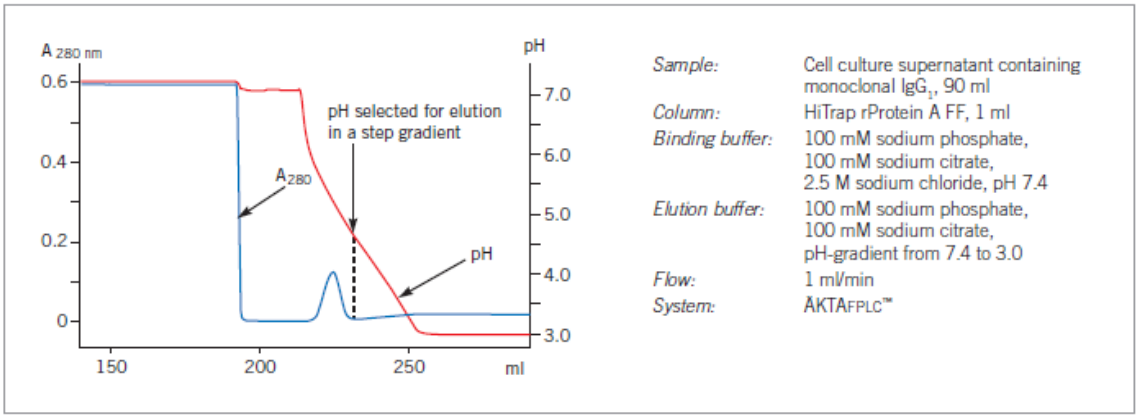


Fig. 8. Scouting for optimal elution pH of a monoclonal IgG_1 from HiTrap rProtein A FF, using a pH gradient.

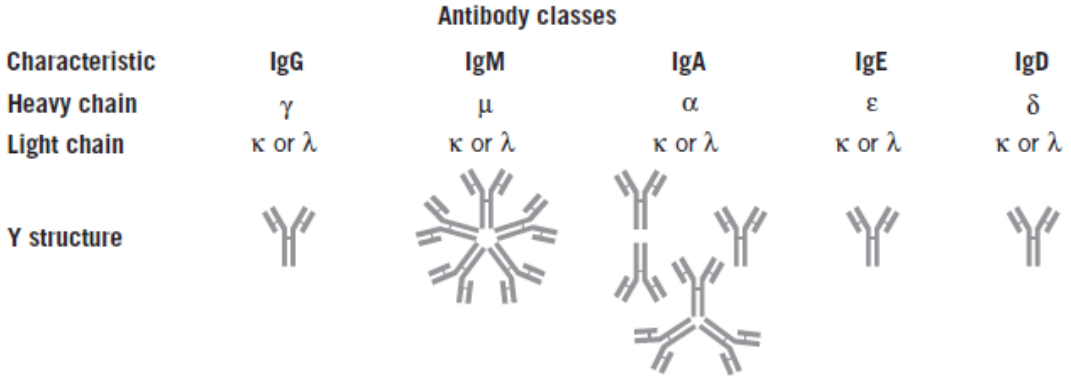


Fig. 10. Antibody classes.

Protein A and protein G are bacterial proteins (from Staphylococcus aureus and Streptococcus, respectively) which, when coupled to Sepharose, create extremely useful, easy to use media for many routine applications. Examples include the purification of monoclonal IgG-type antibodies, purification of polyclonal IgG subclasses, and the adsorption and purification of immune complexes involving IgG. IgG subclasses can be isolated from ascites fluid, cell culture supernatants and serum.

	Binding capacity	Maximum operating flow	Comments
HiTrap Protein G HP	Human IgG, > 25 mg/column Human IgG, >125 mg/column	4 ml/min (1 ml column) 20 ml/min (5 ml column)	Purification of IgG, fragments and subclasses, including human IgG_3. Strong affinity for monoclonal mouse IgG_1 and rat IgG. Prepacked columns.
MAbTrap Kit	Human IgG, > 25 mg/column	4 ml/min	Purification of IgG, fragments and subclasses, including human IgG_3. Strong affinity for monoclonal mouse IgG_1 and rat IgG. Complete kit contains HiTrap Protein G HP (1 x 1 ml), accessories, pre-made buffers for 10 purifications and detailed experimental protocols.
Protein G Sepharose 4 Fast Flow	Human IgG, > 20 mg/ml medium Cow IgG, 23 mg/ml medium Goat IgG, 19 mg/ml medium Guinea pig IgG, 17 mg/ml medium Mouse IgG, 10 mg/ml medium Rat IgG, 7 mg/ml medium	400 cm/h*	Supplied as a suspension ready for column packing.

Table 2. Relative binding strengths of protein A and protein G to various immunoglobulins. No binding: –, relative strength of binding: +, ++, +++, ++++.

Species	Subclass	Protein A binding	Protein G binding
Human	IgA	variable	–
	IgD	–	–
	IgE		
	IgG_1	++++	++++
	IgG_2	++++	++++
	IgG_3	–	++++
	IgG_4	++++	++++
	IgM*	variable	–
Avian egg yolk	IgY**	–	–
Cow		++	++++
Dog		++	+
Goat		–	++
Guinea pig	IgG_1	++++	++
	IgG_2	++++	++
Hamster		+	++
Horse		++	++++
Koala		–	+
Llama		–	+
Monkey (rhesus)		++++	++++
Mouse	IgG_1	+	++++
	IgG_{2a}	++++	++++
	IgG_{2b}	+++	+++
	IgG_3	++	+++
	IgM*	variable	–
Pig		+++	+++
Rabbit	no distinction	++++	+++
Rat	IgG_1	–	+
	IgG_{2a}	–	++++
	IgG_{2b}	–	++
	IgG_3	+	++
Sheep		+/–	++

Purification options

	Binding capacity	Maximum operating flow	Comments
HiTrap Protein A HP	Human IgG, > 20 mg/column Human IgG, > 100 mg/column	4 ml/min (1 ml column) 20 ml/min (5 ml column)	Purification of IgG, fragments and sub-classes. Prepacked columns.
Protein A Sepharose 4 Fast Flow*	Human IgG, > 35 mg/ml medium Mouse IgG, 3–10 mg/ml medium	400 cm/h**	Supplied as a suspension ready for column packing.
HiTrap rProtein A FF	Human IgG, > 50 mg/column Human IgG, > 250 mg/column	4 ml/min (1 ml column) 20 ml/min (5 ml column)	Purification of IgG, fragments and sub-classes. Enhanced binding capacity. Prepacked columns.
rProtein A Sepharose 4 Fast Flow*	Human IgG, > 50 mg/ml medium Mouse IgG, 8–20 mg/ml medium	300 cm/h**	Enhanced binding capacity. Supplied as a suspension ready for column packing.
MabSelect™ (recombinant protein A ligand)	Human IgG, approx. 30 mg/ml medium	500 cm/h**	For fast processing of large sample volumes. Retains high binding capacity at high flow rates. Supplied as a suspension ready for column packing.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
HiTrap Protein G HP (MAbTrap Kit)	2 mg/ml	Ligand coupled to Sepharose HP by N-hydroxysuccinimide activation (gives stable attachment through alkylamine and ether links).	Long term 3–9 Short term 2–9	34 µm
Protein G Sepharose 4 Fast Flow	2 mg/ml	Ligand coupled to Sepharose 4 Fast Flow by cyanogen bromide activation.	Long term 3–9 Short term 2–10	90 µm

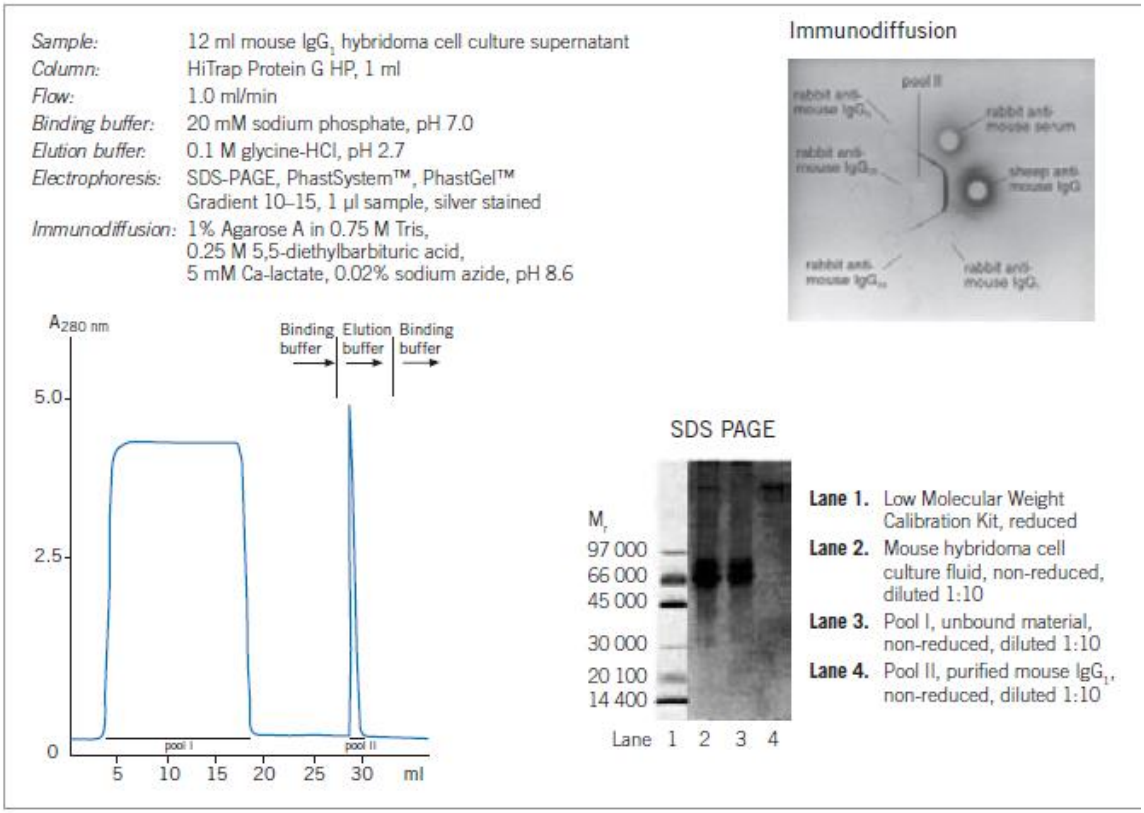


Fig. 11. Purification of monoclonal mouse IgG_1 on HiTrap Protein G HP, 1 ml.

Fig. 16. Using HiTrap Protein G HP with a syringe. A: Dilute buffers and prepare sample. Remove the column's top cap and twist off the end. B: Equilibrate the column, load the sample and begin collecting fractions. C: Wash and elute, continuing to collect fractions.

Chemical stability

Stable in all common aqueous buffers.

Storage

Wash media and columns with 20% ethanol (use approximately 5 column volumes for packed media) and store at +4 to +8 °C.

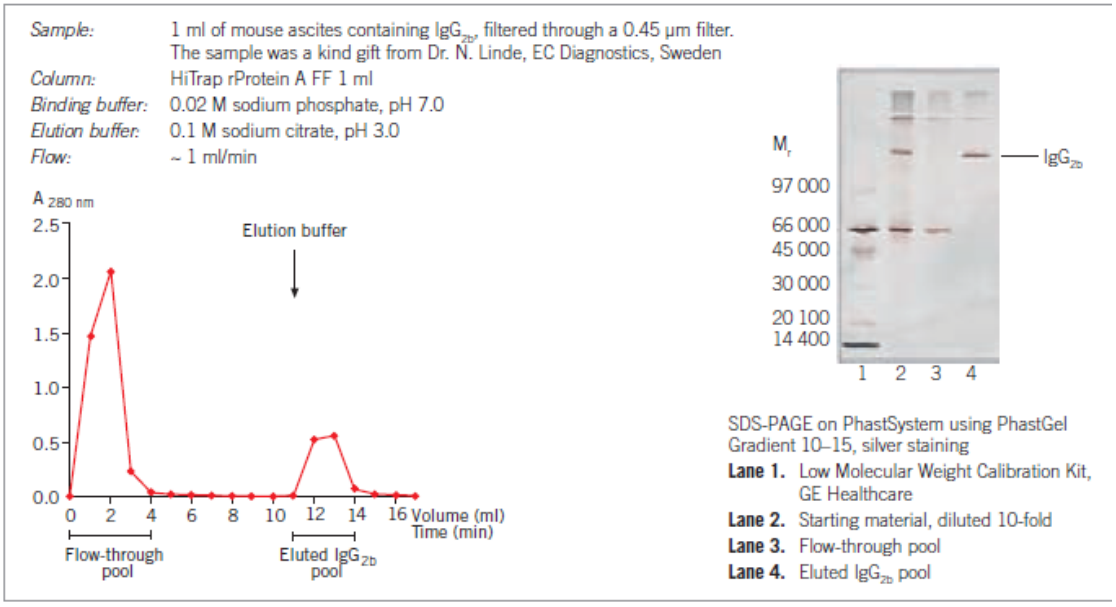


Fig. 17. Purification of mouse IgG_{2b} from ascites on HiTrap rProtein A FF 1 ml column using a syringe.

Table 3 gives examples of some typical binding and elution conditions that have been used with Protein A Sepharose.

Table 3.

Species	Subclass	Binding to free protein A	Protein A Sepharose binding pH	Protein A Sepharose elution pH
				Usually elutes by pH 3
Human	IgG_1	++	6.0–7.0	3.5–4.5
	IgG_2	++	6.0–7.0	3.5–4.5
	IgG_3	–	8.0–9.0	≤ 7.0
	IgG_4	++	7.0–8.0	use step elution
Cow	IgG_2	++		2
Goat	IgG_2	+		5.8
Guinea pig	IgG_1	++		4.8
	IgG_2	++		4.3
Mouse	IgG_1	+	8.0–9.0	5.5–7.5
	IgG_{2a}	+	7.0–8.0	4.5–5.5
	IgG_{2b}	+	7	3.5–4.5
	IgG_3	+	7	4.0–7.0
Rat	IgG_1	+	≥ 9.0	7.0–8.0
	IgG_{2a}	–	≥ 9.0	≤ 8.0
	IgG_{2b}	–	≥ 9.0	≤ 8.0
	IgG_3	+	8.0–9.0	3–4 (using thiocyanate)

HiTrap IgM Purification HP columns are packed with a thiophilic adsorption medium (2-mercaptopyridine coupled to Sepharose High Performance). The interaction between the protein and the ligand has been suggested to result from the combined electron donating- and accepting-action of the ligand in a mixed mode hydrophilic-hydrophobic interaction.

Purification option

	Binding capacity	Maximum operating flow	Comments
HiTrap IgM Purification HP	Human IgM, 5 mg/column	4 ml/min	Purification of monoclonal and human IgM. Prepacked 1 ml column.

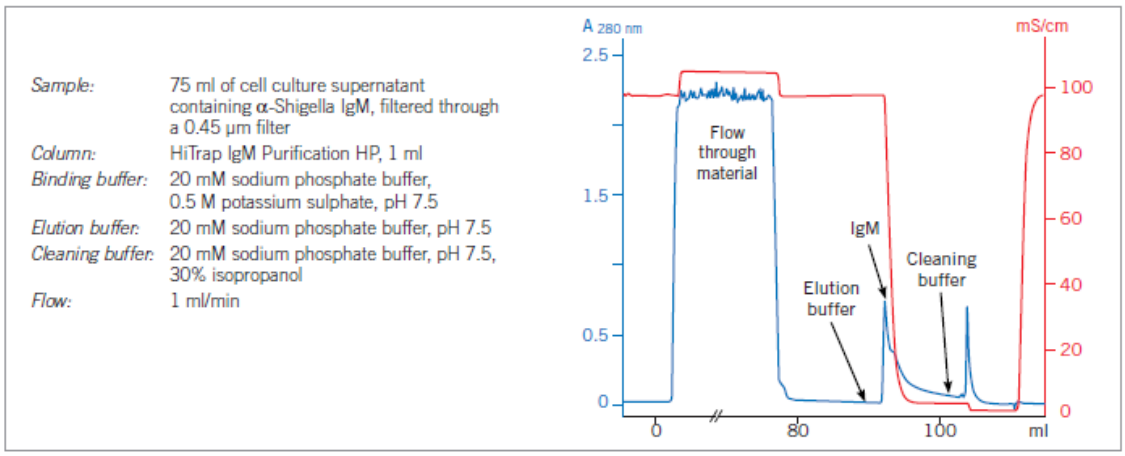


Fig. 19a. Purification of α-Shigella IgM on HiTrap IgM Purification HP.

HiTrap IgY Purification HP columns are packed with a thiophilic adsorption medium (2-mercaptopyridine coupled to Sepharose High Performance). The interaction between the protein and the ligand has been suggested to result from the combined electron donating- and accepting-action of the ligand in a mixed mode hydrophobic-hydrophilic interaction.

Purification option

	Binding capacity	Maximum operating flow	Comments
HiTrap IgY Purification HP	100 mg pure IgY/column (1/4 egg yolk)	20 ml/min	Purification of IgY from egg yolk. Prepacked 5 ml column.

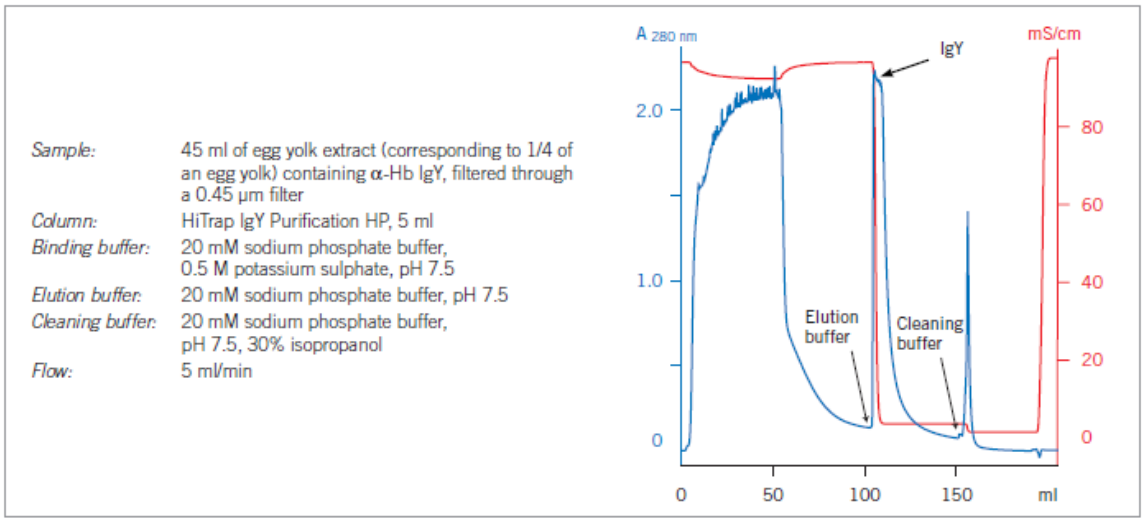


Fig. 20. Purification of avian IgY on HiTrap IgY Purification HP.

Glutathione S-transferase (GST) is one of the most common tags used to facilitate the purification and detection of recombinant proteins and a range of products for simple, one step purification of GST fusion proteins are available (see Purification options).

Purification options

	Binding capacity	Maximum operating flow	Comments
GST MicroSpin™ Purification Module	400 µg/column	n.a.	Ready to use, prepacked columns, buffers and chemicals. High throughput when used with MicroPlex™ 24 Vacuum (up to 48 samples simultaneously).
GSTrap FF 1 ml	10–12 mg recombinant GST/column	4 ml/min	Prepacked column, ready to use.
GSTrap FF 5 ml	50–60 mg recombinant GST/column	15 ml/min	Prepacked column, ready to use.
GSTPrep FF 16/10	>200 mg recombinant GST/column	>10 ml/min	Prepacked column, ready to use.
Glutathione Sepharose 4 Fast Flow	10–12 mg recombinant GST/ml medium	450 cm/h*	For packing high performance columns for use with purification systems and scaling up.
Glutathione Sepharose 4B	8 mg horse liver GST/ml medium	75 cm/h*	For packing small columns and other formats.

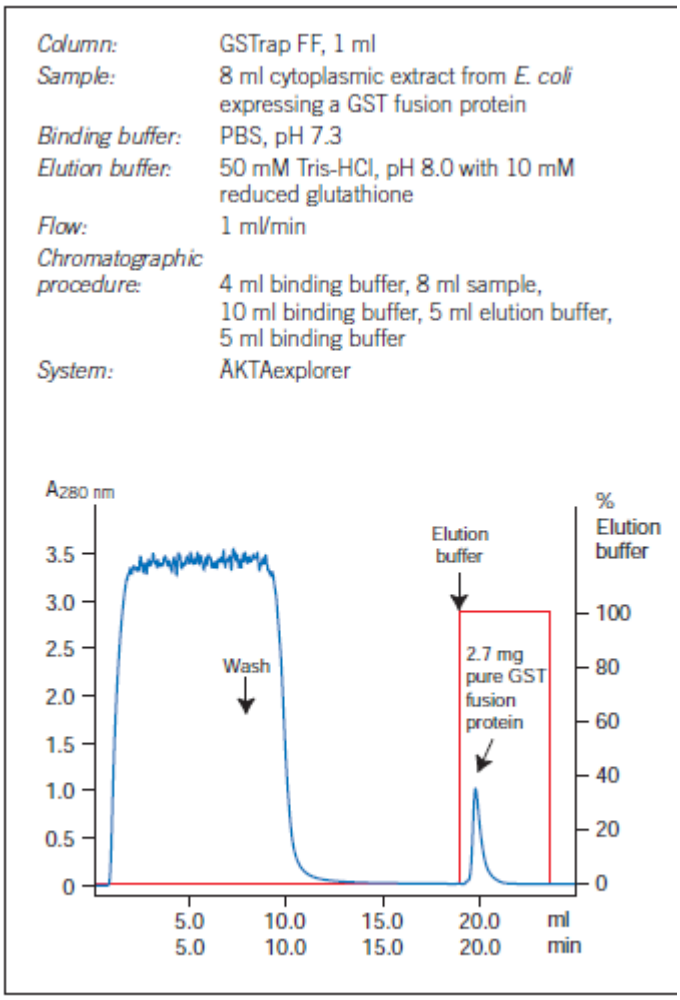


Fig. 22. Purification of a GST fusion protein.

Poly (His) fusion proteins

His MicroSpin Purification Module, HisTrap Kit, HiTrap Chelating HP, Chelating Sepharose Fast Flow The (His)6 tag is one of the most common tags used to facilitate the purification and detection of recombinant proteins and a range of products for simple, one step purification of (His)6 fusion proteins are available (see Purification options). Polyhistidine tags such as (His)4 or (His)10 are also used. They may provide useful alternatives to (His)6 for improving purification results. For example, since (His)10 binds more strongly to the affinity

medium, a higher concentration of eluent (imidazole) can be used during the washing step before elution. This can facilitate the removal of contaminants which may otherwise be co-purified with a (His)6 fusion protein.

Chelating Sepharose, when charged with Ni2+ ions, selectively binds proteins if complex forming amino acid residues, in particular histidine, are exposed on the protein surface. (His)6 fusion proteins can be easily bound and then eluted with buffers containing imidazole. Purification and detection of His-tagged proteins, together with information on how to handle fusion proteins when they are expressed as inclusion bodies, are dealt with in depth in The Recombinant Protein Handbook: Protein Amplication and Simple Purification, available from GE Healthcare.

Purification options

	Binding capacity	Maximum operating flow	Comments
His MicroSpin Purification Module	100 µg/column	n.a.	Ready to use, prepacked columns, buffers and chemicals. High throughput when used with MicroPlex 24 Vacuum (up to 48 samples simultaneously).
HisTrap Kit	12 mg*/column	4 ml/min	As above, but includes buffers for up to 12 purifications using a syringe.
HiTrap Chelating HP 1 ml	12 mg*/column	4 ml/min	Prepacked column, ready to use.
HiTrap Chelating HP 5 ml	60 mg*/column	20 ml/min	Prepacked column, ready to use.
Chelating Sepharose Fast Flow	12 mg*/ml medium	400 cm/h**	Supplied as suspension for packing columns and scale up.

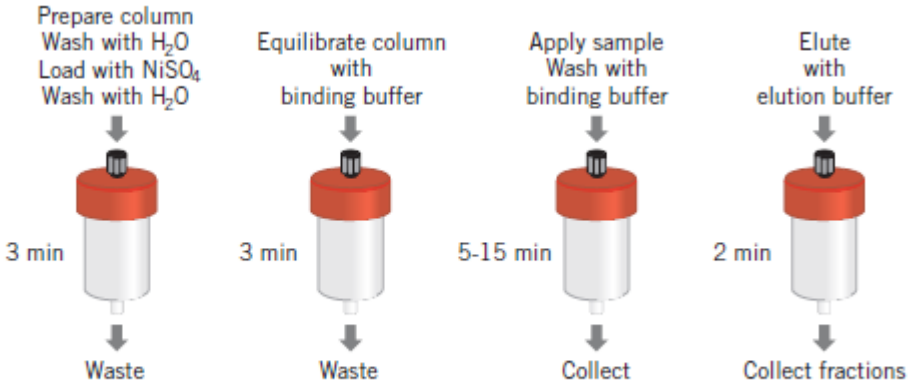


Fig. 28. HiTrap Chelating HP and a schematic overview of poly (His) fusion protein purification.

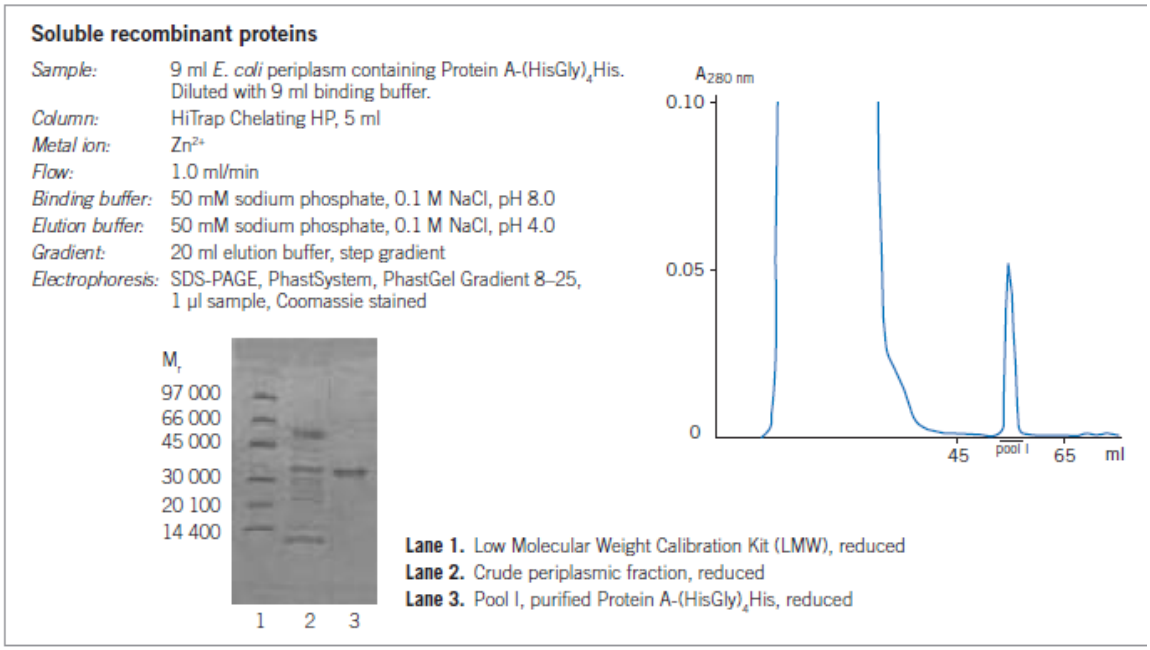


Fig. 25. Purification of recombinant proteins on HiTrap Chelating HP, 5 ml, charged with Zn^{2+}.

操作方法

Nickel solution: 0.1 M $NiSO_4$
Binding buffer: 20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4
Elution buffer: 20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4

1. Wash the column with 5 column volumes of distilled water. Use water, not buffer, to wash away the column storage solution which contains 20% ethanol. This avoids the risk of nickel salt precipitation in the next step. If air is trapped in the column, wash the column with distilled water until the air disappears.
2. Load 0.5 column volumes of the 0.1 M nickel solution onto the column.
3. Wash with 5 column volumes of distilled water.
4. Equilibrate the column with 10 column volumes of binding buffer.
5. Apply sample at a flow rate 1–4 ml/min (1 ml column) or 5 ml/min (5 ml column). Collect the flow-through fraction. A pump is more suitable for application of sample volumes greater than 15 ml.
6. Wash with 10 column volumes of binding buffer. Collect wash fraction.
7. Elute with 5 column volumes of elution buffer. Collect eluted fractions in small fractions such as 1 ml to avoid dilution of the eluate.
8. Wash with 10 column volumes of binding buffer. The column is now ready for a new purification and there is rarely a need to reload with metal if the same (His)6 fusion protein is to be purified.
9. Imidazole absorbs at 280 nm. Use elution buffer as blank when monitoring absorbance. If imidazole needs to be removed, use a desalting column
10. The loss of metal ions is more pronounced at lower pH. The column does not have to be stripped (i.e. all metal ions removed) between each purification if the same protein is going to be purified. In this case, strip and re-charge (i.e. replace metal ions) the column after 5–10 purifications.
11. HisTrap Kit includes everything needed for 12 purifications using a syringe. Three ready to use HiTrap Chelating HP 1 ml columns and ready-made buffer concentrates are supplied with easy-to-follow instructions.

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Removal of nickel ions before re-charging or storage:

1. Wash with 5 column volumes of 20 mM sodium phosphate, 0.5 M NaCl, 0.05 M EDTA, pH 7.4.
2. Wash with 10 column volumes of distilled water.
3. For storage, wash with 5 column volumes of 20% ethanol.

移除沉澱蛋白質

1. Fill column with 1 M NaOH and incubate for 2 hours.
2. Wash out dissolved proteins with 5 column volumes of water and a buffer at pH 7.0 until the pH of the flow-through reaches pH 7.0.

Media characteristics

	Composition	Metal ion capacity	pH stability*	Mean particle size
Chelating Sepharose High Performance (HiTrap Chelating HP)	Iminodiacetic acid coupled to Sepharose High Performance via an ether bond.	23 µmoles Cu^{2+}/ml	Short term 2–14 Long term 3–13	34 µm
Chelating Sepharose Fast Flow	Iminodiacetic acid coupled Sepharose Fast Flow via a spacer arm using epoxy coupling.	22–30 µmoles Zn^{2+}/ml	Short term 2–14 Long term 3–13	90 µm

化學穩定性

Stable in all commonly used aqueous buffers and denaturants such as 6 M guanidine hydrochloride and 8 M urea.

貯藏

Wash media and columns with 20% ethanol at neutral pH (use approximately 5 column volumes for packed media) and store at +4 to +8 °C. The column must be recharged with metal ions after long term storage to reactivate the medium.

Protein A fusion proteins IgG Sepharose 6 Fast Flow

Recombinant fusion proteins containing a protein A tail and protein A can be purified on IgG Sepharose 6 Fast Flow.

Purification option

Product	Binding capacity/ml medium	Maximum operating flow
IgG Sepharose 6 Fast Flow	2 mg protein A at pH 7.5	400 cm/h*

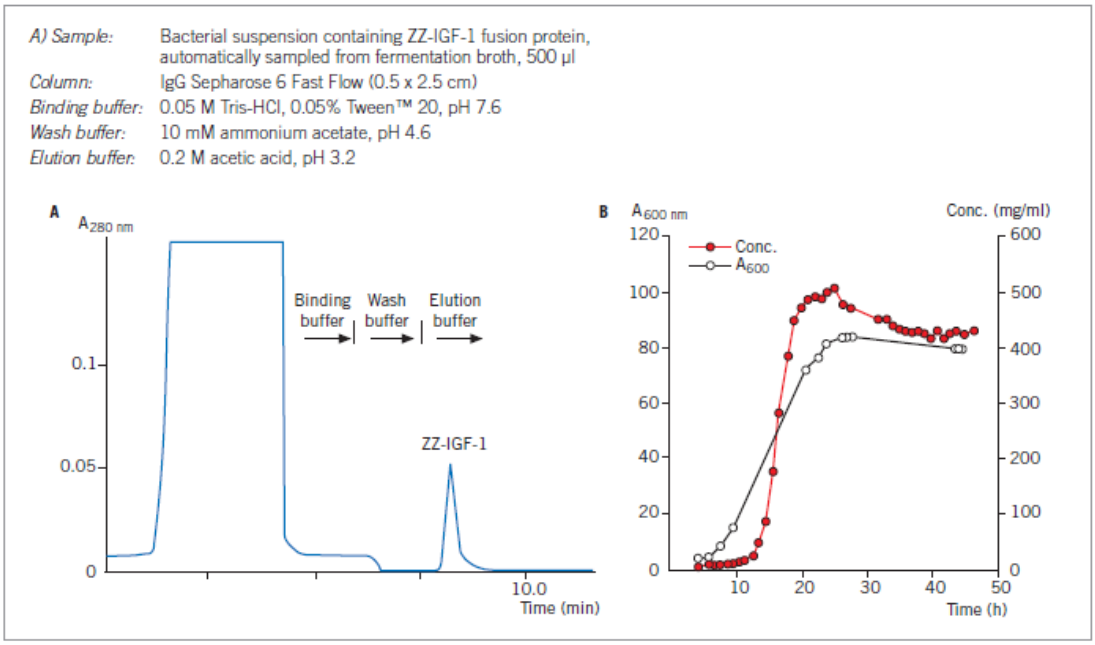
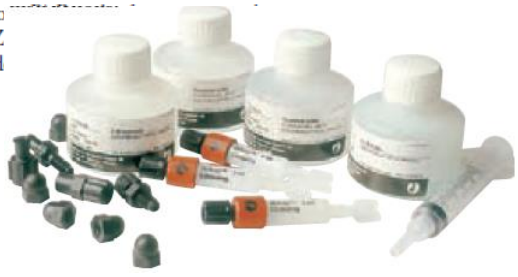


Fig. 29. A) Chromatogram of a sample taken at one time point during fermentatio monitoring of the product concentration during fermentation. Concentration of Z of the ZZ-IGF-1 peak obtained during each chromatographic analysis. Bacterial d



絲氨酸蛋白酶的純化及移除, **e.g. thrombin and trypsin, and zymogens**

HiTrap Benzamidine FF (high sub), Benzamidine Sepharose 4 Fast Flow (high sub) Sample extraction procedures often release proteases into solution, requiring the addition of protease inhibitors to prevent unwanted proteolysis. An alternative to the addition of inhibitors is to use a group specific affinity medium to remove the proteases from the sample. The same procedure can be used to either specifically remove these proteases or purify them. The synthetic inhibitor para-aminobenzamidine is used as the affinity ligand for trypsin, trypsin-like serine proteases and zymogens. Benzamidine Sepharose 4 Fast Flow (high sub) is frequently used to remove molecules from cell culture supernatant, bacterial lysate or serum. During the production of recombinant proteins, tags such

as GST are often used to facilitate purification and detection. Enzyme specific recognition sites are included in the recombinant protein to allow the removal of the tag by enzymatic cleavage when required. Thrombin is commonly used for enzymatic cleavage, and must often be removed from the recombinant product. HiTrap Benzamidine FF (high sub) provides a simple, ready to use solution for this process. Figure 30 shows the partial structure of Benzamidine Sepharose 4 Fast Flow (high sub) and Table 4 gives examples of different serine proteases.

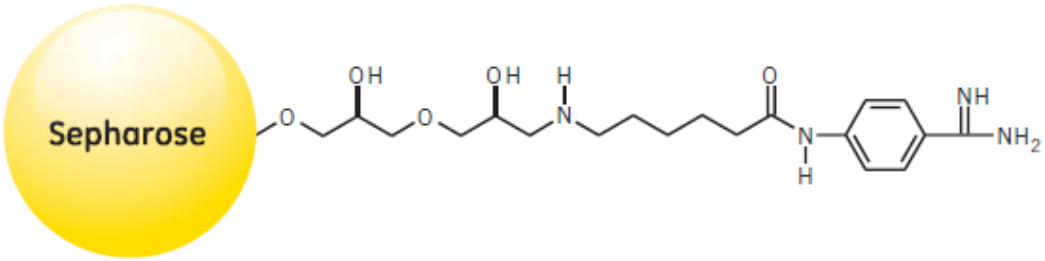


Fig. 30. Partial structure of Benzamidine Sepharose 4 Fast Flow (high sub).

Table 4. Examples of different serine proteases.

	Source	M_r	pI
Thrombin	Bovine pancreas	23 345	10.5
Trypsin	Human plasma chain A Human plasma chain B	5 700 31 000	 7.1
Urokinase	Human urine	54 000	8.9
Enterokinase	Porcine intestine heavy chain Porcine intestine light chain	134 000 62 000	4.2
Plasminogen	Human plasma	90 000	6.4–8.5
Prekallikrein	Human plasma	nd	nd
Kallikrein	Human plasma Human saliva	86 000 nd	nd (plasma) 4.0 (saliva)

Purification options

	Binding capacity	Maximum operating flow	Comments
HiTrap Benzamidine FF (high sub)	Trypsin, > 35 mg/column Trypsin, > 175 mg/column	4 ml/min (1 ml column) 15 ml/min (5 ml column)	Prepacked columns**.
Benzamidine Sepharose 4 Fast Flow (high sub)	Trypsin, > 35 mg/ml medium	300 cm/h*	Supplied as a suspension ready for column packing**.

Purification examples

Figure 31 shows an example of the removal of trypsin-like proteases from human plasma to prevent proteolysis of the plasma components, using a low pH elution. The activity test demonstrated that almost all trypsin-like protease activity is removed from the sample and bound to the column.

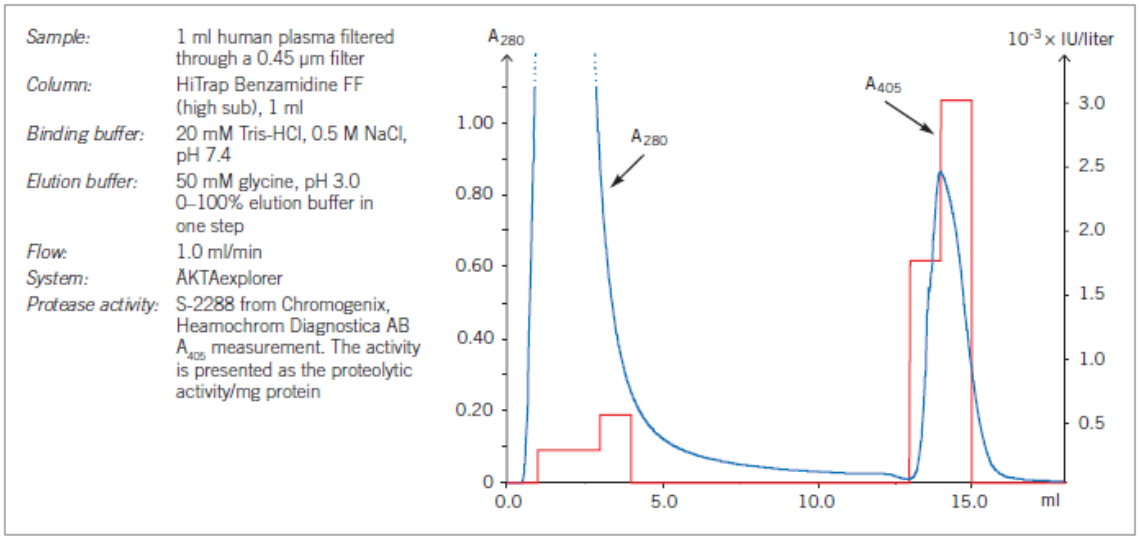


Fig. 31. Removal of trypin-like serine proteases from human plasma using HiTrap Benzamidine FF (high sub), 1 ml.

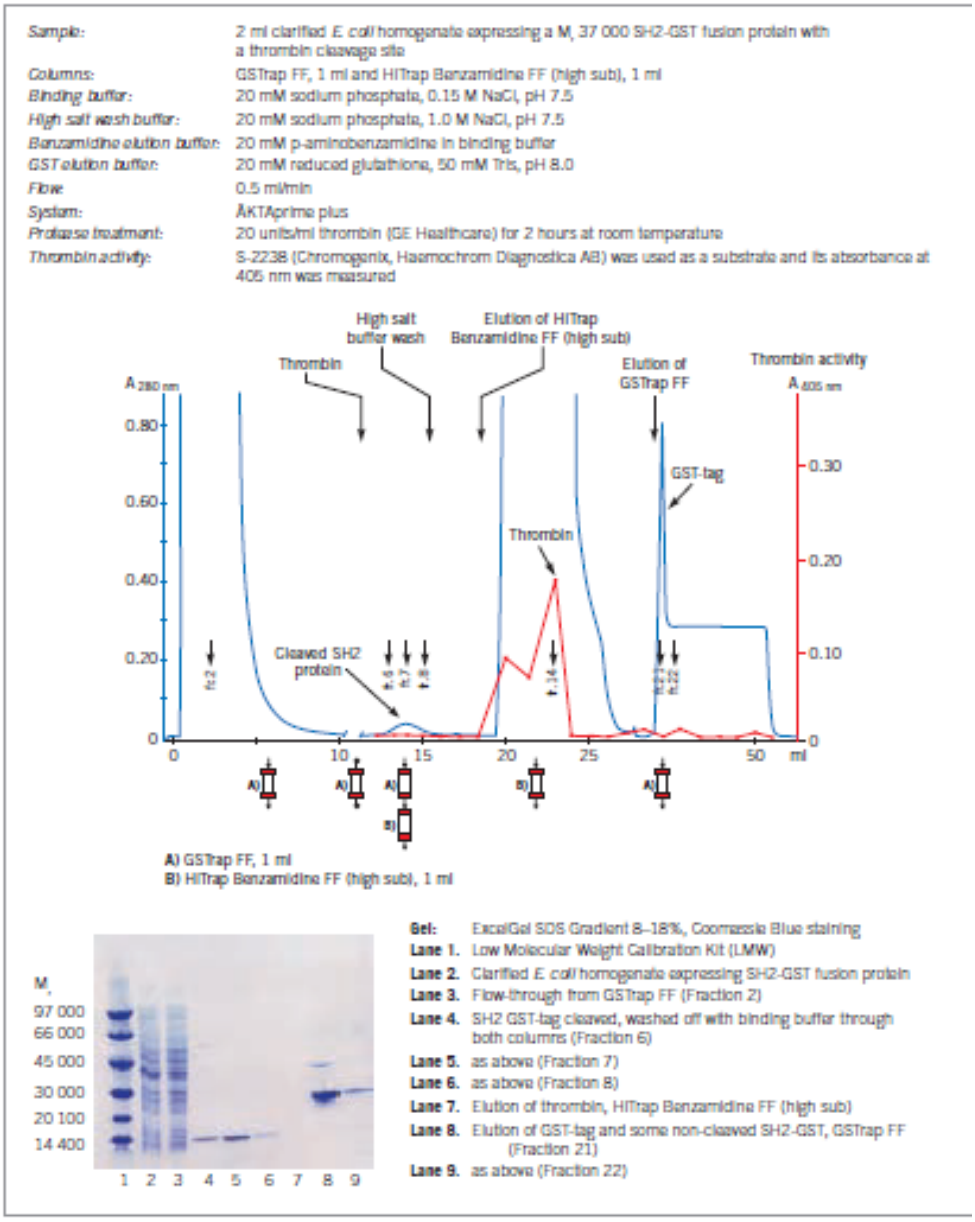


Fig. 32. On-column cleavage of a GST fusion protein and removal of thrombin after on-column cleavage, using GSTrap FF and HiTrap Benzamidine FF (high sub).

Figure 32 shows the effectiveness of using a GSTrap FF column with a HiTrap Benzamidine FF (high sub) for purification of a GST fusion protein, followed by cleavage of the GST tag via the thrombin cleavage site and subsequent removal of the thrombin enzyme. The GST fusion protein binds to the GSTrap

FF column as other proteins wash through the column. Thrombin is applied to the column and incubated for 2 hours. A HiTrap Benzamidine FF (high sub) column, pre-equilibrated in binding buffer, is attached after the GSTrap FF column and both columns are washed in binding buffer followed by a high salt buffer. The cleaved protein and thrombin wash through from the GSTrap FF column, thrombin binds to the HiTrap Benzamidine FF (high sub) column, and the eluted fractions contain pure cleaved protein.

操作方法

Binding buffer: 0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4
Elution buffer alternatives:
- pH elution: 0.05 M glycine-HCl, pH 3.0 or 10 mM HCl, 0.05 M NaCl, pH 2.0
- competitive elution: 20 mM p-aminobenzamidine in binding buffer
- denaturing eluents: 8 M urea or 6 M guanidine hydrochloride

1. Equilibrate the column with 5 column volumes of binding buffer.
2. Apply the sample.
3. Wash with 5–10 column volumes of binding buffer or until no material appears in the eluent (monitored by UV absorption at A280 nm).
4. Elute with 5–10 column volumes of elution buffer. Collect fractions in neutralization buffer if low pH elution isused*. The purified fractions can be buffer exchanged using desalting columns (see page 133).

* Since elution conditions are quite harsh, collect fractions into neutralization buffer (60 µl – 200 µl 1 M Tris-HCl,

pH 9.0 per ml fraction), so that the final pH of the fractions will be approximately neutral. Since Benzamidine Sepharose 4 Fast Flow (high sub) has some ionic binding characteristics,the use of 0.5 M NaCl and pH elution between 7.4–8.0 is recommended. If lower salt concentrations are used, include a high salt wash step after sample application and before elution. The elution buffer used for competitive elution has a high absorbance at 28 0 nm. The eluted protein must be detected by other methods, such as an activity assay, total protein or SDSPAGE analysis. The advantage with competitive elution is that the pH is kept constant throughout the purification.

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Wash with 3–5 column volumes of 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5 followed with 3–5 column volumes of 0.1 M sodium acetate, 0.5 M NaCl, pH 4.5 and re-equilibrate immediately with 3–5 column volumes of binding buffer.
Remove severe contamination by washing with non-ionic detergent such as 0.1% Triton X-100 at +37 °C for 1 minute.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
Benzamidine Sepharose 4 Fast Flow (high sub)	≥ 12 µmoles p-aminobenzamidine/ml	Amide coupling of ligand via a 14 atom spacer to highly cross-linked 4% agarose	Short term 1–9 Long term 2–8	90 µm

化學穩定性

All commonly used aqueous buffers.

貯藏

Wash media and columns with 20% ethanol in 0.05 M sodium acetate, pH 4.0 (use approximately 5 column volumes for packed media) and store at +4 to +8 °C.

Serine proteases and zymogens with an affinity for arginine
-Arginine Sepharose 4B

Arginine Sepharose 4B is an L-arginine derivative of Sepharose 4B that can be used for any biomolecule with a biospecific or charge dependent affinity for arginine, such as serine proteases and zymogens. Specific examples include prekallikrein, clostripain, prothrombin, plasminogen and plasminogen activator. The L-arginine is coupled via its a-amino group, leaving the guanidino and a-carboxyl groups free to interact with samples. Electrostatic and stereospecific effects may contribute to the binding and elution process depending upon the specific sample involved. Figure 33 shows the partial structure of Arginine Sepharose 4B.

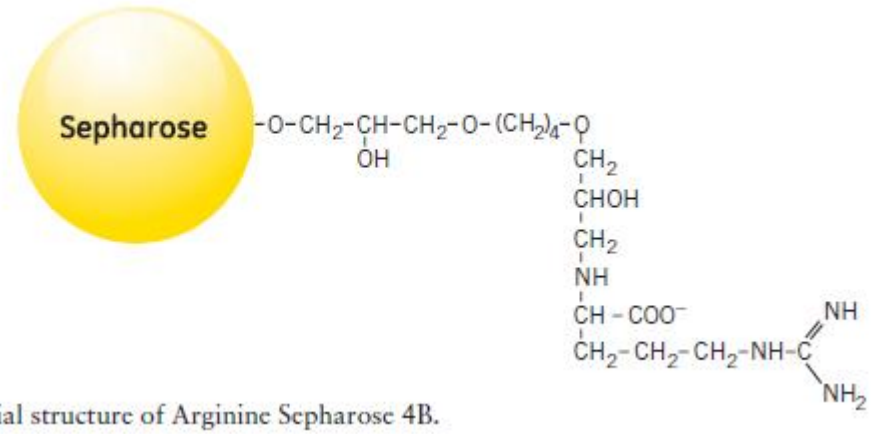

Fig. 33. Partial structure of Arginine Sepharose 4B.

Purification option

	Binding capacity/ml medium	Maximum operating flow	Comments
Arginine Sepharose 4B	No data available	75 cm/h*	Supplied as a suspension ready for column packing.

DNA binding proteins

HiTrap Heparin HP, HiPrep 16/10 Heparin FF, Heparin Sepharose 6 Fast Flow DNA binding proteins form an extremely diverse class of proteins sharing a single characteristic, their ability to bind to DNA. Functionally the group can be divided into those responsible for the replication and orientation of the DNA such as histones, nucleosomes and replicases and those involved in transcription such as RNA/DNA polymerases, transcriptional activators and repressors and restriction enzymes. They can be produced as fusion proteins to enable more specific purification (see page 42), but their ability to bind DNA also enables group specific affinity purification using heparin as a ligand. Heparin is a highly sulphated glycosaminoglycan with the ability to bind a very wide range of biomolecules including:

- DNA binding proteins such as initiation factors, elongation factors, restriction endonucleases, DNA ligase, DNA and RNA polymerases.
- Serine protease inhibitors such as antithrombin III, protease nexins.
- Enzymes such as mast cell proteases, lipoprotein lipase, coagulation enzymes, superoxide dismutase.
- Growth factors such as fibroblast growth factor, Schwann cell growth factor, endothelial cell growth factor.
- Extracellular matrix proteins such as fibronectin, vitronectin, laminin, thrombospondin, collagens.
- Hormone receptors such as oestrogen and androgen receptors.
- Lipoproteins.

The structure of heparin is shown in Figure 34. Heparin has two modes of interaction with proteins and, in both cases, the interaction can be weakened by increases in ionic strength.

1. In its interaction with DNA binding proteins heparin mimics the polyanionic structure of the nucleic acid.

2. In its interaction with coagulation factors such as antithrombin III, heparin acts as an affinity ligand.

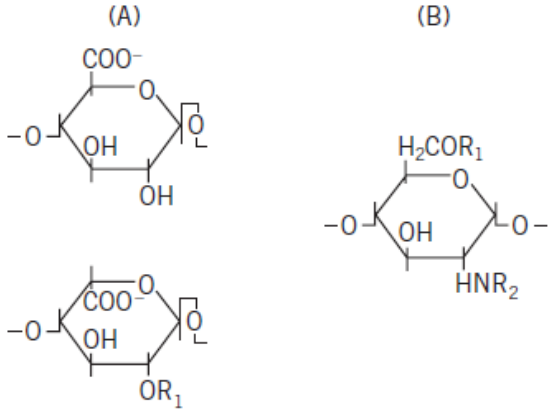

Fig. 34. Structure of a heparin polysaccharide consisting of alternating hexuronic acid (A) and D-glucosamine residues (B). The hexuronic acid can either be D-glucuronic acid (top) or its C-5 epimer, L-iduronic acid (bottom). R_1 = -H or $-SO_3^-$, R_2 = $-SO_3^-$ or $-COCH_3$.

Purification options

	Binding capacity	Maximum operating flow	Comments
HiTrap Heparin HP	Bovine antithrombin III, 3 mg/column Bovine antithrombin III, 15 mg/column	4 ml/min (1 ml column) 20 ml/min (5 ml column)	Prepacked columns.
HiPrep 16/10 Heparin FF	Bovine antithrombin III, 40 mg/column	10 ml/min	Prepacked 20 ml column.
Heparin Sepharose 6 Fast Flow	Bovine antithrombin III, 2 mg/ml medium	400 cm/h*	Supplied as a suspension ready for column packing.

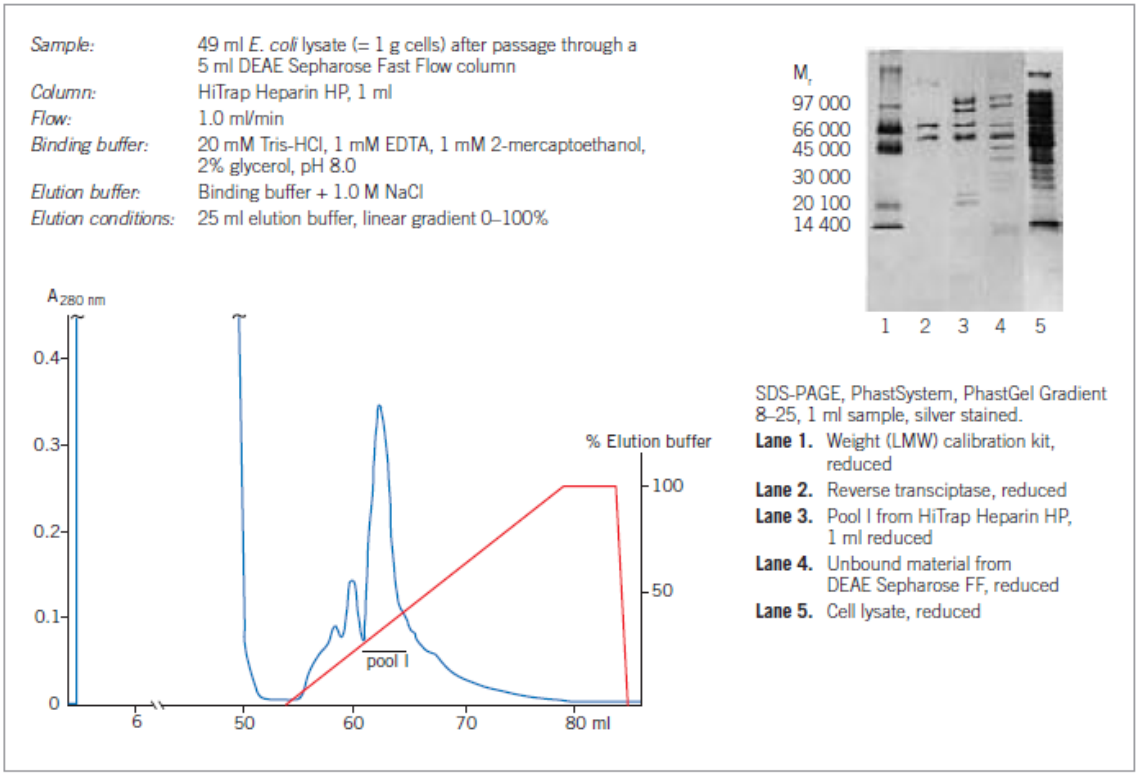

Fig. 35. Partial purification of recombinant HIV-reverse transcriptase on HiTrap Heparin HP.

Purification or removal of fibronectin-Gelatin Sepharose 4B

Fibronectin is a high molecular weight glycoprotein found on the surfaces of many cell typesand present in many extracellular fluids including plasma. Fibronectin binds specifically to gelatin at or around physiological pH and ionic strength.

Purification option

	Binding capacity/ml medium	Maximum operating flow	Comments
Gelatin Sepharose 4B	1 mg human plasma fibronectin	75 cm/h*	Supplied as a suspension ready for column packing.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
Gelatin Sepharose 4B	4.5–8 mg gelatin/ml	Gelatin linked to Sepharose using the CNBr method	Short term 3–10 Long term 3–10	90 µm

操作方法

Binding buffer: PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4
Elution buffer alternatives:
- 0.05 M sodium acetate, 1.0 M sodium bromide (or potassium bromide), pH 5.0
- Binding buffer + 8 M urea
- Binding buffer + arginine

Fibronectin has a tendency to bind to glass. Use siliconized glass to prevent adsorption.

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Wash 3 times with 2–3 column volumes of buffer, alternating between high pH (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5) and low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.5).
Re-equilbrate immediately with 3–5 column volumes of binding buffer. Remove denatured proteins or lipids by washing the column with 0.1% Triton X-100 at +37 °C for one minute.
Re-equilibrate immediately with 5 column volumes of binding buffer.

Purification or removal of albumin-HiTrap Blue HP, Blue Sepharose 6 Fast Flow

The same procedure can be used either to purify albumin or to remove albumin as a specific contaminant before or after other purification steps. Albumin binds to Cibacron™ Blue F3G-A, a synthetic polycyclic dye that acts as an aromatic anionic ligand binding the albumin via electrostatic and/or hydrophobic interactions. Similar interactions are seen with coagulation factors, lipoproteins and interferon. Cibacron Blue F3G-A is linked to Sepharose to create Blue Sepharose affinity media.

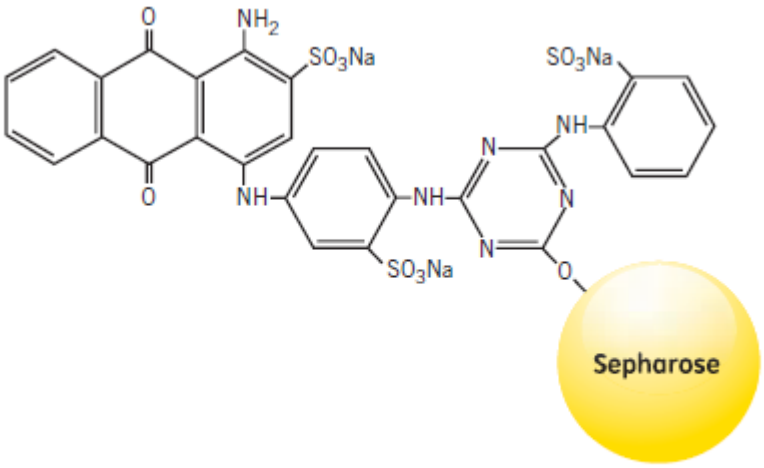

Fig. 41. Partial structure of Blue Sepharose Fast Flow and Blue Sepharose High Performance.

Use HiTrap Blue HP 1 ml or 5 ml columns to remove host albumin from mammalian expression systems, or when the sample is known to contain high levels of albumin that may mask the visualization of other protein peaks seen by UV absorption. Cibacron Blue F3G-A also shows certain structural similarities to naturally occurring molecules, such as the cofactor NAD+, that enable it to bind strongly and specifically to a wide range of proteins including kinases, dehydrogenases and most other enzymes requiring adenylyl-containing cofactors (see page 73).

Purification options

	Binding capacity	Maximum operating flow	Comments
HiTrap Blue HP	Human serum albumin, 20 mg/column Human serum albumin, 100 mg/column	4 ml/min (1 ml column) 20 ml/min (5 ml column)	Prepacked columns.
Blue Sepharose 6 Fast Flow*	Human serum albumin, > 18 mg/ml medium	750 cm/h**	Supplied as a suspension ready for column packing.

Media characteristics

	Ligand and density	Composition	pH stability*	Mean particl size
HiTrap Blue HP	Cibacron Blue F3G-A 4 mg/ml	Ligand coupled to Sepharose High Performance using the triazine method.	Short term 3–13 Long term 4–12	34 µm
Blue Sepharose 6 Fast Flow	Cibacron Blue F3G-A 6.7–7.9 µmoles/ml	Ligand coupled to Sepharose Fast Flw using the triazine method.	Short term 3–13 Long term 4–12	90 µm

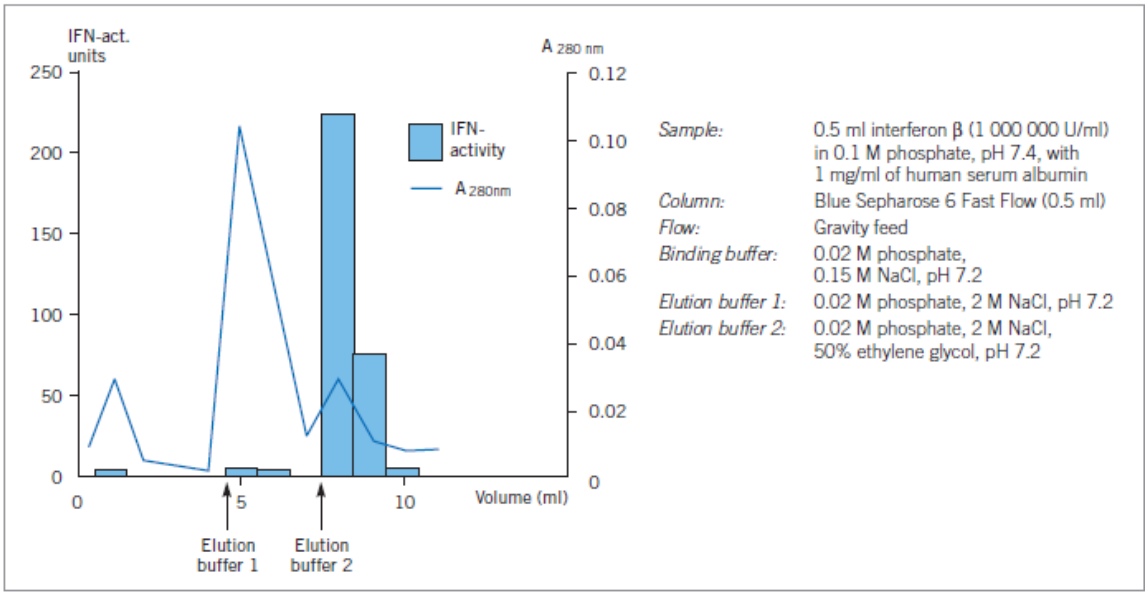

Fig. 43. Purification of human serum albumin and interferon β on Blue Sepharose 6 Fast Flow.

Figure 43 shows the use of Blue Sepharose 6 Fast Flow for the separation of human serum albumin from interferon β.

Glycoproteins or polysaccharides

Con A Sepharose 4B, Lentil Lectin Sepharose 4B, Agarose Wheat Germ Lectin

Glycoproteins and polysaccharides react reversibly, via specific sugar residues, with a group of proteins known as lectins. As ligands for purification media, lectins are used to isolate and separate glycoproteins, glycolipids, polysaccharides, subcellular particles and cells, and to purify detergent solubilized cell membrane components. Substances bound to the lectin are resolved by using a gradient of ionic strength or of a competitive binding substance.

Media screening

To select the optimum lectin for purification, it may be necessary to screen different media. The ligands, Concanavalin A (Con A), Lentil Lectin and Wheat Germ Lectin provide a spectrum of parameters for the separation of glycoproteins. Table 5 gives their specificity.

Table 5. Specificity of lectins.

Lectin	Specificity
Mannose/glucose binding lectins	
Con A, *Canavalia ensiformis*	Branched mannoses, carbohydrates with terminal mannose or glucose (αMan > αGlc > GlcNAc).
Lentil Lectin, *Lens culinaris*	Branched mannoses with fucose linked α(1,6) to N-acetyl-glucosamine, (αMan > αGlc > GlcNAc).
N-acetylglucosamine binding lectins	
Wheat Germ Lectin, *Triticum vulgare*	Chitobiose core of N-linked oligosaccharides, [GlcNAc(β1,4GlcNAc)$_{1-2}$ > βGlcNac].

Purification options

	Binding capacity/ml medium	Maximum operating flow	Comments
Con A Sepharose 4B	Porcine thyroglobulin, 20–45 mg	75 cm/h**	Supplied as a suspension ready for column packing*.

* *Supplied in acetate buffer solution (0.1 M, pH 6) containing 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 20% ethanol.*

Con A for binding of branched mannoses, carbohydrates with terminal mannose or glucose (aMan > aGlc > GlcNAc)

Concanavalin A (Con A) is a tetrameric metalloprotein isolated from *Canavalia ensiformis* (jack bean). Con A binds molecules containing a-D-mannopyranosyl, a-D-glucopyranosyl and sterically related residues. The binding sugar requires the presence of C-3, C-4 and C-5 hydroxyl groups for reaction with Con A. Con A can be used for applications such as:

- Separation and purification of glycoproteins, polysaccharides and glycolipids.
- Detection of changes in composition of carbohydrate-containing substances, e.g. during development.
- Isolation of cell surface glycoproteins from detergent-solubilized membranes.
- Separation of membrane vesicles into “inside out” and “right side out” fractions.

Purification example

Figure 47 shows the purification of a human cell surface alloantigen on Con A Sepharose 4B.

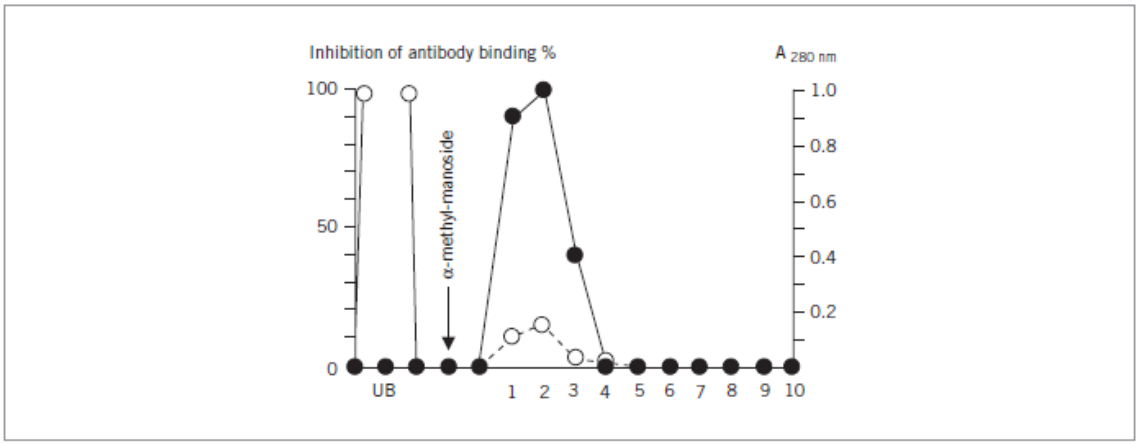

Fig. 47. Purification of a cell surface antigen on Con A Sepharose 4B. Solid circles represent antigen activity and open circles represent protein profile. Reproduced courtesy of the authors and publishers.
Reference: A novel heteromorphic human cell surface alloantigen, gp60, defined by a human monoclonal antibody. Schadendorf, D. *et al.*, *J. Immunol.* **142**, 1621 (1989).

Performing a separation

Binding buffer: 20 mM Tris-HCl, 0.5 M NaCl, 1 mM MnCl2, 1 mM CaCl2, pH 7.4
Elution buffer: 0.1–0.5 M methyl-α-D-glucopyranoside (methyl-α-D-glucoside) or methyl-α-D-mannopyranoside (methyl-α-D-mannoside), 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4

1. Pack the column (see Appendix 3) and wash with at least 10 column volumes of binding buffer to remove preservative.
2. Equilibrate the column with 10 column volumes of binding buffer.
3. Apply the sample, using a low flow from 15 cm/h, during sample application (flow rate is the most significant factor to obtain maximum binding).

4. Wash with 5–10 column volumes of binding buffer or until no material appears in the eluent (monitored by UV absorption at A280 nm).
5. Elute with 5 column volumes of elution buffer.

Recovery from Con A Sepharose 4B is decreased in the presence of detergents. If the glycoprotein of interest needs the presence of detergent and has affinity for either lentil lectin or wheat germ lectin, the media Lentil Lectin Sepharose 4B or Agarose Wheat Germ Lectin may provide a suitable alternative to improve recovery

For complex samples containing glycoproteins with different affinities for the lectin, a continuous gradient or step elution may improve resolution. Recovery can sometimes be improved by pausing the flow for some minutes during elution.

Elute tightly bound substances by lowering the pH. Note that elution below pH 4.0 is not recommended and that below pH 5.0 Mn2+ will begin to dissociate from the Con A and the column will need to be reloaded with Mn2+ before reuse.

Cleaning

Wash with 10 column volumes of 0.5 M NaCl, 20 mM Tris-HCl, pH 8.5, followed by 0.5 M NaCl, 20 mM acetate, pH 4.5. Repeat 3 times before re-equilibrating with binding buffer. Remove strongly bound substances by:

- washing with 0.1 M borate, pH 6.5 at a low flow rate
- washing with 20% ethanol or up to 50% ethylene glycol
- washing with 0.1% Triton X-100 at +37 °C for one minute

Re-equilibrate immediately with 5 column volumes of binding buffer after any of these wash steps.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
Con A Sepharose 4B	10–16 mg/ml	Con A coupled to Sepharose 4B by CNBr method	Short term 4–9 Long term 4–9	90 µm

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Stable to all commonly used aqueous buffers. Avoid 8 M urea, high concentrations of guanidine hydrochloride, chelating agents such as EDTA, or solutions with pH < 4.0 as these remove the manganese from the lectin or dissociate Con A, resulting in loss of activity.

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Wash media and columns with 20% ethanol in 0.1 M acetate, 1 M NaCl, 1 mM CaCl2, 1 mM MnCl2, 1 mM MgCl2, pH 6 (use approximately 5 column volumes for packed media) and store at +4 to +8 °C.

Lentil lectin for binding of branched mannoses with fucose linked a(1,6) to the N-acetyl-glucosamine, (aMan > aGlc > GlcNAc) N-acetylglucosamine binding lectins

Lentil lectin binds a-D-glucose and a-D-mannose residues and is an affinity ligand used for the purification of glycoproteins including detergent-solubilized membrane glycoproteins, cell surface antigens and viral glycoproteins. Lentil lectin is the haemagglutinin from the common lentil, Lens culinaris. When compared to Con A, it distinguishes less sharply between glucosyl and mannosyl residues and binds simple sugars less strongly. It also retains its binding ability in the presence of 1% sodium deoxycholate. For these reasons Lentil Lectin Sepharose 4B is useful for the purification of detergent-solubilized membrane proteins, giving high capacities and extremely high recoveries.

Purification options

	Binding capacity/ml medium	Maximum operating flow	Comments
Lentil Lectin Sepharose 4B	Porcine thyroglobulin, 16–35 mg	75 cm/h*	Supplied as a suspension ready for column packing.

Performing a separation

Binding buffer: 20 mM Tris-HCl, 0.5 M NaCl, 1 mM MnCl2, 1 mM CaCl2, pH 7.4.
Elution buffer: 0.1–0.5 M methyl-a-D-glucopyranoside (methyl-a-D-glucoside), 20 mM Tris-HCl, 0.5 M

NaCl, pH 7.4

Buffers for soluble glycoproteins:
Binding buffer: 20 mM Tris-HCl, 0.5 M NaCl, 1 mM MnCl2, 1 mM CaCl2, pH 7.4
Elution buffer: 0.3 M methyl-a-D-mannopyranoside, 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4

Buffers for detergent-solubilized proteins:
Equilibrate column with the buffer 20 mM Tris-HCl, 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4, to ensure saturation with Mn^{2+} and Ca^{2+}.
Binding buffer: 20 mM Tris-HCl, 0.5 M NaCl, 0.5% sodium deoxycholate, pH 8.3
Elution buffer: 0.3 M methyl-α-D-mannopyranoside, 20 mM Tris-HCl, 0.5 M NaCl, 0.5% sodium deoxycholate, pH 8.3

1. Pack the column (see Appendix 3) and wash with at least 10 column volumes of binding buffer to remove preservative.
2. Equilibrate the column with 10 column volumes of binding buffer.
3. Apply the sample, using a low flow from 15 cm/h, during sample application (flow rate is the most significant factor to obtain maximum binding).
4. Wash with 5–10 column volumes of binding buffer or until no material appears in the eluent (monitored by UV absorption at A280 nm).
5. Elute with 5 column volumes of elution buffer using a step or gradient elution.

Below pH 5, excess Mn^{2+} and Ca^{2+} (1 mM) are essential to preserve binding activity. It is not necessary to include excess Ca^{2+} or Mn^{2+} in buffers if conditions that lead to their removal from the coupled lectin can be avoided.

For complex samples containing glycoproteins with different affinities for the lectin, a continuous gradient or step elution may improve resolution. Recovery can sometimes be improved by pausing the flow for some minutes during elution Elute tightly bound substances by lowering the pH, but not below pH 3. In some cases strongly bound substances can be eluted with detergent, for example 1.0% deoxycholate.

Media characteristics

	Ligand density	Composition	pH stability*	Mean particle size
Lentil Lectin Sepharose 4B	2.5 mg/ml	Lentil lectin coupled to Sepharose 4B by CNBr method.	Short term 3–10 Long term 3–10	90 µm

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Wash with 10 column volumes of 0.5 M NaCl, 20 mM Tris-HCl, pH 8.5, followed by 0.5 M NaCl, 20 mM acetate, pH 4.5. Repeat 3 times before re-equilibrating with binding buffer. Remove strongly bound substances by:

- washing with 0.1 M borate, pH 6.5 at a low flow rate
- washing with 20% ethanol or up to 50% ethylene glycol
- washing with 0.1% Triton X-100 at +37 °C for one minute

Re-equilibrate immediately with 5 column volumes of binding buffer after any of these wash steps.

Calmodulin binding proteins: ATPases, adenylate cyclases, protein kinases, phosphodiesterases, neurotransmitters

Calmodulin Sepharose 4B

Calmodulin is a highly conserved regulatory protein found in all eukaryotic cells. This protein is involved in many cellular processes such as glycogen metabolism, cytoskeletal control, neurotransmission, phosphate activity and control of NAD+/NADP+ ratios. Calmodulin Sepharose 4B provides a convenient method for the isolation of many of the calmodulin binding proteins involved in these pathways. Calmodulin binds proteins principally through their interactions with hydrophobic sites on its surface. These sites are exposed after a conformational change induced by the action of Ca^{2+} on separate Ca^{2+}-binding sites. The binding of enzymes may be enhanced if the enzyme substrate is present and enzyme-substrate-calmodulin-Ca^{2+} complexes are particularly stable.

Purification options

	Binding capacity/ml medium	Maximum operating flow	Comments
Calmodulin Sepharose 4B	No data available	75 cm/h*	Supplied as a suspension ready for column packing.

Performing a separation

Binding buffer: 50 mM Tris-HCl, 0.05–0.2 M NaCl, 2 mM CaCl2, pH 7.5
Elution buffer: 50 mM Tris-HCl, 0.05–0.2 M NaCl, 2 mM EGTA, pH 7.5

Proteins and peptides with exposed amino acids: His, Cys, Trp, and/or with affinity for metal ions (also known as IMAC, immobilized metal chelate affinity chromatography)

HiTrap Chelating HP, Chelating Sepharose Fast Flow,
His MicroSpin Purification Module, HisTrap Kit

Proteins and peptides that have an affinity for metal ions can be separated using metal chelate affinity chromatography. The metals are immobilized onto a chromatographic medium by chelation. Certain amino acids, e.g. histidine and cysteine, form complexes with the chelated metals around neutral pH (pH 6–8) and it is primarily the histidine-content of a protein which is responsible for its binding to a chelated metal. Metal chelate affinity chromatography is excellent for purifying recombinant (His)6 fusion proteins (see page 47) as well as many natural proteins. Chelating Sepharose, the medium used for metal chelate affinity chromatography, is formed by coupling a metal chelate forming ligand (iminodiacetic acid) to Sepharose. Before use the medium is loaded with a solution of divalent metal ions such as Ni^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Co^{2+} or Fe^{2+}. The binding reaction with the target protein is pH dependent and bound sample is, most commonly, eluted by reducing the pH and increasing the ionic strength of the buffer or by including EDTA or imidazole in the buffer. The structure of the ligand, iminodiacetic acid, is shown in Figure 48.

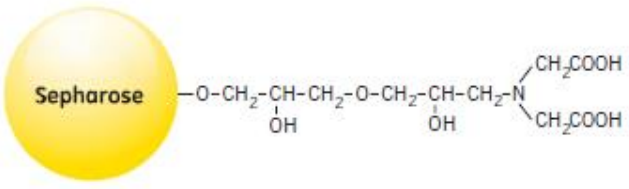

Fig. 48. Partial structure of Chelating Sepharose High Performance and Chelating Sepharose Fast Flow.

Metalloproteins are not usually suitable candidates for purification by chelating chromatography since they tend to scavenge the metal ions from the column.

Purification options

	Binding capacity	Maximum operating flow	Comments
His MicroSpin Purification Module	100 µg/column	Not applicable	Ready to use, prepacked columns, buffers and chemicals for purification of $(His)_6$ fusion proteins.
HiTrap Chelating HP 1 ml	12 mg/column	4 ml/min	Prepacked column, ready to use.
HiTrap Chelating HP 5 ml	60 mg/column	20 ml/min	Prepacked column, ready to use.
HisTrap Kit	12 mg/column*	4 ml/min	Ready to use, prepacked columns, buffers and chemicals for purification of $(His)_6$ fusion proteins for up to 12 purifications using a syringe.
Chelating Sepharose Fast Flow	12 mg/ml medium	400 cm/h**	Supplied as suspension for packing columns and scale up.

* *Estimate for a $(His)_6$ fusion protein of M_r 27 600, binding capacity varies according to specific protein.*

Purification example

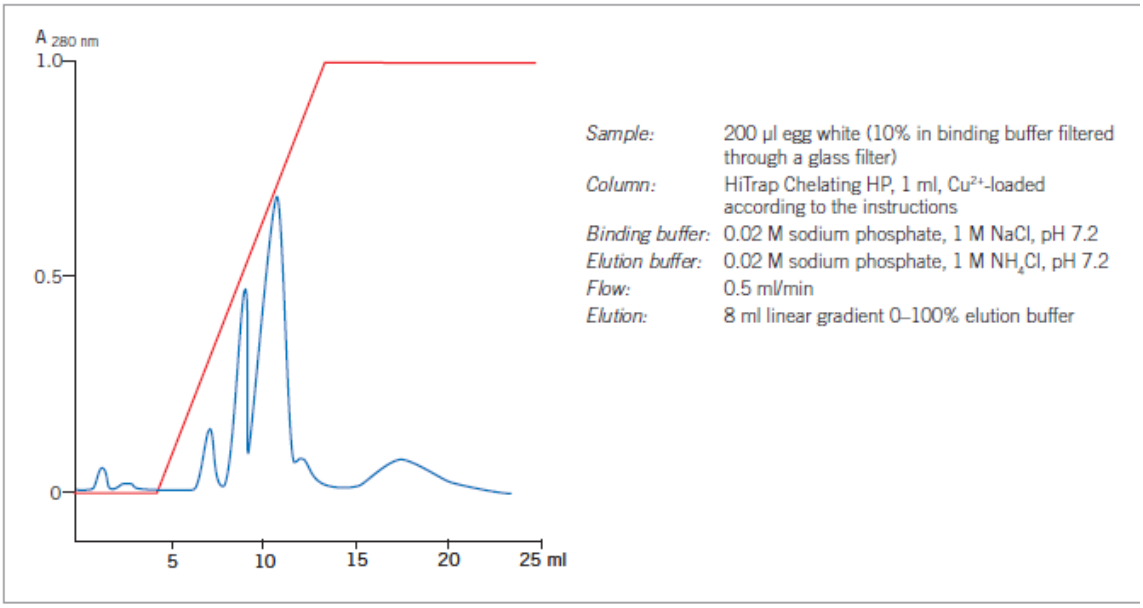

Fig. 49. Purification of egg white proteins on HiTrap Chelating HP 1 ml, using the metal ion Cu^{2+}.

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The following guidelines may be used for preliminary experiments to select the metal ion that is most useful for a given separation:

- Cu^{2+} gives strong binding and some proteins will only bind to Cu^{2+}. Load solution equivalent to 60% of the packed column volume to avoid leakage of metal ions during sample application. Alternatively, the medium can be saturated and a short secondary uncharged column of HiTrap Chelating HP or packed Chelating Sepharose Fast Flow should be connected in series after the main column to collect excess metal ions.
- Zn^{2+} gives a weaker binding and this can, in many cases, be exploited to achieve selective elution of a protein mixture. Load solution equivalent to 85% of the packed column volume to charge the column.
- Ni^{2+} is commonly used for poly (His) fusion proteins. Ni^{2+} solution equivalent to half the column volume is usually sufficient to charge the column.
- Co^{2+} and Ca^{2+} are also alternatives. Charge the column with metal ions by passing through a solution of the appropriate salt through the column, e.g. 0.1 M $ZnCl_2$, $NiSO_4$ or $CuSO_4$ in distilled water. Chloride salts can be used for other metals.

Several methods can be used to determine when the column is charged. If a solution of metal salt in distilled water is used during charging, the eluate initially has a low pH and returns to neutral pH as the medium becomes saturated with metal ions. The progress of charging with Cu2+ is easily followed by eye (the column contents become blue). When charging a column with zinc ions, sodium carbonate can be used to detect the presence of zinc in the eluate. Wash the medium thoroughly with binding buffer after charging the column.

Choice of binding buffer

A neutral or slightly alkaline pH will favor binding. Tris-acetate (0.05 M), sodium phosphate (0.02–0.05 M) and Tris-HCl (0.02–0.05 M) are suitable buffers. Tris-HCl tends to reduce binding and should only be used when metal-protein affinity is fairly high. High concentrations of salt or detergents in the buffer normally have no effect on the adsorption of protein and it is good practice to maintain a high ionic strength (e.g. 0.5–1 M NaCl) to avoid unwanted ion exchange effects. Chelating agents such as EDTA or citrate should not be included, as they will strip the metal ions from the medium.

Choice of elution buffers

Differential elution of bound substances may be obtained using a gradient of an agent that competes for either the ligand or the target molecules. An increased concentration of imidazole (0–0.5 M), ammonium chloride (0–0.15 M), or substances such as histamine or glycine with affinity for the chelated metal can be used. The gradient is best run in the binding buffer at constant pH. Since pH governs the degree of ionization of charged groups at the binding sites, a gradient or step-wise reduction in pH can be

used for non-specific elution of bound material. A range of pH 7.0–4.0 is normal, most proteins eluting between pH 6.0 and 4.2. Deforming eluents such as 8 M urea or 6 M guanidine hydrochloride can be used. Elution with EDTA (0.05 M) or other strong chelating agents will strip away metal ions and other material bound. This method does not usually resolve different proteins. If harsh elution conditions are used, it is recommended to transfer eluted fractions immediately to milder conditions (either by collecting them in neutralization buffer or by passing directly onto a desalting column for buffer exchange (see page 133).

The loss of metal ions is more pronounced at lower pH. The column does not have to be stripped between consecutive purifications if the same protein is going to be purified, as shown in Figure 50.

Cleaning

Remove metal ions by washing with 5 column volumes 20 mM sodium phosphate, 0.5 M NaCl, 0.05 M EDTA, pH 7.4. Remove precipitated proteins by filling the column with 1 M NaOH and incubate for 2 hours. Wash out dissolved proteins with 5 column volumes of water and a buffer at pH 7.0 until the pH of the flow-through reaches pH 7.0. Alternatively wash with a non-ionic detergent such as 0.1% Triton X-100 at +37 °C for 1 min. Remove lipid and very hydrophobic proteins by washing with 70% ethanol, or with a saw-tooth gradient 0%–30%–0% isopropanol/water.

Media characteristics

	Composition	Metal ion capacity	pH stability*	Mean particle size
Chelating Sepharose High Performance	Iminodiacetic acid coupled to Sepharose High Performance via an ether bond.	23 µmoles Cu^{2+}/ml	Short term 2–14 Long term 3–13	34 µm
Chelating Sepharose Fast Flow	Iminodiacetic acid coupled Sepharose Fast Flow via a spacer arm using epoxy coupling.	22–30 µmoles Zn^{2+}/ml	Short term 2–14 Long term 3–13	90 µm

Chemical stability

Stable in all commonly used aqueous buffers and denaturants such as 6 M guanidine hydrochloride, 8 M urea and other chaotropic agents.

Storage

Wash media and columns with 20% ethanol at neutral pH (use approximately 5 column volumes for packed media) and store at +4 to +8 °C. Before long term storage, remove metal ions by washing with five column volumes 20 mM sodium phosphate, 0.5 M NaCl, 0.05 M EDTA, pH 7.4. The column must be recharged with metal ions after long term storage.

Although metal leakage is very low, the presence of any free metal in the purified product can be avoided by connecting an uncharged HiTrap Chelating HP column in series after the first column and before the protein is eluted. This column will bind any metal ions removing them from the protein as it passes through the second column.

Thiol-containing substances (purification by covalent chromatography)

Activated Thiol Sepharose 4B, Thiopropyl Sepharose 6B

Thiol-containing substances can be isolated selectively by covalent binding to an activated thiolated matrix via thiol-disulphide exchange to form a mixed disulphide bond. After washing away unbound material, the thiol-containing substance is eluted by reducing the disulphide bond. This technique is also known as covalent chromatography. The reaction scheme is shown in Figure 51.

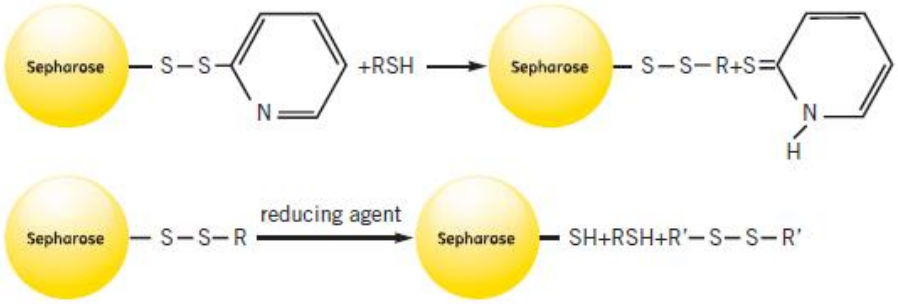

Fig. 51. Reaction scheme purification of a thiolated substance (RSH) on Activated Thiol Sepharose 4B or Thiopropyl Sepharose 6B. The reducing agent is a low molecular weight thiol such as dithiothreitol.

In Thiopropyl Sepharose 6B the 2-hydroxypropyl residue acts as a hydrophilic spacer group.
The partial structure of Thiopropyl Sepharose 6B is shown in Figure 53.

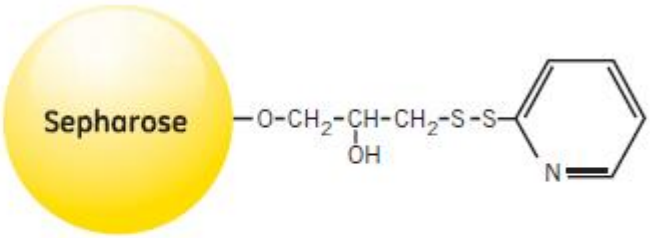

Fig. 53. Partial structure of Thiopropyl Sepharose 6B.

Purification options

	Binding capacity/ml medium	Coupling conditions	Maximum operating flow	Comments
Activated Thiol Sepharose 4B	Mercaptalbumin, 2–3 mg	pH 4–8, 3–16 hours, +4 °C - room temp.	75 cm/h*	Low capacity derivative suitable for coupling of high molecular weight substances. Supplied as dry powder, rehydration required.
Thiopropyl Sepharose 6B	Ceruloplasmin, 14 mg	pH 4–8, 3–16 hours, +4 °C - room temp.	75 cm/h*	High capacity derivative suitable for coupling of low molecular weight substances. Supplied as dry powder, rehydration required.

Media characteristics

	Density of thiol groups	Composition	pH stability*	Mean particle size
Thiopropyl Sepharose 6B	25 µmoles/ml	Mixed disulphide containing 2-thiopyridyl protecting groups attached to Sepharose 6B through a chemically stable ether linkage.	Short term 2–8 Long term 2–8	90 µm
Activated Thiol Sepharose 4B	1 µmole/ml	Mixed disulphide formed between 2,2'-dipyridyl disulphide and glutathione coupled to CNBr-activated Sepharose 4B.	Short term 2–8 Long term 2–8	90 µm

Performing a separation

Binding buffer:

20 mM Tris-HCl, 0.1–0.5 M NaCl, pH 7.0. If required, include 8 M urea or 6 M guanidine HCl to ensure that the protein is denatured and all thiol groups are accessible for the reaction.
1 mM EDTA can be added to remove trace amounts of catalytic heavy metals.

Elution buffer alternatives:

For covalently bound proteins: 0.025 M cysteine, 50 mM Tris-HCl, pH 7–8. To minimize reduction of intramolecular disulphide bridges: 5–20 mM L-cysteine, 50 mM Tris-HCl, 1 mM EDTA, pH 8.0 or 20–50 mM 2-mercaptoethanol, 50 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Note:

When using Thiopropyl Sepharose, 2-thiopyridyl groups must be removed after the protein has bound. Wash the column with sodium acetate 0.1 M, 2-mercaptoethanol 5 mM, pH 4.0 before beginning elution.

If the proteins to be purified contain disulphide bonds, the disulphide bridges must be reduced, for example with 2-mercaptoethanol (5 mM). Analyze the thiol content of the sample by thiol titration to ensure that the capacity of the medium will not be exceeded. Use preliminary titration studies with 2,2'-dipyridyl disulphide to provide a guide to optimal coupling conditions. A spectrophotometer can be

used to determine the release of 2-thiopyridone (absorbance coefficient = 8.08 x 103 M-1 cm-1 at 343 nm) when the sample (1–5 mg in 1–3 ml binding buffer) reacts with 2, 2'-dipyridyl disulphide. Choose the conditions to suit the specific sample. Under standard conditions at pH 7.5, a few minutes is usually enough for a complete reaction.

Reactivation

Pass one to two column volumes of a saturated solution (approximately 1.5 mM) of 2,2'-dipyridyl disulphide, pH 8.0 through the medium. Prepare 2,2'-dipyridyl disulphide:

1. Make a stock solution by adding 40 mg 2,2'-dipyridyl disulphide to 50 ml buffer at room temperature and stirring the suspension for several hours.
2. Filter off insoluble material.
3. Adjust the pH. The solution will be approximately 1.5 mM with respect to 2,2'-dipyridyl disulphide.

Chemical stability

Stable to all commonly used aqueous buffers and additives such as detergents. Avoid azides.

Storage

Store freeze-dried powders below +8 °C. Wash media and columns with 20% ethanol at neutral pH (use approximately 5 column volumes for packed media) and store at +4 to +8 °C. Storage under nitrogen is recommended to prevent oxidation of thiol groups by atmospheric oxygen. **Avoid using sodium azide, merthiolate or phenyl mercuric salts as bacteriostatic agents.** Azide ions will react with the 2,2'-dipyridyl disulphide groups, although low concentrations (0.04%) have been used.

Do not store the suspension for long periods in the free thiol form. Thiol groups are susceptible to oxidation by atmospheric oxygen, especially at alkaline pH. Figure 54 shows the decrease in free thiol content of Thiopropyl Sepharose 6B on storage for moderate periods at three different pH values. The thiol content of partially oxidized medium is restored by treatment with reducing agent under conditions used for removing protecting groups (see below).

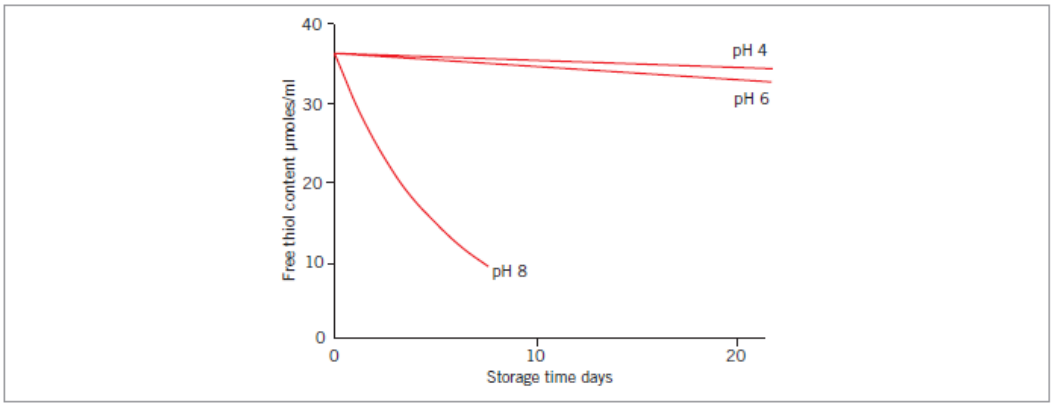

Fig. 54. Loss of free thiol content of reduced Thiopropyl Sepharose 6B on storage at +4 °C. The reduced medium was stored in 0.1 M sodium acetate or phosphate, 0.3 M NaCl, 1 mM EDTA at the indicated pH values.

Removal of protecting groups

Activated Thiol Sepharose 4B and Thiopropyl Sepharose 6B may easily be converted into the free thiol form (i.e. reduced) by removing the 2-thiopyridyl protecting groups with a reducing agent.

1. Prepare the medium as described earlier. Gently remove excess liquid on a glass filter (porosity G3).
2. Suspend the medium in a solution containing 1% (w/v) dithiothreitol or 0.5 M 2-mercaptoethanol, 0.3 M sodium bicarbonate, 1 mM EDTA, pH 8.4.
3. Use 4 ml of solution per gram of freeze-dried powder.
4. React for 40 minutes at room temperature, mixing gently.
5. Wash the medium thoroughly with 0.5 M NaCl, 1 mM EDTA in 0.1 M acetic acid. Use a total of 400 ml of solution per gram of original freeze-dried powder. Perform the washing in several steps.

Estimate the content of free thiol groups by measuring the absorption increase at 343 nm (see above) due to the 2-thiopyridone liberated in the wash solutions. The amount of thiol groups on the medium can be estimated by reacting an excess of 2,2'-dipyridyl disulphide with the medium and measuring the liberated 2-thiopyridone at 343 nm.